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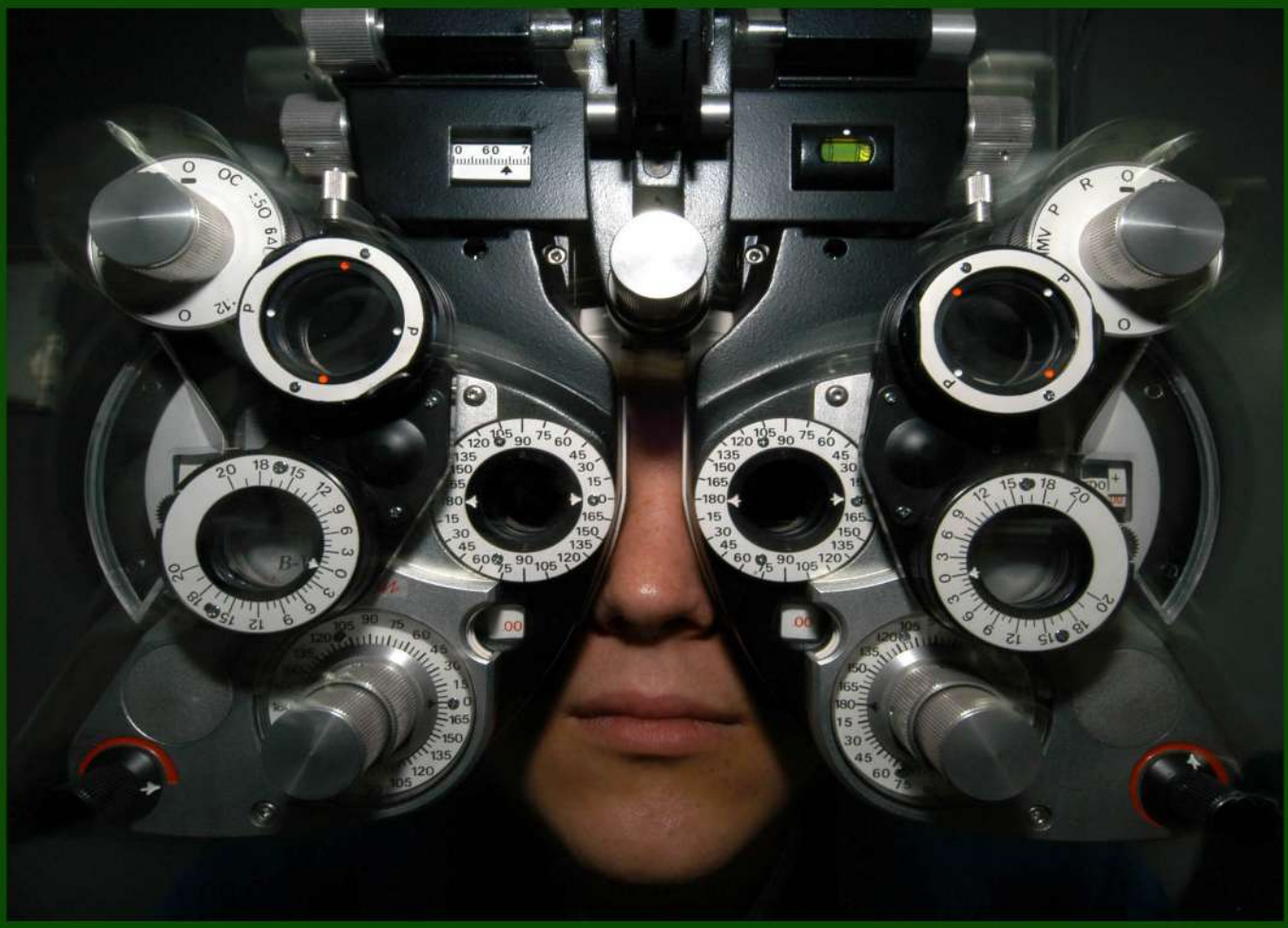

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Case Report

A Case Report on Vogt-Koyanagi-Harada Disease Seen at a Tertiary Hospital in the Philippines

Tathiana T. Marcelo, MD*; Eleonore B. Iguban, MD*

Department of Ophthalmology, Rizal Medical Center, Pasig Boulevard, Pasig City, Philippines

Corresponding authors
Tathiana T. Marcelo, MD
Department of Ophthalmology, Rizal Medical Center, Pasig Boulevard, Pasig City, Philippines; E-mail: tathianamarcelo@yahoo.com

Eleonore B. Iguban, MD
Department of Ophthalmology, Rizal Medical Center, Pasig Boulevard, Pasig City, Philippines; E-mail: eleonore_i@yahoo.com

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ABSTRACT

Vogt-Koyanagi-Harada disease (VKHD) is a defined as a bilateral granulomatous panuveitis that affects pigmented structures, such as the eye, inner ear, meninges, skin and hair. Up until this point, the exact pathogenesis is still a matter of inquiry. The most accepted mechanism involves autoimmune reaction among tissues that contains melanocytes. This disease has been described to have four stages: prodromal, acute uveitic, convalescent and chronic/recurrent stage, all of which affecting pigmented structures in the body. Early diagnosis and meticulous use of steroids has remained as the mainstay treatment, however, poor prognosis for individuals who presented with complications in the initial consultation has been associated with a poor final visual acuity. The main objective of presenting this classic case of Vogt-Koyanagi-Harada disease is describing its chronic systemic course, and the possible medical and surgical management for the disease and its complications.

Keywords
Vogt-Koyanagi-Harada Disease (VKHD); Vitiligo; Bilateral granulomatous panuveitis; Autoimmune disorder.

INTRODUCTION

Vogt-Koyanagi-Harada disease (VKHD), initially described as an uveomeningoencephalitic syndrome, is a constellation of clinical symptoms and signs that involves the eye, inner ear, meninges, skin and hair. It is a systemic granulomatous autoimmune disease that targets melanocyte-rich tissues.

It was in the 19th century when Vogt, Harada and Koyanagi first described the disease. VKH disease occurs more frequently among individuals of pigmented skin, such as Asians, Middle Easterners, Hispanics and Native Americans. However, it is interesting to note that it is infrequent among persons of African descent.[1] Different studies point out to the sex predilection of this disease, some stating that is has a sexual predilection and the others stating that it does not. In the Philippines, in an article published by the Philippine Journal of Ophthalmology by Castillo et al, they were able to note that among patients diagnosed to have VKH disease in the Uveitis Clinic of the Philippine General Hospital from 1985 to 1987, the sex ratio was 2:3 (M:F).[2] Women account for 55 to 78% of VKHD patients in the United States and approximately 38% in Japan, showing a global variation in gender predilection.[3]

Theories revolve around the possibility that a T-cell mediated autoimmune reaction against one or more antigens associated with melanocytes, melanin, and retinal pigment epithelium (RPE) may play a major role in the disease.

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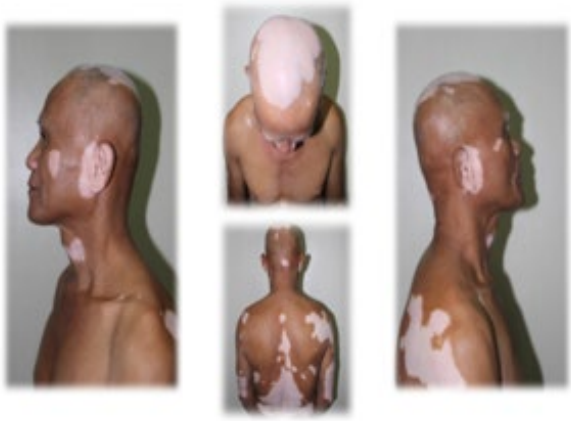
CASE REPORT

We are presented with a case of a 59-years-old-male who came in with a chief complaint of blurring of vision described to be gradual and progressive, more noted on the right eye than on the left eye, 3-years prior to consult.

The history of present illness started 14-years prior to consult when the patient noted patches of lighter skin that appeared on the patient's lower extremities and small round and oval spot baldness. He denies any history of joint pain, vascular diseases, and recurrent wounds in all parts of neither the body nor taking any of maintenance medication. There was no noted ringing in the ears, decrease in hearing and episodes of persistent headache. Patient does not recall having blurring of vision at this point. He also denies any history of eye trauma (Figure 1).

Figure 1. *Generalized Vitiligo (pictures were taken with patient's permission through a written consent)*

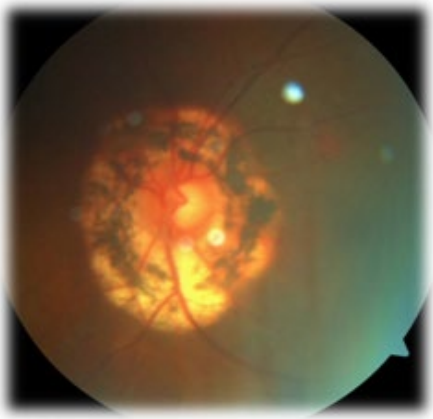


Seven-years prior to consult, still with the aforementioned signs and symptoms, the spot baldness and patches of lighter skin increased in size and number, now involving the upper extremities. Patches of white lashes were also present. There was a noted loss of axillary and pubic hair. Photophobia was also noted. There was no change in the patient's hearing and refutes any incident of persistent headache. Still, no consult was done and no medications were taken.

Three-years prior to consult, patient noticed hearing loss and ringing in the ears. He also experienced gradual and progressive blurring of vision on both eyes, both at near and distance described to be cloudy in character and still associated with photophobia.There was no eye pain noted. Patient denies any neck stiffness or recurrent headache. Persistence of these symptoms prompted the patient to seek consult at our institution.

On initial ocular examination, the visual acuity on the right eye was hand movement with good light projection, and 6/60 best corrected to 6/30 on the left eye. Poliosis was present in both eyes. The cornea has multiple keratic precipitates and multiple stromal opacities bilaterally however; there was no noted anterior chamber reaction. Perilimbal vitiligo was also noted. Both eyes had significant cataractous lenses. The intraocular pressure for both eyes was normal (Figure 2).

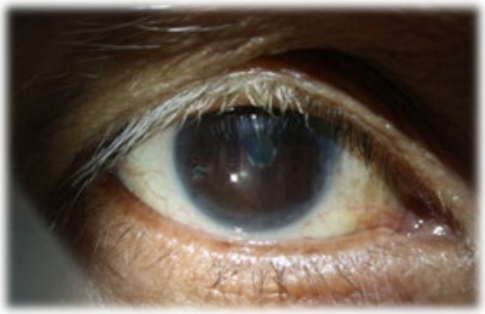
Figure 2. *Fundus Photo of the Left Eye Revealing a Hazy Media and the Classic Sunset Glow Fundus (pictures were taken with patient's permission through a written consent)*



On indirect ophthalmoscopy, sunset glow fundus was noted on the left eye with multiple hyperpigmented nummular lesions with hypopigmented border at the mid periphery, seen mostly at the superior nasal and temporal quadrants. There was no view on the right eye and B-scan was done which revealed a thickened choroid, however the retina was attached.

The patient was initially diagnosed with VKH syndrome, cataract senile mature oculus dexter (OD), cataract senile immature oculus sinister (OS) (Figure 3).

