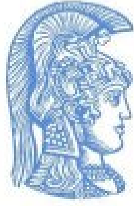


National and Kapodistrian University of Athens

Health Sciences Division

Medical School

1st Pathology Laboratory

Director: Professor Nikolaos Kavantzas, MD

Investigative analysis of the combined cellular expression of survivin and PTEN, in ophthalmic pterygium, with the application of liquid face cytology

Kallirroi V. Konstantopoulou, MD

Ophthalmic Surgeon

Athens 2022

Άδεια χρήσης ψηφιακού αρχείου:
CC BY-NC-SA

Εθνικό και Καποδιστριακό Πανεπιστήμιο Αθηνών

Τμήμα Επιστημών Υγείας

Ιατρική Σχολή

Α' Εργαστήριο Παθολογικής Ανατομικής

Διευθυντής : Καθηγητής Νικόλαος Καβαντζάς

Διερεύνηση της συνδυασμένης έκφρασης των survivin και PTEN σε αλλοιώσεις του επιπεφυκότα, επι πτερυγίου, με την εφαρμογή κυτταρολογίας υγρής φάσης

Καλλιρρόη Κωνσταντοπούλου

Χειρουργός Οφθαλμίατρος

Αθήνα 2022

Acknowledgments

For his valuable guidance at every step, towards the completion of this endeavor and his endless patience and contribution, I would like to thank my supervising professor, Nikolaos Kavantzas, MD.

Also, I would like to thank my co-workers on this project, who patiently and efficiently assisted me and guided me towards the realization of this laborious endeavor.

Dr Evaggelos Tsiambas MD, for the meticulous and expert analysis of the samples, that allowed me to reach conclusions and support this thesis and Mr George Vilaras for, without his excellent processing of the samples, it would have been impossible to draw any results.

Furthermore, I would like to thank Mr Sotirios Maipas, for the diligent and thorough evaluation of the manuscript, leading me to the fruition of a presentable and readable essay.

Finally, I would like to thank professor Efstathios Patsouris and professor Andreas Lazaris for their continuous assistance and support, through the years.

Curriculum Vitae

Name: Kallirroi Konstantopoulou

Address: Cholargos, Athens, Greece

Date of Birth: 02/10/69, Athens

Nationality: Greek

E-Mail: kalikonsta@yahoo.gr

Elementary studies: Papagou Gymnasium-Lyceum 1982-1988

Foreign languages: -English, proficiency certification of Cambridge University

-French, French language certificate, Sorbonne University

Graduate studies: Medical School of Aristotle University of Thessaloniki, 1989-1995

Medical specialty: Ophthalmology, 25/1/05

Member of the Medical Association of Athens, 1995-2009

Member of the Medical Association of England, 1998-2008

Member of the Medical association of Kalamata, 2010- today

Member of the Hellenic Ophthalmological Society, January 2005 – today

Clinical experience:

-02/19 - today, NHS Consultant, Ophthalmology Department of the Army Fund General Hospital, NIMTS 417

-03/08 - 01/19, NHS Consultant, Ophthalmology Department of the General Prefectural Hospital of Kalamata, Messinia

-03/06 - 03/08, with scholarship of the State Scholarship Foundation, clinical research in Ophthalmology specializing in intraocular inflammation and in

external diseases, Uveitis and External Diseases Clinics Moorfields Eye Hospital, London, England

-03/05 - 01/06, Locum Consultant, Ophthalmology Department of the General Prefectural Hospital of Kalamata, Messinia

-03/03 - 11/04, Specialist Registrar, 2nd Ophthalmology Department, "Red Cross" Hospital ", Athens

-08/00 - 08/02, Senior House Officer in Ophthalmology, Ophthalmology Department, Countess of Chester Hospital, Chester, England

-05/00 - 08/00, locum Senior House Officer in Ophthalmology, Queens Medical Center University Hospital, Nottingham, England

-9/98 - 11/99, Senior House Officer in Ophthalmology, Ophthalmology Department of Children's Hospital, Penteli, Athens