Figure 3. *Poliosis on both Upper and Lower Lashes (pictures were taken with patient's permission through a written consent)*



The patient had serial follow-ups from the initial consult. He was started on atropine sulfate and prednisolone acetate eye drops which was gradually tapered throughout the treatment. Intraocular pressure for both eyes were normal, however, it was oc-

cludable in one quadrant and the service entertained the possibility of occlusio pupillae, therefore, a laser iridotomy on both eyes had to be done as he was also diagnosed with primary angle closure suspect, oculus uterque (OU). He was referred to ears, nose, and throat (ENT) service wherein puro tone audiometry was done which revealed sensorineural hearing loss on the left ear. He was then prescribed with Mecobalamin 500 mg/tab, 1 tablet twice daily and was advised to do a repeat pure tone audiometry after 1-year. Complete blood count, urinalysis, erythrocyte sedimentation rate and C-reactive protein were all within normal limits. To rule out any pulmonary pathology, chest X-ray was done, however it revealed normal results. Rapid plasma reagin was also nonreactive. Electroretinography and fluorescein angiography were requested, however, due to the unavailability of these tests in our institution and the financially inability of the patient to have these examination done, the aforementioned tests were not made.

After the topical medications were discontinued, the patient was observed for four months without anterior chamber (AC) reaction noted. The patient then underwent an uneventful cataract surgery *via* phacoemulsification on the right eye with a final best-corrected visual acuity (BCVA) of 6/48. Optical coherence tomography (OCT) was requested after the cataract extraction; however, the patient was already lost to follow-up.

CASE DISCUSSION

It is believed that an autoimmune aggression is the nature of VKHD. Sugita et al described those T-cells from peripheral blood and intraocular fluid from patients with VKHD cross-reacted with tyrosinase protein and with highly homologous cytomegalovirus specific sequences. Studies conducted by Matsuda and Hammer were able to find evidences linking the interaction among lymphocytes, peripheral blood mononuclear cells and melanocytes on this disease process.[4,5] In separate studies, Damico and Imai sited the role of helper T-cells, Th1 cytokines, interferon gamma and interleukin-2 in the acute phase of VKHD.[6,7] As these new studies have surfaced, still, the primary pathological feature of this disease would be a diffuse thickening of the uveal tissues that is more noted in the choroid.

Histologic findings vary at the different stages of VKHD. Lymphocytic dominance with epithelioid cells and multinucleated giant cells is noted in the acute uveitic stage.[8] The phenomena of Dalen-Fuchs nodules may be explained by the focal collections of retinal pigment epithelium (RPE), macrophages, epithelioid cells and lymphocytes found along the RPE and Bruch's membrane.[8]

Nongranulomatous inflammation is said to be found during the convalescent stage. The classic sunset glow fundus was described by Rao to be a choroidal melanocyte aggression wherein there is a loss of melanin granules that results in a pale and depigmented choroid. Nummular, small hypopigmented lesions especially noted in the periphery of the fundus was further believed to be produced by focal chorioretinal adhesions and subsequent atrophy.[8]

Granulomatous damage to the choriocapillaries is observed during the chronic recurrent stage. Subretinal neovascularization, fibrosis and involvement of the choriocapillaries can be seen in this stage.[8]

Nevertheless, the diagnosis if VKHD is still primarily clinical. As in this case, the attending service had to rely heavily on clinical examination due to unavailability of the tests that can contribute to the diagnosis of the disease. Ancillary examinations such as OCT, fluorescein angiography, ocular ultrasonography and indocyanine green angiography can help one with the diagnosis of VKHD cases.

The mainstay of treatment of VKHD is the meticulous use of steroids to control the damage induced by the autoimmune reaction. It is believed that aggressive treatment with steroids can result to less structural damage to the melanocyte containing tissues, thus somehow preventing complications and recurrence.[9] As in this case, we caught the disease 14-years later since the commencement of its course, where there were already multiple keratic precipitates with a quiet anterior chamber, except that the complications such as cataract and the possibility of glaucoma is already present. Use of immunomodulatory has also been considered in the treatment of VKHD.

The visual prognosis of this disease lies in the early diagnosis and consequent use of corticosteroids and immunomodulators. Complications such as cataract, glaucoma and choroidal neovascularization (CNV) are not unusual in this syndrome. These complications, together with its recurrence and chronicity, are believed to instigate the sight-threatening illness that Vogt-Koyanagi-Harada disease can bring about.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

CONSENT

The authors have received written informed consent from the patient.

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Review

Do You Still Use Topical Antibiotics after Intravitreal Injections?

Banu Turgut Öztürk, MD, MSc*

Department of Ophthalmology, Selcuk University, Tıp Fakültesi GözHastalıkları, A.D. Kampüs 42075, Konya, Turkey

Corresponding author
Banu Turgut Öztürk, MD, MSc
Proffesor, Faculty of Medicine, Department of Ophthalmology, Selcuk University, Tıp Fakültesi GözHastalıkları, A.D. Kampüs 42075, Konya, Turkey;
Tel. 00 90 332 2244559; Fax. 00 90 332 2244544; E-mail: ozturkbanuturgut@yahoo.com

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ABSTRACT

Nowadays intravitreal drug injection is the most frequent treatment for retinal diseases. Despite widely use endophthalmitis is already most feared complication of every intravitreal injection in each patient. In clinical setting topical antibiotics have been widely used as a precaution to prevent endophthalmitis however recent published evidence showed it to be unnecessary. Furthermore repeated use of topical antibiotics might give rise to antibiotic resistance in conjunctival flora and thus more aggressive endophthalmitis. Strict asepsis has been awarded as the main rule for endophthalmitis prophylaxis intravitreal injection.

Keywords
Intravitreal injection; Steroid; Anti-vascular endothelial growth factor (VEGF); Topical antibiotic; Enodphthalmitis; Antibiotic resistance.

INTRODUCTION

Intravitreal injections have become the main treatment modality for retinal diseases all over the world. Anti-vascular endothelial growth factor (VEGF) drugs and to a lesser extent steroid implants have made intravitreal injection the most frequent procedure in ophthalmology. The anti-VEGF era began in 2004 with the approval of the first pharmacologic agent for inhibition of VEGF.[1,2] Till that time the intravitreal injection was an uncommon procedure and many guidelines were advicing the use of pre- and post-injection topical antibiotics to prevent endophthalmitis which was the most feared complication.[3] The initial randomized clinical trial protocols evaluating the efficacy of anti-VEGF agents mandated also topical antibiotics following intravitreal drug injection. Additionally, product informations of anti-VEGF agents included recommendations for topical antibiotic use as well.[4] However, the exponential increase in the number of intravitreal injections evidenced a lower incidence of endophthalmitis than expected ranging between 0.01-0.26% in several studies.[5-12] This raises questions about the necessity of pre- or post-injection topical antibiotic use.

The practice of using topical antibiotics was actually adopted from other intraocular procedures like cataract surgery. In surgical procedures there exists a surgical wound that may not be completely sealed. However, in intravitreal injections there isn't such a wound as a 30-gauge or at the largest 27-gauge needdle is used. This was probably the main reason for the lower endophthalmitis rate.

PRE-INJECTION ANTIBIOTIC USE

Most endophthalmitis cases related to intravitreal injections showed that the causative organism in post-injection endophthalmitis is usually inoculated at the time of injection rather than subsequent entry to the eye. Therefore, needle penetration into a nutrient-rich body cavity warrants pre-cautions that should be taken during the procedure to avoid contamination.[13] Pre-injection antibiotic use was found logical untill studies showed that povidone-iodine immediately prior to injection revealed less positive bacterial cultures compared to pre-treatment antibiotic use.[14,15] Two studies also revealed higher risk for endophthalmitis in patients using prophylactic antibiotics. The mechanism has been thought to be the increased ratio of antibiotic-resistant surface bacteria or the detrimental effect of repeated fluoroquinolone use on ocular surface health.[6,16]

POST-INJECTION ANTIBIOTIC USE

As mentioned above post-injection topical antibiotic use was also a query. First in 2004 Aeillo et al[3] postulated that post-injection antibiotics did not decrease endophthalmitis incidence. Recently the outcome of numerous large retrospective studies evaluating thousands of injections presented in Table 1 showed no benefit to prevent endophthalmitis.[6,8,11,12,16] In contrast, some of them found higher rates of endophthalmitis in patients using topical antibiotics.[5,6,9,17-22] A www.DRCR.net study reported endophthalmitis rate to be 0.13% in the group receiving topical antibiotics and 0.03% in the group without antibiotic.[18] This was attributed to resistant organisms due to antibiotic overuse.[23] The comparison of age-related macular degeneration treatments trials (CATT) study also showed no significant difference in endophthalmitis rates between groups with and without antibiotic use.[24]

Table 1. *Summary of Studies Evaluating Endophalmitis Group*

Study	Prophylaxis	Drug	Endophthalmitis Rate	
			With AB	Without AB
Meyer[45]	Post-injection	Anti-VEGF, TA	2/860	0/984
Bhatt[18]	Post-injection	Anti-VEGF, TA	5/2287	5/2480
Cheung[19]	Post-injection	Anti-VEGF, TA	5/8259	4/7636
Falavarjani[20]	Post-injection	Anti-VEGF	6/3975	01926
Casparis[46]	Post-injection	Anti-VEGF	2/13234	1/26777
Ramel[47]	Post-injection	Anti-VEGF, TA	3/10144	3/1306
Stranak[48]	Pre+post-injec.	Anti-VEGF, TA	2/2651	1/2355
Li[28]	Pre+post-injec.	Anti-VEGF, TA, Dex, Ocriplasmin	6/16984	11/53345
Falavarjani[49]	Post-injection	Anti-VEGF	1/2771	0/5266
Meredith[24]	Pre+post-injec.	Anti-VEGF	8/16509	3/2000
Pachuo[50]	Post-injection	Anti-VEGF	0/310	0/310
Bhavsar[51]	Pre+post-injec.	Anti-VEGF, TA	6/11565	3/17208
Storey[21]	Post-injection	Anti-VEGF	28/57654	24/89825

STEROID INJECTION

Among intravitreally injected drugs steroids have distinctive features than anti-VEGFs. The most frequently used steroid-dexamethasone intravitreal implant (Allergan, Inc., Irvine, CA, USA) has a 22-Gauge needle and additionally steroids give rise to the tendency for infections.[25] Therefore, steroid injections are expected to require a different approach than anti-VEGF drugs. However, retrospective studies and meta-analysis were surprising. A retrospective study evaluating 3593 dexamethasone implant injections without pre- or post-antibiotic use suggests that an endophthalmitis is a rare event as anti-VEGF injections.[26] Another meta-analysis evaluating 13 studies and approximately 350.000 injections assessed that type of antibiotic, type of drug injected or antibiotic prophylaxis regimen neither influence endophthalmitis rate nor reduce its incidence.[27] A significant number of triamcinolone and dexamethasone injections were enrolled in this meta-analysis. Many retrospective studies also reported similar endophthalmitis risks for both anti-VEGF and steroid implants. However, most of them evaluated a smaller number of steroid injections compared to anti-VEGF injections.[9,28] This should be kept in mind when evaluating this outcome.

WHY ARE TOPICAL ANTIBIOTICS UNNECESSARY?

The distinctive feature of intravitreal injections compared to other invasive ophthalmic procedures is the repeated application. The ocular surface was thought to be sterile for years due to the presence of lysozyme, antimicrobial peptides, immunoglobulin A (IgA) complement and other substances. Local bacteria on the ocular surface maintain ocular immunity but transient disruption of bacteria *via* antibiotics results in a reduction in immune-related mechanisms.[29] Additionally, the repeated use of short term topical antibiotics increases the resistance of the ocular surface organism to antibiotics.[19,30] This has been clearly demonstrated in the study of Kim et al.[31] After 1-year monthly intravitreal injection, the treated eyes using topical antibiotics had increased bacterial resistance compared to untreated fellow control eyes.[31] Moss et al[15] reported the rate of positive bacterial culture as 8% in patients using pre-operative antibiotic and povidone-iodine and 4% in the group receiving povidone-iodine only. Another study compared conjunctival cultures of the injected eye following the use of several topical antibiotics with the fellow untreated eye. Cultures proved multi-drug resistance of coagulase-negative staphylococci between 67.5-81.8% to antibiotics used. As conjunctival flora is presumed the source of post-injection endophthalmitis, this outcome has severe implications.[23] The drug resistance caused by repeated antibiotic use is not only limited to the eye. It has also impact on the drug resistance in nasopharyngeal flora which may give rise to soft tissue infections and pneumonia.[31]

A confounding factor related to antibiotic use is it's limited penetration to vitreous. Only about 1/100.000th of the drug observed in tear fluids reaches the retina and choroid.[32] So it is impossible to reach the minimum inhibitory concentration in vitreous required to prevent microorganism proliferation after intravitreal injection.[33]

MEASURES TO PREVENT ENDOPHTHALMITIS

Certainly, some prophylactic measures are warranted to minimize the risk of endophthalmitis following intravitreal injection. Recent evidence shows the use of povidone-iodine installation as the safest way for ocular surface preparation before injection.[34] Povidone-iodine is a disinfectant and antiseptic agent commonly used for pre-operative preparation in ophthalmic procedures and provides broad-spectrum fast-acting microbicidal activity. It is applied to lids and lashes in 5% concentration. In contrast to repeated topical antibiotics ocular surface preparation using povidone-iodine 5% without antibiotic use does not promote bacterial resistance.[35] A study evaluating daily use of povidone-iodine for peritoneal dialysis exit sites reported no resistance.[36] Hsu et al[34] also reported no antibiotic resistance even no significant alteration resulting from povidone-iodine 5% for ocular preparation. Povidone-iodine is ad-

viced to be instilled as the last drop before injection and should be allowed to stay for at least 30-seconds or more.[37] Dropping it after lid retraction has been shown to decrease the risk of endophthalmitis 7 folds.[38] Povidone-iodine with 10% showed no significant difference with povidone-iodine 5% in endophthalmitis risk and it may cause greater corneal toxicity and discomfort (Table 2).[39]

Table 2. *Precautions Before Intravitreal Injection Procedure*

Use of Povidone-iodine for Intravitreal Injection
%5 concentration preferred
Last drop before injection
Put a drop after lid retraction
Wait at least for 30-seconds or more

CONCLUSION

In light of these informations most of the authorities are trending away from topical antibiotic use following intravitreal injections.[40] The www.DRCR.net and CATT advice to abandon the use of topical antibiotics as frequent use of antibiotics appear to promote the emergence of microbial resistance.[24,41] American Academy of Ophthalmology (AAO) and also Royal College of Ophthalmology (RCOphth) also discourage the use of antibiotics after intravitreal injections in their guidelines.[42-44] These are strong scientific evidence to justify a jury that antibiotics are no longer required post-injection if an injection-related endophthalmitis would be brought to a court of law as a medicolegal suit. Using povidone-iodine 5% without topical antibiotic appears to be the safest approach to avoid the widespread problem of increasing antibiotic resistance. We have to keep in mind to instill it as the last drop possibly after lid retraction and wait for at least 30-seconds.

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Systematic Review

Complications Related to Implants Used in Anterior Bleb Forming Glaucoma Surgery: A Systematic Review of the Literature

Alejandra Salinas, MD[1*]; Fabián Rodríguez, MD, MSc[2]; Javier Bardavío, MD, FRCSEd[3]

[1]*Department of Ophthalmology, Hospital Universitario de Torrevieja, Spain*
[2]*Department of Ophthalmology, Hospital Verge de la Cinta, Tortosa, Spain*
[3]*St Mary's Treatment Centre, Portsmouth, UK*

[*]***Corresponding author***
Alejandra Salinas, MD
Ophthalmologist, FEBO, Department of Ophthalmology, Hospital Universitario de Torrevieja, Spain; E-mail: san.salinas.ss@gmail.com

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ABSTRACT

Purpose
To make an account of published implant-related complications (IRC) by a systematic review of the literature.

Methods
A systematic search of Pubmed and Scopus databases and Google Scholar engine was performed with selection criteria to detect papers on IRC. We excluded unrelated papers and reviewed selected ones. We considered papers that did not explicitly state about occurrence or not occurrence of IRC as non-IRC reporting. Main outcome measures were the number of papers reporting on complications, IRC, and types of IRC.

Results
After the search, selection, and addition, we studied 109 papers. Incidence of IRC was 4.5%, half required explantation. While 26 implant studies found IRCs (23%), 13 case reports on surgical complications, 8 (61.5%) of them reported IRC. Frequent complications were conjunctival erosion, blockage of the tube, migration to anterior chamber or damage to surrounding tissues.

Conclusion
Most papers did not report on IRC. Length or nature of studies may skew finding IRC. The incidence of IRC was 4.5%. Hard and sharp implants carry a greater risk of IRC and explantation.

Keywords
Implant-related complications (IRC); Glaucoma surgery; Anterior bleb forming.

INTRODUCTION

Glaucoma is a chronic progressive optic neuropathy that may cause severe visual loss and affect the quality of life. The front line of treatment consists of reducing intraocular pressure. Most patients achieve good medical control: some require one or more surgical procedures. Although conventional filtering surgery is effective has only moderate success and are subject to risks. Some devices have been designed and introduced to improve success and risks. Some of these implants are associated with classical conventional fistulizing surgery or to newer filtering procedures that intend the construction of a paralimbal bleb (subconjunctival, Subtenon's or intra-scleral). Regulatory bodies have studied the security and effectiveness of these implants and authorized for human application. Some systematic reviews or meta-analysis show that support for the use of these devices may be limitated.[1-4] This study aimed to make an account of published implant-related complications (IRC), this is complications directly caused by the implant, by a systematic review of the literature up to April 2015. Only implants that contribute to creating a paralimbal bleb were considered since those are the ones that are used as an alternative to trabeculectomy or non-penetrating deep sclerectomy and are

potentially avoidable. We excluded equator bleb creating implants, angular devices or anterior chamber to supraciliary space shunts from the scope of this study. We were also interested to know how many of the papers referred to complications related to the implants.

METHODS

Search Strategy

Three different search engines were used: Pubmed, Google Scholar and Scopus in April 2015. The search was performed over all fields of bibliographic references. We applied three levels of search restriction and combined controlled Mesh and natural languages. Language filters were applied. Inclusion criteria: any series of patients, whether prospective or retrospective and case reports in English, Spanish or French (filtered) found introducing the selected keywords. The word "complication" was extended to the abstract search also. Exclusion criteria: reports on other glaucoma drainage devices like Ahmed or Krupin Valve, Baerveltd or Molteno drainage devices. Any anterior chamber to supraciliary or suprachoroidal shunts like Gold Micro Shunt or Esnoper V-2000. Papers in any other languages. Keywords: "prostheses and implants/complications", "glaucoma drainage implants", implant, ex-press, esnoper, SK gel, aqua flow, collagen implant, Hem Acrylic, reticulated hyaluronic acid, T flux, thiobarbituric acid-reactive (T BAR), filtering surgery, filtering surgeries,deep sclerectomy, non-penetrating deep sclerectomy, viscocalanostomy, trabeculectomy, sclerostomy. Mendeley reference manager (www.mendeley.com,[5] Elsevier) detected duplications. Refer to Table 1 for search strategy.

Initial Revision Strategy:The Selection of Papers for Revision

All papers' titles were reviewed by a team of trained or in training ophthalmologist in search of papers that reported on paralimbal bleb forming surgical procedures with the implantation of a device. When in doubt, we reviewed the abstract. We excluded papers reporting on surgical procedures without an implant, different pro-

Table 1. *Search Strategy Details*

#	Searches on Google Scholar	Results
1	((implant* OR collagen implant OR collagen implants OR "Hemacrylic Metha Stealth" OR "reticulated hyaluronic acid" OR "T flux" OR "T bar") AND complication) AND (glaucoma)	11600
2	(implant* OR esnoper OR skgel OR aquaflow OR "ex-press" OR "collagen implant" OR "collagen implants" OR "Hemacrylic" OR "reticulated hyaluronic acid" OR "T flux" OR "T bar") AND complication* AND Glaucoma-"Molteno Implants"-Molteno-Ahmed-"Krupin valve"-"krupin valves"-Baerveldt)	2300
3	("Filtering Surgery" OR "Trabeculectomy" OR "Filtering Surgeries" OR "deep sclerectomy" OR "Sclerostomy" OR "deep sclerectomies" OR viscocanalostomy) AND (implant* OR esnoper OR skgel OR aquaflow OR "ex-press" OR "collagen implant" OR "collagen implants" OR "Hemacrylic" OR "reticulated hyaluronic acid" OR "T flux" OR "T bar") AND complication* AND Glaucoma-"Molteno Implants"-Molteno-Ahmed-"Krupin valve"-"krupin valves"-Baerveldt)	1572
#	**Searches on Scopus**	
4	(TITLE(implant* OR esnoper OR sk gel OR aqua flow OR "express implant*" OR "collagen implant*" OR "Hemacrylic Metha Stealth" OR "reticulated hyaluronic acid" OR "T flux" OR "T bar") OR ABS(implant* OR esnoper OR sk gel OR aqua flow OR "express implant*" OR "collagen implant*" OR "Hemacrylic Metha Stealth" OR "reticulated hyaluronic acid" OR "T flux" OR "T bar")) AND (TITLE(complication*) ORABS(complication*)) AND (TITLE(glaucoma) OR ABS(glaucoma)) AND (LIMIT-TO(LANGUAGE,"English") OR LIMIT-TO(LANGUAGE, "French") OR LIMIT-TO(LANGUAGE,"Spanish"))	1533
5	(TITLE(implant* OR esnoper OR skgel OR aquaflow OR "express implant*" OR "collagen implant*" OR "Hemacrylic Metha Stealth" OR "reticulated hyaluronic acid" OR "T flux" OR "T bar") OR ABS(implant* OR esnoper OR skgel OR aquaflow OR "express implant*" OR "collagen implant*" OR "Hemacrylic Metha Stealth" OR "reticulated hyaluronic acid" OR "T flux" OR "T bar")) AND (TITLE(complication*) OR ABS(complication*)) AND (TITLE(glaucoma) OR ABS(glaucoma)) AND NOT TITLE-ABS-KEY(Molteno* OR Ahmed OR "Krupin valve*" OR baerveldt) AND (LIMIT-TO(LANGUAGE,"English") OR LIMIT-TO(LANGUAGE,"French") OR LIMIT-TO(LANGUAGE,"Spanish"))	1106
6	(TITLE("Trabeculectom*" OR "Filtering Surger*" OR "deep sclerectom*" OR "Sclerostomy" OR viscocanalostom*) OR ABS("Trabeculectom*" OR "Filtering Surger*" OR "deep sclerectom*" OR "Sclerostomy" OR viscocanalostom*)) AND (TITLE(implant* OR esnoper OR skgel OR aquaflow OR "express implant*" OR "collagen implant*" OR "Hemacrylic Metha Stealth" OR "reticulated hyaluronic acid" OR "T flux" OR "T bar") OR ABS(implant* OR esnoper OR skgel OR aquaflow OR "express implant*" OR "collagen implant*" OR "Hemacrylic Metha Stealth" OR "reticulated hyaluronic acid" OR "T flux" OR "T bar")) AND (TITLE(complication*) OR ABS(complication*)) AND (TITLE(glaucoma) OR ABS(glaucoma)) AND NOT TITLE-ABS-KEY(Molteno* OR Ahmed OR "Krupin valve*" OR baerveldt) AND(LIMIT-TO(LANGUAGE,"English")ORLIMIT-TO(LANGUAGE,"French") OR LIMIT-TO(LANGUAGE,"Spanish"))	273
#	**Searches on Pubmed/Medline**	
7	("Prostheses and Implants/complications"[Mesh] OR ((implant*[tiab] OR esnoper[tiab] OR skgel[tiab] OR aquaflow[tiab] OR "express implant"[tiab] OR "express implants"[tiab] OR "collagen implant"[tiab] OR "collagen implants"[tiab] OR "HemacrylicMetha Stealth"[tiab] OR "reticulated hyaluronic acid"[tiab] OR "T flux"[tiab] OR "T bar"[tiab]) AND complication*[tiab]))) AND ("Glaucoma"[Mesh] OR glaucoma*[tiab])	1249
8	(TITLE(implant* OR esnoper OR skgel OR aquaflow OR "express implant*" OR "collagen implant*" OR "Hemacrylic Metha Stealth" OR "reticulated hyaluronic acid" OR "T flux" OR "T bar") OR ABS(implant* OR esnoper OR skgel OR aquaflow OR "express implant*" OR "collagen implant*" OR "Hemacrylic Metha Stealth" OR "reticulated hyaluronic acid" OR "T flux" OR "T bar")) AND (TITLE(complication*) OR ABS(complication*)) AND (TITLE(glaucoma) OR ABS(glaucoma)) AND NOT TITLE-ABS-KEY(Molteno* OR Ahmed OR "Krupin valve*" OR baerveldt) AND(LIMIT-TO(LANGUAGE,"English") OR LIMIT-TO(LANGUAGE,"French") OR LIMIT-TO(LANGUAGE,"Spanish"))	967
9	(TITLE("Trabeculectom*" OR "Filtering Surger*" OR "deep sclerectom*" OR "Sclerostomy" OR viscocanalostom*) OR ABS("Trabeculectom*" OR "Filtering Surger*" OR "deep sclerectom*" OR "Sclerostomy" OR viscocanalostom*)) AND (TITLE(implant* OR esnoper OR skgel OR aquaflow OR "express implant*" OR "collagen implant*" OR "Hemacrylic Metha Stealth" OR "reticulated hyaluronic acid" OR "T flux" OR "T bar") OR ABS(implant* OR esnoper OR skgel OR aquaflow OR "express implant*" OR "collagen implant*" OR "Hemacrylic Metha Stealth" OR "reticulated hyaluronic acid" OR "T flux" OR "T bar")) AND (TITLE(complication*) OR ABS(complication*)) AND (TITLE(glaucoma) OR ABS(glaucoma)) AND NOT TITLE-ABS-KEY(Molteno* OR Ahmed OR "Krupin valve*" OR baerveldt) AND (LIMIT-TO(LANGUAGE,"English") ORLIMIT-TO(LANGUAGE,"French")ORLIMIT-TO(LANGUAGE,"Spanish"))	314