-9/95 - 6/98, Rural service, Valira primary clinic, General Prefectural Hospital of Kalamata, Messinia

Surgical experience:

- strabismus surgeries
- catheterization of the nasolacrimal duct
- ocular perforation injuries
- extracapsular cataract removal
- trabeculectomy
- posterior core vitrectomy
- penetrating wounds
- minor eyelid surgery, entropion and ectropion correction
- tumors and cysts removal
- YAG laser peripheral iridotomies, posterior capsulotomies, xanthelasmas
- Argon laser photocoagulations

Scientific experience:

Clinical Research in Moorfields Eye Hospital, London, U.K., with State scholarship from the Greek Foundation of State Scholarships, for the purpose of clinical research on the subject of ocular inflammation, post obtained after successfully passing the examinations, in April 2005 1st for one post, 08/06/2005

Research studies

1. Inflammatory Cystoid Macular Oedema and Sodium Pegaptanib as treatment option

2. The use of Laser Flaremeter and differences in the evaluation of inflammation of the anterior chamber by the examiners, in relation to the device

3. Serpiginous Choroidopathy, Eales disease and Ocular Tuberculosis

Publications

in Greek magazines: 2

in foreign language magazines: 5

Announcements, in conferences, workshops: 22

Educational experience:

Postgraduate training/specialization, at Moorfields Eye Hospital London. Commencement 03/06, as clinical research fellow at ocular inflammations, with scholarship of the Greek State Scholarship Foundation. Participation in clinics and surgery, respectively. Research projects and in-hospital classes, twice a week, training courses, bibliographic information, audits.

Participation in training courses, during my specialty (SHO) training in England, twice a week and in Greece with the SHO annual program of lessons, of the University Ophthalmology Department of Athens, the clinical lessons at the Red

Cross Hospital and responsible of the SHO training program, at the hospital of Kalamata.