cedures all together (posterior forming blebs, angular surgery, anterior chamber to supraciliary or suprachoroidal shunts), different languages or animal studies. We included systematic reviews and meta-analysis of implanting surgical procedures studies to secure relevant studies that our search could have missed out, but carefully made sure we did not duplicate the study papers by doing so.

Full text of all included papers was requested for revision. New references were found during revision and added under the same criteria.

Ethical Committee approval was not requested as no patient information was searched at any time.

Outcome Measures

The following outcomes were measured: total number of papers after exclusions, number of papers that report complications, number of papers that report IRC, type of implant, type of IRC found as extrusion, migration, damage to surrounding tissue, dysfunctional implants, explanted cases, relocated cases, other (Table 2).

Table 2. *Definition of Outcomes to be Measured*

Outcomes to be Measured
• Number of papers found once the non-related are excluded
• Number of papers that report complications
• Number of papers that report implant-related complications
• Type of implant-related complications found with every implant
o Extrusion
o Migration
o Damage of surrounding tissue
o Obstructed
o Explanted cases
o Relocated cases
o Other

Papers'revision was directed to find a statement on complications within the method and the results sections of the article. We did not look for complications reports within the discussion section nor conclusions of the papers. Since the presence or absence of complications report is not interpretable, only one reviewer read through every paper. A paper was considered to report on IRC if the actual complication was reported or it explicitly stated that no IRC was found during the study. Papers that did not state one thing or the other were considered not to report on IRC. In case of doubt, the paper was consulted with the senior ophthalmologist in the study group.

A register of complications was established to include both studies with and without complications' reports.

RESULTS

The result from the search of the three engines was 253 papers from Scopus, 314 from Pubmed and 1572 from Google Scholar that were reduced to 1102 after removing redundant papers. All titles of those papers were reviewed to discard unrelated ones when in doubt the abstract was used. Finally, 120 full-text articles were requested for review. From systematic revisions and meta-analysis, we found 28 new unknown references that were also requested. From a total of 148 papers, 23 were excluded due to belonging to animal or experimental implants studies, reporting on the different procedure, German language, or unfound full text. Of the 125 reviewed papers, 9 were systematic reviews, and 7 were meta-analysis that included studies already reviewed by this research team.[1-16] Therefore, the final number of papers considered for study purposes was 109 (Figure 1).[17-126]

All these papers correspond to studies or case reports that report on glaucoma implant surgery and also on surgical complications (100% of the 109 papers reviewed). Out of the 109 papers reviewed, 75 (68.8%) did not report on IRC, 34 (31.2%) reported on IRC, 6 (17.6%) of the IRC reporting papers) explicitly reporting an absence of IRC (4 prospective and 2 retrospective studies). Of the 59 prospective studies (54.1%, of 109), 17 reported on IRC (28.8%, of 59). Of the 37 retrospective studies (33.9%, of 109), 9 (24.3%, of 37) reported IRC. Thirteen of the papers (11.9%, of

Figure 1. *Final Number of Papers Considered for Study Purposes*

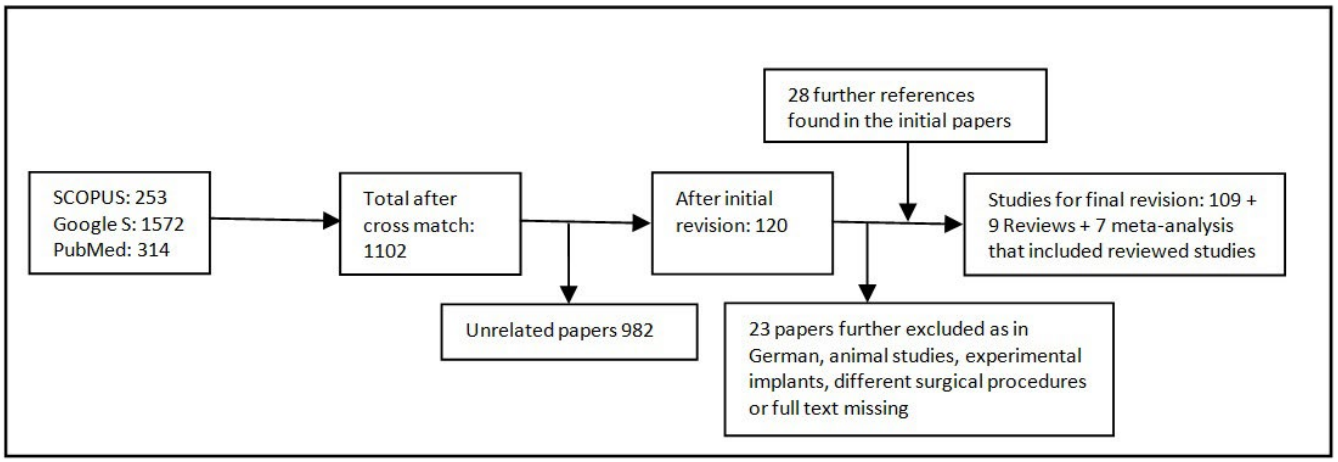

Table 3. *Understudy Implants, IRC Reporting Papers, Number of Study Eyes (Eyes with Implants in IRC Reporting Papers) and Number of Complicated Cases*

Implant	N° of Papers*	N° IRC Papers**	N° of Study Eyes	N° Complicated Cases
Express (Steel)	38	22 (57,9%)	940	54 (5,7%)
Collagen Implant	25	5 (16,6%)	208	9 (4,3%)
SK-gel (Hyualuronic acid)	25	3 (12%)	185	2 (1,07%)
T-flux (Acrylic)	15	1 (7,14%)	25	1 (4%)
Ethicon 1/0 Chromic Catgut	1	1 (100%)	23	1 (4,3%)
PMMA	1	1	30	0
ePTFE	1	1	35	0
Ologen (Collagen)	9	1	33	0
HEALA Flow	1	0	0	0
Aquaflow (Collagen)	5	0	0	0
Total	109	35	1479	67 (4,5%)

**Twelve papers tested two or three implant devices; **One paper tested two devices PMMA and Collagen implant*

Table 4. *Reported Complications*

Implant Complication	SK-gel	Collagen Implant	T-flux	Express	Ethicon 1/0 chromic catgut
Migration or malposition	1 migration to subconjunctival space		1 migration into anterior chamber	1 misplacement 1 migration to subconjunctival space 5 into anterior chamber	1 into anterior chamber after blunt trauma
Extrusion	1 conjunctival erosion and extrusion that did not require explants or repositioning			20 conjunctiva erosions (15 without scleral flap, 4 with sclera flap, 1 unknown)	
Damage to surrounding tissues		1 Corneal abrasion 5 Descemet mb. detachment		5 iris touch 2 endothelial touch 1 Corneal abrasion 1 crystalline lens touch 1 scleral flap erosion only	
Obstructed				14	
Explanted		3 During hypotony repair surgery		2 unreported reasons 1 due to infection	
Explanted as treatment of complication				26	
Repositioned				3 relocated due to other complication Other causes	

109) were case reports, 8 (61.5%, of 13) reported IRC.

The total population of eyes considered in this study was 5831 (population range per study: 1-345), 4576 of which had been implanted (78.4%), the others belonging to control groups. The population of eyes with an implant included in studies that reported on IRC was 1479. Of those, the number of eyes that suffered an IRC was 67 (4.5%). Table 3 shows the implants understudied, the number of IRC reporting papers, the number of study eyes and the number of complicated cases.

A summary of the complications is in Table 4. The most common IRC was conjunctival erosion; 20 cases after Express implantation (15 through a full-thickness scleral incision, four under a partial-thickness scleral flap and one unreported technique) that required implant removal, and one case of SK-gel that healed under observation. Obstruction of Express tube occurred in 14 cases. Four Express tubes migrated postoperatively into the anterior chamber (AC) and another one was introduced in the AC during the procedure. Another Express tube migrated to the subconjunctival space, as it occurred to an SK gel implant, and a further Express tube was found misplaced. A T flux implant migrated into the patient's anterior chamber. A fragment of Ethicon 1/0 chromic catgut, placed under a deep sclerectomy flap, migrated into de AC also. Sixteen cases of damage to surrounding tissues were found, 5 Descemet membrane detachments and one corneal abrasion after collagen implant, five iris touch, two endothelial touches, one corneal abrasion, one crystalline lens touch that induced a white cata-

ract and one scleral flap extrusion, without conjunctival erosion, after Express implantation. Three primary explantation of Express tubes, one due to a bleb infection and two for unreported reasons, as well as three collagen implant removal during the hypotony repair. A total of 29 Express tubes (3% of all tubes implanted) and three collagen implants (1.4% of all implanted) were explanted.

DISCUSSION

The market offers implants in an attempt to improve post-operative survival, efficacy or safety for patients requiring glaucoma surgery. Some studies, however, suggest that traditional trabeculectomy or newer deep sclerectomy without implants might be just as effective in reducing intraocular pressure. Devices, therefore, attempt to improve prognosis, but its mere physical presence may also increase risks. This study aimed to make an account of published IRC by a systematic review of the literature up to April 2015. We only studied implants that contribute to creating a paralimbal bleb. Equator bleb creating implants, angular devices or anterior chamber to supraciliary space shunts are not within the scope of this study.

Our search produced a total of 1102 papers. The use of natural language and mesh terms explain such a large number of papers found. The initial selection resulted in 120 papers of which 23 were further excluded, and another 28 were added during revision. Seven systematic reviews, nine meta-analysis, 13 case reports, 37 retrospective and 59 prospective studies were reviewed. Only an explicit account of IRC was considered as a report. All papers reviewed (109/109) reported surgical complications. Only 31.2% reported on IRC: 6 (17.6%) of those (4 prospective and two retrospectives), reported an absence of IRC. Only 28.8% of all prospective and 24.4% of all retrospective studies reported on IRC. It was therefore interesting to find that of 13 case reports dealing with complications, 8 (61.5%) were about IRC. We suspect that the discrepancy in IRC incidence reported in retrospective and prospective studies compared to case reports might be related to the length of the studies and although inconclusive, could be relevant. Issues related to reporting could justify further research.

Sixty-seven cases of IRC of 1479 (4.5%) patients were found, of which 31 required implant removal. Our estimation of IRC incidence might be biased by the search or the selection method. The incidence of Express removal was 3% before the tube was guarded under a scleral flap: that reduced the risk but did not eliminate it. The initial design of the technique may show that the risk of extrusion was underestimated. Risk of misplacement or injury to surrounding tissues like Descemet membrane, corneal endothelium or the crystalline lens is also relevant as well as migration into the anterior chamber of mainly the express tubes.

The physical properties of the implant are relevant: harder and sharper implants that remain longer, increase the risk of complications, mainly extrusion. In our study, the steel implant Express accounts for most cases of explanted, extruded or migrated cases. Also, non-absorbable acrylic T flux tends to migrate if left unfixed. Soft and absorbable implants tend to be more respectful. We speculated that tensile vectors generated by scaring tissue might tend to move rigid pieces and to fold soft material: this could explain the migration of implants or simple orientation changes. The intermittent robbing effect of the eyelid over the conjunctiva exerts an external pressure (tissue towards implant) that injures the tissue in the opposite direction (implant towards sclera, conjunctiva or both). Both mechanisms are active in all operated eyes, so the risk to induce complications exists.

Study limitations include missing relevant papers by the search (no excerpta medica dataBASE (EMBASE) search) or the selection method: the initial search failed to detect 28 relevant papers added later. It is possible that relevant data from studies was filtered out by title or abstract selection. A further limitation is late publication of this study that was finalized in 2016 and sent for publication in 2019. Finally, this review relies on what authors report on their papers, under or overreporting issues would modify these results. However, the study remains useful to stress the fact that an implant might induce complications by itself. Also, the type and shape of the material are relevant and worth bearing in mind at the time of assessing the purpose and usefulness of the implant. Further, the importance of considering IRC at the time of designing studies to make them sensible to complication detection and reporting.

CONCLUSION

An IRC incidence of 4.5% resulted from this study, of which about a half required removal. Harder and sharper implants carry a higher risk. It is worth considering the implant's function and the risk it may carry its implantation before making the clinical decision of using them. Studies should consider this risk and be methodologically designed to detect them.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Sirtuin Inhibitor as a Novel Cell Cycle Checkpoint and Regulator of the TP53-MDM2 Pathway in Uveal Melanoma

Zachary K. Goldsmith, PhD[1,#]; Matthew W. McEwen, MD[1,#]; Mercy W. Kibe, MD[1,#]; Kelley Yuan, BS[1]; Hua Yang, MD, PhD[2]; Qing Zhang, MD, PhD[2]; Sumana R. Chintalapudi, PhD[1]; Bradley T. Gao, MD[1]; Monica M. Jablonski, PhD[1,3,4]; Benjamin King, MD[1]; Ryan P. Lee, MD[1]; Abigail Lepsch, MD[1]; Neel Patel, MD[1]; Xiang Di Wang, MD, PhD[1]; Hans E. Grossniklaus, MD, MBA[2,##]; Matthew W. Wilson, MD, FACS[1,5,##*]; Vanessa M. Morales-Tirado, MS, PhD[1,6,##*]

[#]Equal Author Contribution
[##]Co-senior Authors

[1]Department of Ophthalmology, Hamilton Eye Institute, The University of Tennessee Health Science Center, Memphis, TN, USA
[2]Department of Ophthalmology, Emory Eye Center, Emory University, Atlanta, GA, USA
[3]Department of Anatomy and Neurobiology, The University of Tennessee Health Science Center, Memphis, TN, USA
[4]Department of Pharmaceutical Sciences, The University of Tennessee Health Science Center, Memphis, TN, USA
[5]Department of Surgery, St. Jude Children's Research Hospital, Memphis, TN, USA
[6]Department of Microbiology, Immunology and Biochemistry, The University of Tennessee Health Science Center, Memphis, TN, USA

Corresponding authors
Matthew W. Wilson, MD, FACS
Professor, Department of Ophthalmology, Hamilton Eye Institute, The University of Tennessee Health Science Center, 930 Madison Ave, Suite 100, Memphis, TN 38163, USA; Tel. (901) 448-6550; E-mail: mwilson5@uthsc.edu

Vanessa M. Morales-Tirado, MS, PhD
Senior Scientist III, Department of Microbiology, Immunology and Biochemistry, The University of Tennessee Health Science Center, Memphis, TN, USA;
E-mail: vmoralestirado@gmail.com

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ABSTRACT

Purpose
The liver is the most common site of uveal melanoma (UM) metastasis with approximately 50% of UM patients being affected. With no proven therapies that mitigate metastases the mortality rate is 85% within the first year after detection of the liver disease. In this study, we provide a mechanistic understanding of the de-regulation of the *TP53*-MDM2 pathway in UM, which plays a central role in tumor biology.

Methods
We investigated the *TP53*-MDM2 signaling pathway in the microenvironment of liver metastases taken from both a murine orthotopic xenograft and post-mortem metastatic UM human liver. These findings were studied in-depth using both primary and metastatic UM cell lines treated with the MDM2 antagonist Nutlin-3a and the sirtuin inhibitor and transcriptional activator of *TP53*, Tenovin-6.

Results
De-regulation of the *TP53*-MDM2 signaling pathway is specific to the liver microenvironment, providing a survival mechanism for UM metastases. Tenovin-6, not Nutlin-3a, reduced UM cell survival by increasing the percentage of cell death and reducing the percentage of proliferating cells. Tenovin-6 increased acetylation of p53, reduced ubiquitination of the protein, and acted as a cell cycle regulator.

Conclusion
Our findings suggest that in patients with metastatic UM de-regulation of *TP53*-MDM2 signaling pathway promotes growth of the liver metastases and provides pre-clinical information on the potential of targeting of the *TP53*-MDM2 signaling pathway *via* Tenovin-6.

Keywords
Uveal melanoma; Ocular tumors; Nutlin-3a; Tenovin-6; *TP53*; MDM2.

INTRODUCTION

The liver is a primary metastatic site for several cancers including uveal melanoma (UM).[1,2] Despite effective, prompt local control of the primary tumor, 50% of UM patients still develop liver metastasis. This is due to early hematogenous dissemination of cancer cells (micrometastases) to the liver prior to diagnosis of primary ocular disease.[3-5] Micrometastases may remain dormant for years. Even with current advances in therapeutic options for cutaneous melanoma, there are no proven therapies for metastatic UM. Metastatic UM patients have a 1-year overall mortality rate of 85%, and 2-year overall of 92%.

Current efforts in the UM field focus on inherent properties of the tumor independent of tumor location.[6-8] However, the interaction of tumor cells with the hepatic microenvironment may determine the metastasis' fate, whether growth is promoted or suppressed. Using an established orthotopic xenograft model[9] we recently demonstrated *in vivo* an increase in metastatic UM clearance in the liver by augmentation of the natural killer (NK)-cell population by triggering the Toll-like Receptor 5 signaling agonist entolimod. This study provided pre-clinical evidence of the efficacy of modulation of the microenvironment against liver metastases.[10]

We hypothesized that interaction with the host liver microenvironment plays a role in shaping UM tumor properties, leading to tumor survival and growth. It has been shown that p53 mutations are infrequent in UM[11] compared to other cancers.[12] These findings have not yet been studied in-depth in an orthotopic xenograft system. In our study, we sought to further characterize the changes that take place in metastatic UM after interaction with the liver microenvironment.

In support of our hypothesis, we discovered differences between UM cells prior to inoculation and after seeding into the liver microenvironment in our orthotopic xenograft. We validated these findings by examination of a cohort of samples taken post mortem from the liver of a patient with metastatic UM. Our work revealed that the *TP53*-MDM2 signaling axis is de-regulated in metastatic UM. Despite overexpression of MDM2, inhibition with Nutlin-3a did not decrease cellular proliferation. However, the sirtuin inhibitor and transcriptional activator of the *TP53* signaling, Tenovin-6, increased the percentage of apoptotic cells in UM primary and metastatic cell lines, increased acetylation of p53 while reducing ubiquitination of the protein, and acted as a cell cycle regulator.

METHODS

Cell Lines and *in Vitro* Cell Culture

Established human UM cell lines derived from primary (Mel 270, Mel 290) and metastatic tissue (OMM 2.5) were kindly given by Dr. Bruce Ksander (Schepens Eye Research Institute, Boston, MA, USA; Massachusetts Eye and Ear, Boston, MA, USA) to HEG and cultured at a cell density of 1.0×10^5 cells per well. The next day, cells were harvested and supernatants collected for cytokine secretion assays. Routinely, cells were cultured in UM media, as described.[9] Cell lines were treated with Hepatocyte Growth Factor (20 ng/mL, Peprotech, Rocky Hill, NJ, USA), Nutlin-3a (PubChem CID: 11433190, CalBioChem, EMD Millipore, San Diego, CA, USA), and Tenovin-6 (PubChem CID: 24772043, ChemCruz™, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at different concentrations ranging from 0.1 μM-5.0 μM.

Orthotopic Xenograft Model

All experiments were conducted according to the Declaration of Helsinki and Guiding Principles in the Care and Use of Animals conform to the Association for Research in Vision and Ophthalmology (ARVO) for animal use in ophthalmic and vision research, the Institutional Animal Care and Use Committee at Emory University and adherence to the National Institutes of Health (NIH) guide for the care and use of laboratory animals. Details of the orthotopic xenograft model are published.[9] Briefly, mice were anesthetized with ketamine and xylazine (45 and 4.5 μg/g, respectively) prior to inoculation with 1.0×10^6 cells in a 2.5 μL final volume into the choroid. The inoculated eye was enucleated 7-days after inoculation. Mice were sacrificed 5-weeks after enucleation. Liver and spleen tissues were removed.

Liver Tissue

The UTHSC Institutional Review Board (IRB) approved the present study, which aimed at molecular and genomic investigation of post-mortem UM liver tissue. This is in full compliance with and adheres to the tenets of the Declaration of Helsinki and the ARVO statement on human subjects. Different sections of liver tissue were obtained from a metastatic UM patient post-mortem. Tissue was macerated and cells were lysed in RIPA Buffer (Thermo Scientific). As controls, we purchased healthy liver cell lysates and RNA from Zyagen, Life Science Products (San Diego, CA, USA).