Seminars-Courses: 28

Participation in Conferences: 29

ΙΠΠΟΚΡΑΤΕΙΟΣ ΟΡΚΟΣ
ΚΕΙΜΕΝΟ

ΟΜΝΥΜΙ ΑΠΟΛΛΩΝΑ ΙΗΤΡΟΝ ΚΑΙ ΑΣΚΛΗΠΙΟΝ ΚΑΙ ΥΓΕΙΑΝ ΚΑΙ ΠΑΝΑΚΕΙΑΝ ΚΑΙ ΘΕΟΥΣ ΠΑΝΤΑΣ ΤΕ ΚΑΙ ΠΑΣΑΣ ΙΣΤΟΡΑΣ ΠΟΙΕΥΜΕΝΟΣ, ΕΠΙΤΕΛΕΑ ΠΟΙΗΣΕΙΝ ΚΑΤΑ ΔΥΝΑΜΙΝ ΚΑΙ ΚΡΙΣΙΝ ΕΜΗΝ ΟΡΚΟΝ ΤΟΝΔΕ ΚΑΙ ΞΥΓΓΡΑΦΗΝ ΤΗΝΔΕ. ΗΓΗΣΕΣΘΑΙ ΜΕΝ ΤΟΝ ΔΙΔΑΞΑΝΤΑ ΜΕ ΤΗΝ ΤΕΧΝΗΝ ΤΑΥΤΗΝ ΙΣΑ ΓΕΝΕΤΗΣΙΝ ΕΜΟΙΣΙ, ΚΑΙ ΒΙΟΥ ΚΟΙΝΩΣΕΣΘΑΙ ΚΑΙ ΧΡΕΩΝ ΧΡΗΙΖΟΝΤΙ ΜΕΤΑΔΟΣΙΝ ΠΟΙΗΣΕΣΘΑΙ ΚΑΙ ΓΕΝΟΣ ΤΟ ΕΞ ΑΥΤΟΥ ΑΔΕΛΦΕΟΙΣ ΙΣΟΝ ΕΠΙΚΡΙΝΕΕΙΝ ΑΡΡΕΣΙ ΚΑΙ ΔΙΔΑΞΕΙΝ ΤΗΝ ΤΕΧΝΗΝ ΤΑΥΤΗΝ, ΗΝ ΧΡΗΙΖΩΣΙ ΜΑΝΘΑΝΕΙΝ, ΑΝΕΥ ΜΙΣΘΟΥ ΚΑΙ ΞΥΓΓΡΑΦΗΣ ΠΑΡΑΓΓΕΛΙΗΣ ΤΕ ΚΑΙ ΑΚΡΟΗΣΙΟΣ ΚΑΙ ΤΗΣ ΛΟΙΠΗΣ ΑΠΑΣΗΣ ΜΑΘΗΣΙΟΣ ΜΕΤΑΔΟΣΙΝ ΠΟΙΗΣΕΣΘΑΙ ΥΙΟΙΣΙ ΤΕ ΕΜΟΙΣΙ ΚΑΙ ΤΟΙΣΙ ΤΟΥ ΕΜΕ ΔΙΔΑΞΑΝΤΟΣ ΚΑΙ ΜΑΘΗΤΑΙΣΙ ΞΥΓΓΕΓΡΑΜΜΕΝΟΙΣ ΤΕ ΚΑΙ ΩΡΚΙΣΜΕΝΟΙΣ ΝΟΜΩ ΙΗΤΡΙΚΩ ΑΛΛΩ ΔΕ ΟΥΔΕΝΙ. ΔΙΑΙΤΗΜΑΣΙ ΤΕ ΧΡΗΣΟΜΑΙ ΕΠ' ΩΦΕΛΕΙΗ ΚΑΜΝΟΝΤΩΝ ΚΑΤΑ ΔΥΝΑΜΙΝ ΚΑΙ ΚΡΙΣΙΝ ΕΜΗΝ, ΕΠΙ ΔΗΛΗΣΕΙ ΔΕ ΚΑΙ ΑΔΙΚΙΗ ΕΙΡΞΕΙΝ. ΟΥ ΔΩΣΩ ΔΕ ΟΥΔΕ ΦΑΡΜΑΚΟΝ ΟΥΔΕΝΙ ΑΙΤΗΘΕΙΣ ΘΑΝΑΣΙΜΟΝ, ΟΥΔΕ ΥΦΗΓΗΣΟΜΑΙ ΞΥΜΒΟΥΛΙΗΝ ΤΟΙΗΝΔΕ ΟΜΟΙΩΣ ΔΕ ΟΥΔΕ ΓΥΝΑΙΚΙ ΠΕΣΣΟΝ ΦΘΟΡΙΟΝ ΔΩΣΩ. ΑΓΝΩΣ ΔΕ ΚΑΙ ΟΣΙΩΣ ΔΙΑΤΗΡΗΣΩ ΒΙΟΝ ΤΟΝ ΕΜΟΝ ΚΑΙ ΤΕΧΝΗΝ ΤΗΝ ΕΜΗΝ. ΟΥ ΤΕΜΕΩ ΔΕ ΟΥΔΕ ΜΗΝ ΛΙΘΙΩΝΤΑΣ, ΕΚΧΩΡΗΣΩ ΔΕ ΕΡΓΑΤΗΣΙΝ ΑΝΔΡΑΣΙΝ ΠΡΗΞΙΟΣ ΤΗΣΔΕ. ΕΣ ΟΙΚΙΑΣ ΔΕ ΟΚΟΣΑΣ ΑΝ ΕΣΙΩ, ΕΣΕΛΕΥΣΟΜΑΙ ΑΠ' ΩΦΕΛΕΙΗ ΚΑΜΝΟΝΤΩΝ, ΕΚΤΟΣ ΕΩΝ ΠΑΣΗΣ ΑΔΙΚΙΗΣ ΕΚΟΥΣΙΗΣ ΚΑΙ ΦΘΟΡΙΗΣ ΤΗΣ ΤΕ ΑΛΛΗΣ ΚΑΙ ΑΦΡΟΔΙΣΙΩΝ ΕΡΓΩΝ ΕΠΙ ΤΕ ΓΥΝΑΙΚΕΙΩΝ ΣΩΜΑΤΩΝ ΚΑΙ ΑΝΔΡΕΙΩΝ, ΕΛΕΥΘΕΡΩΝ ΤΕ ΚΑΙ ΔΟΥΛΩΝ. Α Δ' ΑΝ ΕΝ ΘΕΡΑΠΕΙΗ Η ΙΔΩ Η ΑΚΟΥΣΩ, Η ΚΑΙ ΑΝΕΥ ΘΕΡΑΠΕΙΗΣ ΚΑΤΑ ΒΙΟΝ ΑΝΘΡΩΠΩΝ, Α ΜΗ ΧΡΗ ΠΟΤΕ ΕΚΛΑΛΕΕΣΘΑΙ ΕΞΩ, ΣΙΓΗΣΟΜΑΙ, ΑΡΡΗΤΑ ΗΓΕΥΜΕΝΟΣ ΕΙΝΑΙ ΤΑ ΤΟΙΑΥΤΑ. ΟΡΚΟΝ ΜΕΝ ΟΥΝ ΜΟΙ ΤΟΝΔΕ ΕΠΙΤΕΛΕΑ ΠΟΙΕΟΝΤΙ ΚΑΙ ΜΗ ΞΥΓΧΕΟΝΤΙ ΕΙΗ ΕΠΑΥΡΑΣΘΑΙ ΚΑΙ ΒΙΟΥ ΚΑΙ ΤΕΧΝΗΣ, ΔΟΞΑΖΟΜΕΝΩ ΠΑΡΑ ΠΑΣΙΝ ΑΝΘΡΩΠΟΙΣ ΕΣ ΤΟΝ ΑΙΕΙ ΧΡΟΝΟΝ ΠΑΡΑΒΑΙΝΟΝΤΙ ΔΕ ΚΑΙ ΕΠΙΟΡΚΕΟΝΤΙ, ΤΑΛΑΝΤΙΑ ΤΟΥΤΕΩΝ.