Ex-vivo Cell Culture

Single cell suspensions of excised tissues were prepared by mechanical disruption of the tissue as previously described.[13] RBCs were removed using the RBC Lysis Buffer (BioLegend, San Diego, CA, USA).

Cell Proliferation Assay

Quantitation of percentage inhibition was performed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, Wisconsin, USA) as before.[14] Briefly, 1.0×10^4 UM cells per well were cultured under the following conditions: untreated control, HGF, Nutlin-3a, Tenovin-6. Cell Titer reagent was added at a concentration of 20 μL per 100 μL volume per well at specific time points of 0-, 48-, 72-, and 96-hrs after culture. Cells were incubated at 37 °C for 2-hrs before absorbance was read at 490 nm using a 96-well plate reader. The percentage of inhibition

was calculated based on the 72-hrs time point using the following formula: %inhibition=100-(100×absorbance sample–absorbance blank)/absorbance untreated–absorbance blank.

Cell Cycle Analysis

UM cells were labeled with DRAQ5™ (1:200, BioLegend) prior to analysis in a quantitative imager cytometer (Flow Sight®, Amnis, EMD Millipore, CA, USA). Cell cycle analysis was based on deoxyribonucleic acid (DNA) content by a histogram of DRAQ5™ Intensity versus normalized frequency. Samples were acquired using INSPIRE® software. Analysis of the acquired data was performed using IDEAS® software. Using the statistics tool the percentage of cells in each phase was determined. Three independent experiments were done.

Apoptosis Analysis

Flow cytometry: UM cells were resuspended in Annexin V Binding Buffer (BioLegend) at a concentration of 1.0×10^6 cells/100 µL. We added 10 µL of PI (BioLegend) and gently vortexed. The cell suspension was incubated for 15 min at RT, protected from light prior to flow cytometry analysis. Data acquisition was done in a ZE5 Cell Analyzer (aka YETI), from Propel Labs (Fort Collins, CO, USA). Analysis was done using FlowJo software v10.0.8 (FlowJo, LLC, Ashland, OR, USA).

Imaging flow cytometry: We followed the analysis guidelines using IDEAS® software. A Gradient rhabdomyosarcoma (RMS) histogram of the collected population was selected to choose the cells with better focus. The focused cells were gated and analyzed into a scatter plot of Area *versus* Aspect ratio. Single cells were plotted into a nucleated cells scatter plot of the Brightfield Contrast *versus* the Area of the thresholded nucleus. Apoptotic cells are defined as cells with low nuclear area and high bright field contrast.

Ki-67 Immunolabeling

UM cell lines were cultured at a 2.5×10^5 cells/ well in a 6-well plate overnight at different conditions: untreated control, HGF control, Nutlin-3a +HGF, and Tenovin-6 + HGF at 37 °C. Cells were fixed with paraformaldehyde followed by permeabilization with 0.01% Triton X-100 for 20 min. Cells were washed 3x for 10 min each time with phosphate-buffered saline (PBS) followed by blocking with PBS/1% body surface area (BSA) for 1hr. Biotin Ki-67 antibody (SolA 15, 13-5698-82, eBioscience) was diluted 1:250 in PBS/1% BSA. Cells were incubated for 1 hr followed by PBS washes as before. Streptavidin Alexa Fluor 488® (405235, BioLegend) was added at 1:250 diluted in PBS/1% BSA. Incubation proceeded for 1hr at RT. About 15min prior to wash cells we added a 1:4000 dilution of 4',6-diamidino-2-phenilindole, dihydrochloride (DAPI), FluoroPure™ grade (D21490, ThermoScientific). Cells were washed 3x as before. Images were taken using an EVOS FLoid® Cell Imaging Station (ThermoScientific), at 20x.

Percentage of acetylated-p53$^+$ and Ubiquitinated Cells

Cultured cells were harvested, washed in PBS and immediately fixed in 2% paraformaldehyde prior to permeabilization in 0.01% Triton X-100. Samples were blocked with PBS/1% BSA before addition of a rabbit anti-acetyl-p53 (K382, CST, 1:50) antibody for 1hr in ice. Following primary antibody labeling, cells were washed with PBS and a goat anti-rabbit Alexa Fluor® 488 (ThermoScientific, 1:100) secondary antibody was added for 1hr; nuclei were labeled with DRAQ5™ for the final 15 min of incubation with secondary antibody (BioLegend, 1:500). Cells were analyzed using the AmnisFlowSight®. To assess the percentage of acetylated-p53$^+$ cells we followed the analysis guidelines using IDEAS® software. Gradient RMS histogram was selected to choose the cells with better focus followed by a scatter plot of Area *versus* Aspect ratio to select single cells. We chose the two channels representing our sub-populations (acetyl, nucleus) and gated co-localized events using a histogram of Bright Detail Similarity and a histogram of intensity. The percentage of ubiquitinated-p53$^+$ (anti-ubiquitin rabbit, EPR8830, Abcam, 1:100) cells was assessed in a similar fashion.

qPCR Analyses

RNA was extracted from cells or tissue using RNeasy® Mini Kit (Qiagen Inc., Valencia, CA, USA) following manufacturer's conditions and published protocols.14-16 We used 100 ng of RNA material for complementary DNA (cDNA) synthesis. Resulting cDNA material was pre-amplified prior to assay set up. Samples were run in Roche® Light Cycler 480 and analyzed using the Comparative ΔCT Method. Gene expression assays: (ThermoScientific): *TP53* (Hs01034249_m1), MDM2 (Hs01066930_m1), and MDMX (Hs00910358_s1).

Western Blot Assays

Cells were lysed in RIPA Buffer as described.[13,14] Protein concentrations were determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific). A total of 50 µg of denatured protein were loaded in a Bolt™ 4-12% Bis-Tris Plus Gel (ThermoScientific). After transfer, the polyvinyl difluoride (PVDF) membrane was blocked with 20 mL of SuperBlock™ Blocking Buffer (ThermoScientific) and incubated overnight at 4 °C in primary antibody solution, followed by secondary antibody solution for 2-hrs at real time (RT). The membrane was washed and probed for β-actin as control (Cell Signaling Technologies-CST, Dancers, MA, USA). The following primary antibodies were used: p53 mouse monoclonal antibody (1:1000, 1C12, CST), phosphorylated-p53 rabbit polyclonal (1:1000, Ser15, CST), acetyl-p53 rabbit polyclonal (K382, CST, 1:1000), MDM2 mouse monoclonal IgG_1 antibody (1:100, SMP14, Scbt), phosphorylated-MDM2 rabbit polyclonal antibody (1:100, Ser166, Scbt), and anti-mutant p53 rabbit monoclonal antibody (1:1000, E47, Abcam, Cambridge, MA, USA). The following secondary antibodies were used at 1:1000: anti-mouse IgG HRP-linked antibody and anti-rabbit IgG HRP-linked antibody, both from CST. SuperSignal™ West Pico Chemiluminescent Substrate (ThermoScientific) was used for development. Densitometry analysis was done using Kodak Molecular Imager.

Confocal Imaging

Liver samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 6-hrs. Samples were embedded in low melting

point agarose (Sigma) and sections (50 µm thickness) were cut on a vibratome (VT1000S; Leica, Wetzlar, Germany). Immunofluorescence staining for p/tMDM2 and p/*TP53* was performed as before.[15] Tissue sections were blocked with 10% goat serum for 30 min and permeabilized with 2.5% Triton X-100 (ThermoScientific) to identify intracellular localization patterns. Tissue sections were then separately labeled with anti-MDM2 (1:200), anti-p53 antibody (1:200), anti-pMDM2 (1:200) and anti-pP53 antibody (1:200). Sections were incubated in Alexa Fluor 488® conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA; 1:500) to detect the antigen of interest. TO-PRO3 iodide (1:4000, Invitrogen) was used to label the nuclei. Sections were viewed and images were obtained using a Nikon C1 confocal microscope in HEI, UTHSC. All microscope settings, including laser levels and gain, were held constant and images were collected in identical conditions to allow for relative comparisons of signal intensity within and between experiments.

Statistical Analysis

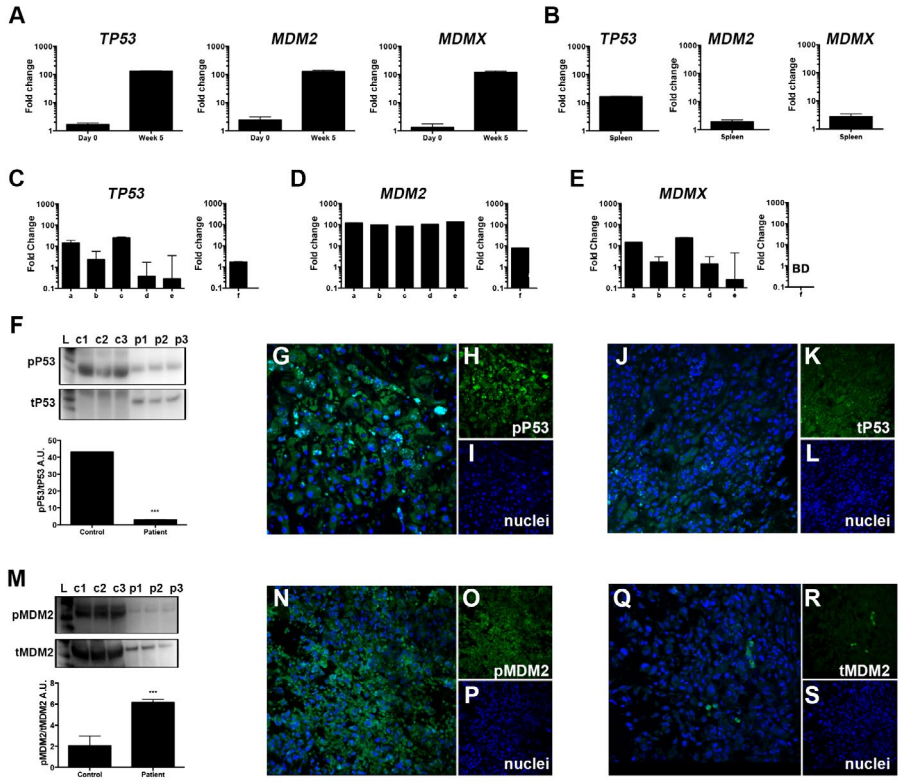
Data were analyzed using Prism 6 for Mac OS X (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance determined using the Holm-Sidak method, with alpha=5.000%. Values of $p<0.05$ were considered significant.

RESULTS

Regulation of the *TP53*-MDM2 Signaling Axis in the Metastatic Liver Microenvironment

We investigated the *TP53*-MDM2 signaling pathway in our orthotopic xenograft model as a potential aberrant mechanism in metastatic UM. Details on the orthotopic xenograft have been previously published.[9] The primary UM cell line Mel270 was inoculated into the posterior compartment of immunocompromised Nu/Nu mice. Clinically detectable metastases were found after 5-weeks post inoculation. To investigate the role the microenvironment plays in shaping the tumor response, we compared *TP53* messenger ribonucleic acid (mRNA) expression of cells prior to inoculation to those of cells cultured ex vivo from liver metastases 5-weeks post inoculation (Figure 1A, left). We discriminated between human UM-derived cells and the murine microenvironment using species-specific gene expression assays. We measured upregulated *TP53* mRNA expression in the liver compared to the *in vitro* cultures on Day 0. Similarly, MDM2 (Figure 1A, middle) and MDMX (Figure 1A, right) were overexpressed ex vivo. To

Figure 1. *Expression of the TP53-MDM2 Signaling axis within the Metastatic Tumor Microenvironment*



A. TP53, MDM2, and MDMX mRNA expression in Mel270 cells prior to inoculation (Day 0) and the recovered liver (5 weeks post inoculation) of an UM orthotopic xenograft model. **B.** mRNA expression of in spleen-derived cell cultures. **C-E**. Tissue was removed from 5-different regions of a metastatic UM patient (samples a-e) to compare with healthy liver (sample f). Examination of TP53(C), MDM2(D), and MDMX(E) mRNA levels. Western blot analyses of TP53 signaling **F.** F in liver samples from healthy controls (c1-c3) and three regions from the mUM liver (p1-p3) with representative immunohistochemical analysis of phosphorylated-p53 (active) and total-p53 **G-L**. MDM2 signaling in healthy and mUM liver **M.** Immunohistochemical analysis of phosphorylated- and total MDM2 **N-S.** All results from bar graphs represent mean±SEM, ***$p<0.0005$.

investigate if this response was limited to the metastatic site, we compared these results to spleen-derived cell cultures. *TP53* was upregulated, but not to the extent of that in the liver,suggesting the presence of circulating tumor cells in the spleen. Additionally, we measured low expression of MDM2 and MDMX mRNA (Figure 1B, Table 1) in the spleen, suggesting that the *TP53*-MDM2 deregulation is specific to the liver microenvironment.

Table 1. *Gene Expression Analyses on Tissues Retrieved from the Orthotopic Murine Model and UM Patient post-mortem*

Tissue		Murine Model	Human
Liver	TP53	+++	+
	MDM2	+++	+++
	MDMX	+++	++
Spleen	TP53	++	N/A
	MDM2	+	N/A
	MDMX	+	N/A

(+) symbol indicates fold change expression. Murine model comparison of week 5 compared to Day 0; human tissue expression relative to endogenous control. N/A, not available.

Tumor Survival Mechanisms in Liver Metastases

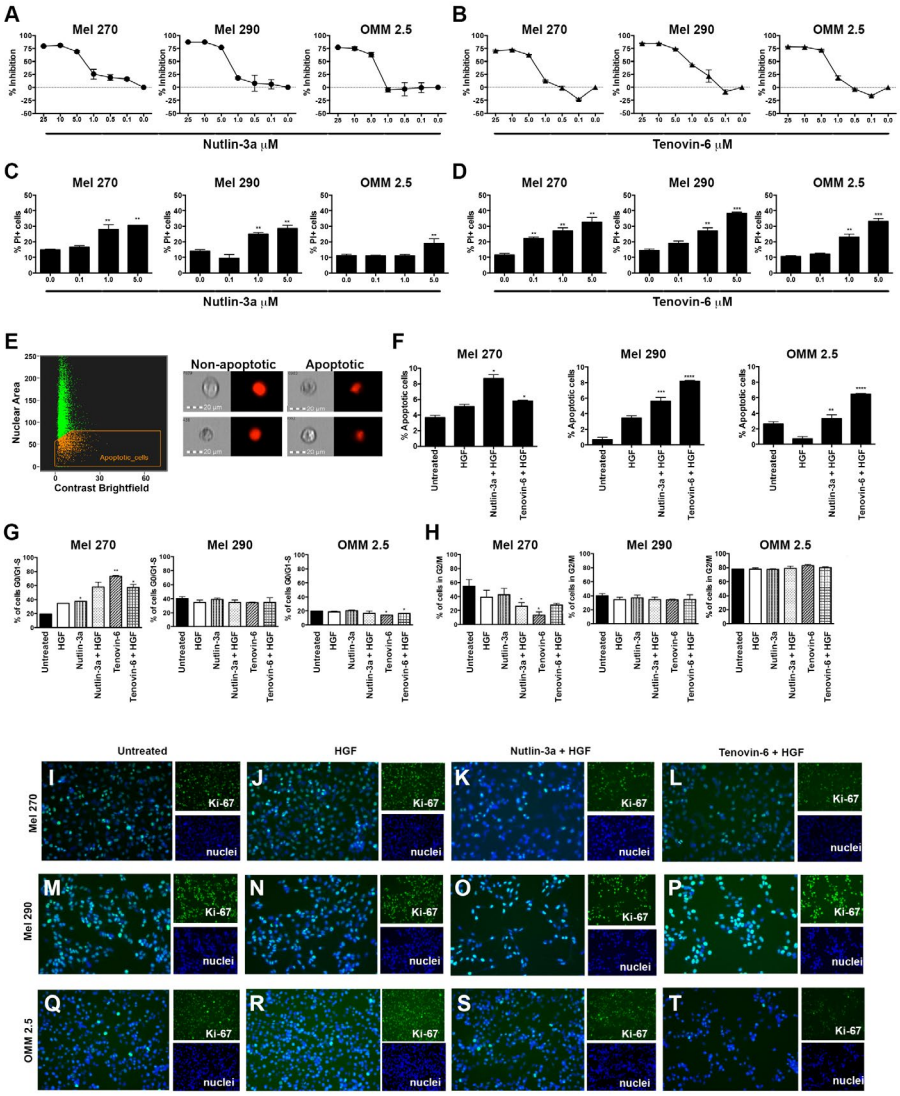
We sought to confirm the results from Figure 1A-Busing post mortem liver samples obtained from a UM patient with liver metastases. Because these cells may have varying levels of pro-survival molecules allowing them to subsist, we addressed the potential heterogeneity of the microenvironment by sampling 5 different regions of the metastatic UM liver. We compared mRNA and protein levels to samples taken from a healthy liver using qPCR analysis and Western blotting (Wb), respectively. We measured a transcriptional enhancement of *TP53*, MDM2 and MDMX mRNA in the cohort of samples from the metastatic liver compared to the healthy liver (Figure 1C-E, Table 1). Next, we assessed the protein activation status as it could provide clinically valuable information for the development of protein inhibitors targeting phosphorylation-residues. Figure 1F shows markedly decreased *TP53* signaling in metastatic UM compared to the healthy liver as reflected by a decreased ratio of phosphorylated to total *TP53*. However, immunohistochemical analyses still demonstrated the expression of both phosphorylated- and total-p53 protein (Figure 1G-L, representative figures) in the metastatic liver. We then evaluated the expression of MDM2 and measured higher MDM2 signaling in the metastatic liver compared to the healthy liver (Figure 1M). We also measured expression of the p-MDM2 localized primarily in the nucleus (Figure 1N-S) and low expression of total MDM2 protein. These results highlight the molecular aberrations present in metastatic UM providing a survival mechanism for the tumor.

Enhanced *TP53* Signaling in UM Cell Lines After Treatment with Nutlin-3a and Tenovin-6

We investigated the molecular defects of the *TP53*-MDM2 signaling pathway using a series of well characterized UM cell lines. We cultured cells in the presence of Nutlin-3a17 or Tenovin-618, as they are considered potential therapeutic targets for the *TP53*-MDM2 signaling pathway. First, we tested the percentage of cell growth inhibition in each of the UM cell lines of interest. Three different UM cell lines were tested, Mel270, a primary tumor derived cell line from an individual with metastatic disease; Mel290, a primary tumor derived cell line; and OMM2.5, a metastatic cell line. Figure 2A depicts the percentage of cell growth inhibition after 72 hrs of culture in the presence of Nutlin-3a relative to untreated cells. About 50% inhibition was achieved between 1-5 μM concentrations. A similar approach was utilized to test the percentage of cell growth inhibition after 72 hrs of culture in the presence of Tenovin-6 (Figure 2B). Next, we tested the cytotoxic effects of Nutlin-3a and Tenovin-6 in UM cell lines by measuring cell death as the percentage of Propidium Iodide (PI)-positive cells by flow cytometry analysis. Primary tumor-derived cell lines displayed a dose dependent response as shown in Figure 2C. In contrast, we measured a significant increase of OMM2.5 PI^+cells only at high concentrations of Nutlin-3a. These cells showed sensitivity against Tenovin-6 at smaller dosage (Figure 2D). As a next step, we investigated cell death *via* apoptosis by measuring the nuclear morphology at the single cell level using imaging flow cytometry. Apoptotic cells are defined as cells with low nuclear area and high bright field contrast, as shown in Figure 2E, left. Figure 2E, right, shows representative images of the non-apoptotic and apoptotic phenotypes. UM cells were cultured in 4 different conditions: untreated; in the presence of hepatocyte growth factor (HGF), mimicking physiological components of the *in vivo* liver microenvironment; Nutlin-3a + HGF; Tenovin-6 + HGF. Quantitation analyses on each of the UM cell lines are shown in Figure 2F. Collectively, we measured a significant increase in the percentage of apoptotic cells in cell cultures containing either Nutlin-3a or Tenovin-6 across all tested UM cell lines. These results support our hypothesis that the de-regulation in the *TP53*-MDM2 signaling pathway plays a role in the control of metastatic UM.

Next, we tested the hypothesis that de-regulation in the *TP53*-MDM2 signaling pathway controls UM cell proliferation. Cell cycle studies were done using the live cell permeant DNA probe DRAQ5™ (Figure 2G-H). The cell cycles studies were done without cellular synchronization to investigate the effects of Nutlin-3a and Tenovin-6 in a microenvironment similar to an *in vivo* setting. Samples were divided into those that are completing cell cycle by measuring the G2/M phase versus those that do not. We measured significant reduction in the percentage of cells in the G2/M phase in Mel270 and OMM2.5 cell lines upon treatment with Tenovin-6, but not with Nutlin-3a treatment. We labeled the cells with Ki-67, as a confirmation of the detection of the growth fraction of the UM cells. Highest intensity of Ki-67 is shown in cells treated with HGF (Figure 2J, N, R). Two distinct features are observed in cells treated with either Nutlin-3a + HGF (Figure 2K, O, S) or Tenovin-6 + HGF (Figure 2L, P, T). First, we observed a reduction in the intensity of Ki-67 labeling in cells treated with either Nutlin-3a + HGF or Tenovin-6 + HGF. Additionally, we observed a reduction in the Nutlin-3a + HGF- and Tenovin-6 + HGF –treated cells compared to the untreated and HGF cells, confirming the results shown in Figure 2C-F.

Figure 2. *Tenovin-6 Increases UM Cell Death and Decreases Cell Proliferation*



A-B. Percentage cell growth inhibition, Mel 270, Mel 290, and OMM 2.5 UM cells were cultured in the presence of Nutlin-3a (A) or Tenovin-6 (B) at different concentrations ranging from 0.1-5.0 μM. Results represent 72 hrs time point. **C-D**. Bar graphs representing flow cytometry results of the percentage of PI+ UM cells after treatment with Nutlin-3a (C) or Tenovin-6 **D**. Drug concentrations ranging from 0.1-5.0 μM. (**E**, left)Scatter plot of the Brightfield Contrast versus the Area of the thresholded nucleus using FlowSight® imaging flow cytometer. (**E**, right) Representative images displayingthe non-apoptotic and apoptotic phenotypes. Apoptotic cells are defined as cells with low nuclear area and high bright field contrast. Quantitation of the percentage of apoptotic cells shown in F. Cell cycle progression analysis is shown in **G-H**. Percentage of cells in G0/G1-S (**G**) and G2/M (**H**) phases for each tested UM cell line. (**I-T**) Immuofluorescence analyses of Ki-67 immunopositivity in UM cell lines treated with Nutlin-3a + HGF and Tenovin-6 + HGF compared to untreated and HGF controls. Analyses performed in EVOS FLoid® Cell Imaging System at 20x. Ki-67 labeling shown in green channel (Alexa Fluor 488) and nuclei shown in blue (DAPI). The light settings, brightness and contrast were kept constant across all images. All results from bar graphs represent mean ±SEM, $^{**}p<0.005$; $^{***}p<0.0005$.

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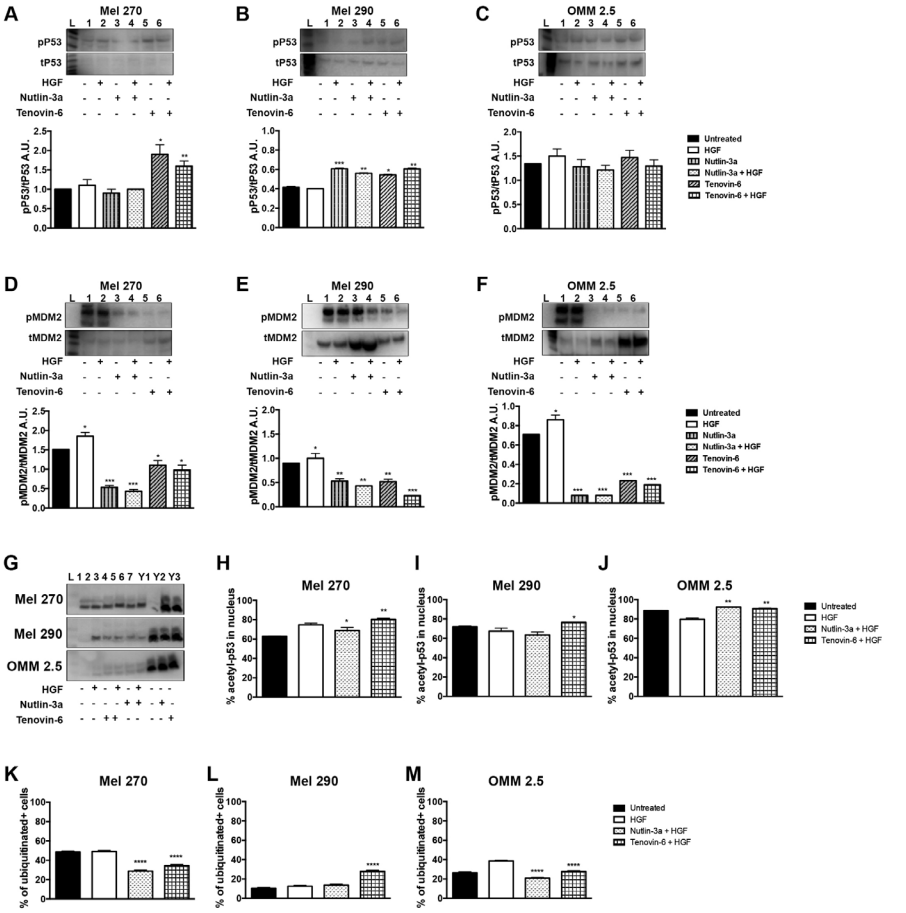
The next step in our investigation was to assess the effects of Nutlin-3a and Tenovin-6 on *TP53*-MDM2 signaling by Wb. Mel270 showed increased *TP53* signaling, defined as the ratio of phosphorylated-p53 over total p53, after treatment with Tenovin-6 (Figure 3A). Mel290 showed increase in *TP53* signaling upon treatment with Nutlin-3a or Tenovin-6 (Figure 3B). The metastatic cell line OMM2.5 exhibited no significant change in *TP53* signaling when treated either with Nutlin-3a or Tenovin-6 (Figure 3C). We measured MDM2 signaling using a similar approach. All investigated cell lines (Figure 3D-F) exhibited a significant reduction of MDM2 signaling after treatment with either Nutlin-3a or Tenovin-6.

Tenovin-6, Not Nutlin-3a, Increased *TP53* Acetylation and Interfered with MDM2-Mediated Ubiquitination

Acetylation of *TP53* enhances its DNA-binding activity *in vitro* and controls its stability. We evaluated acetylation of *TP53* by Wb analysis in Figure 3G and revealed enhancement across all tested UM cell lines. We used imaging flow cytometry to test the percentage

Figure 3. *Increase in the TP52-MDM2 Signaling Pathway in UM Cells by Nutlin-3a and Tenovin-6*



A-B. Wb results showing p53 signaling in Mel270 (**A**), Mel 290 (**B**), and OMM 2.5 cells (**C**). **D-F**. Wb analyses of MDM2 signaling in Mel270 (**D**), Mel 290 (**E**), and OMM 2.5 cells (**F**). Representative results of Wb showing acetylated p53. Lanes Y1-Y3 indicates Y79 Retinoblastoma cells, used as controls. (**H-M**) Quantitation analyses performed using imaging flow cytometry. Quantitation of the percentage of acetylated p53 us showing in **H-J**. Quantitation of the percentage of ubiquitination is shown in **K-M**. All results represent mean±SEM, *p<0.05, **p<0.005, ***p<0.0005, ****p<0.00005.

of acetylated *TP53* after treatment with Nutlin-3a or Tenovin-6. We measured an increase in acetyl *TP53* in all cell lines after treatment with Tenovin-6, as shown in Figure 3H-J. This enhancement in acetylated *TP53* is concomitant to a reduction in the percentage of ubiquitination, as demonstrated in Figure 3K-M.

DISCUSSION AND CONCLUSION

Our findings suggest that in patients with metastatic UM, de-regulation of the *TP53*-MDM2 signaling pathway promotes growth of the liver metastases and may be a novel target to reduce tumor cell survival. Two decades ago Tobal et al[17] reported the presence of abnormalities in the *TP53* gene in malignant choroidal melanomas. Jay et al[18] confirmed these findings by comparing choroidal melanomas with choroidal nevi. These studies paved the way for discrimination between mutations in the *TP53* pathway versus functional inactivation. Brantley et al[11] suggested inactivity of the *TP53*-MDM2 signaling pathway by immunohistochemical analysis of UM from enucleated eyes. While these investigations evolved, novel small molecule inhibitors against this signaling pathway were developed.[19,20] Inhibition of *in vivo* tumor growth in the B16F10 murine ocular melanoma model was demonstrated by concomitant administration of Topotecan and Nutlin-3a.[21] Despite the interesting findings, this has not yet been translated into a clinical application.

The *TP53* gene is considered the most frequently mutated gene in human cancer becoming the target for drug development efforts against cancer.[22] *TP53* undergoes post-transcriptional and post-translational modifications for its regulation and activation. Work by Kruse and Gu demonstrated post-translational modifications are imperative for *TP53*-dependent cell growth arrest and apoptosis to occur.[23] These modifications have effects in the stability and function of *TP53*. Among them, acetylation of *TP53* is indispensable for *TP53* transcriptional activity. *TP53* was the first non-histone substrate shown to be acetylated by histone acetyl transferases. The enhancement of *TP53* acetylation correlated with protein stabilization and activation in response to cellular stress. Under normal physiological conditions MDM2, by its E3 ubiquitin ligase, ubiquitinates *TP53* to the proteasome for degrada-

tion. Conversely,acetylation of MDM2 can inhibit *TP53* acetylation by suppressing the acetyltransferase activity and recruiting the histone deacetylase 1 (HDAC1) to *TP53*. Mammalian sirtuins are HDACs that play a role in chromatin regulation, cell survival under stress, metabolic homeostasis regulation, and developmental and cell differentiation. Bifunctional roles are attributed to sirtuins in cancer, as some can protect DNA from oxidative stress, maintaining genomic stability, while others are involved in tumorigenesis.[24]

The results of our work suggested de-regulation of *TP53* is not at the transcriptional level. We hypothesized de-regulation of the *TP53*-MDM2 complex, which favors the tumor, could be due to a post-translational modification of *TP53*. We tested this hypothesis by investigating the acetylation of *TP53*, which enhances its DNA-binding activity *in vitro* and controls its stability, using the sirtuin inhibitor Tenovin-6. While performing our series of investigations, Pan et al[25] reported the use of Tenovin-6 as an apoptosis inducer in UM cell lines. Our data confirmed Pan et al findings and addresses new questions that rise from this investigation. In our work, we investigated for the first time the *TP53*-MDM2 signaling pathway in an *in vivo* human UM orthotopic xenograft system. Our work revealed the specificity of the liver microenvironment for the accumulation of UM cells, but also revealed the presence, albeit small, of UM tumor cells in the spleen. We demonstrated the ratio of *TP53* to MDM2, is much higher in the spleen compared to the liver microenvironment, providing an explanation for the lack of clinically detectable metastases in the spleen. Additionally, we sampled the heterogeneity observed in metastatic UM, as demonstrated with a cohort of 5-samples taken from distant regions of a post-mortem human liver. We measured a dose-dependent apoptotic effect after Tenovin-6 treatment. In our work, we examined Tenovin-6 and Nutlin-3a as both are considered to activate *TP53* signaling: the former *via* transcriptional activation of *TP53* and the latter by inhibition of MDM2. Nutlin-3a induces cell death in UM cell lines. This effect was overcome when using Nutlin-3a in combination with HGF, an abundant protein in the liver microenvironment. Because of these data, it is clear that it is critically important to pre-clinically test these potential targets in a physiologically relevant model.

Prognosis for metastatic UM is poor with a 1-year overall mortality rate of 85%, and 2-year overall of 92%. Our limited understanding of the mechanisms underlying metastatic UM survival have led to the use of systemic therapies designed to target cutaneous melanoma, despite different genetic and molecular characteristics between the two cancers. Chemotherapeutics including, dacarbazine, temozolomide, cisplatin, treosulfan, and fotemustine, have failed in treatment of metastatic UM.[26,27] Immunotherapy has also shown limited activity in metastatic UM. Data from a Phase II clinical study investigating ipilimumab (NCT01355120) demonstrated limited activity in treatment-naïve and pre-treated patients with metastatic UM.[28] Pembrolizumab is being clinically tested (NCT02359851) in metastatic UM. A recent Phase III clinical study using selumetinib in combination with dacarbazine (NCT01974752) showed no improved clinical outcomes compared to dacarbazine alone. Therefore, we must continue pursuing this "holy grail".

We acknowledge the limitations of our study and these may serve as an initiation point for subsequent studies. Our *in vivo* animal system is an orthotopic xenograft using UM cell lines. Current efforts in generating patient-derived xenografts (PDX) are underway. Recently, Kageyama et al[29] reported an orthotopic PDX model where hepatic metastases are transplanted directly into the liver of immunocompromised mice. We must also be cognizant of the effects prior treatment may have had on modulating the metastases and microenvironment. However, we believe our results to be reliable as they were reproduced in both our xenograft and post mortem liver. Ultimately, our data suggests the de-regulation in the liver microenvironment is the result of both functional inactivation and inhibition of *TP53*.

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DATA AVAILABILITY STATEMENT

The chemical structure and compound summary of Nutlin-3A and Tenovin-6 are available in PubChem, https://pubchem.ncbi.nlm.nih.gov CIDs: 11433190 and 2477204, respectively.

ETHICS APPROVAL AND CONSENT

This study was approved by the Emory University School of Medicine Institutional Animal Care and Utilization Committee (IACUC) and The University of Tennessee Health Science Center Institutional Review Board (IRB).

AUTHORS CONTRIBUTIONS

ZKG, MWM, MWK: Performed experiments, data collection and analysis; KY, HY, QZ, SRC, BTG, BK, RPL, AL, NP, XDW: performed experiments; MMJ: participated in data interpretation and wrote the manuscript; HEG: conceived and designed the experiments, performed data analysis; VMT, MWW:conceived and designed the experiments, performed data analysis and supervised study. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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