For my parents and my husband Nikos,

and all those, who made it worthwhile

Application for PhD candidacy Date: 06/06/2013

Nomination of Advisory Committee Date: 12/06/2013

Advisory Committee:

Professor Nikolaos Kavantzas, MD (Supervising)
Professor Andreas Lazaris, MD
Professor Efstathios Patsouris, MD

Submission Date of Thesis Proposal: 27/01/2014

Nomination of Examination Committee Date: 22/12/2021

Examination Committee:

Professor Nikolaos Kavantzas, MD (Supervising)
Professor Andreas Lazaris, MD
Professor Efstathios Patsouris, MD
Professor Ioannis Panagiotidis, MD
Associate Professor Afrodite Nonni, MD
Associate Professor Xarikleia Gakiopoulou, MD
Assistant Professor Irene Thymara, MD

CONTENTS

PREFACE 1

PART I 2

1. Embryology 2

2. The conjunctiva 4

   2.1 Anatomy of the conjunctiva 4

   2.2 Histology of the conjunctiva 5

3. The cornea 8

   3.1 Anatomy of the cornea 8

   3.2 Histology of the cornea 9

4. Degenerations of the conjunctiva and the corneoscleral coat 13

   4.1 Vogt's white limbal girdle 13

   4.2 Terrien's marginal degeneration 14

   4.3 Spheroidal degeneration (climatic droplet keratopathy, Labrador keratopathy) 15

   4.4 Salzmann's nodular degeneration 16

   4.5 Senile Scleral Plaques 17

   4.5 Pingueculae 18

   4.6 Pseudopterygium 20

5. Pterygium 21

   5.1 Incidence and Prevalence 21

   5.2 Clinical Features 22

   5.3 Clinical Course 23

   5.4 Associated Findings and Symptoms 25

   5.5 Histopathology 26

   5.6 Pathogenesis 30

5.7 Differential Diagnosis 39
5.8 Treatment 40
5.9 Adjunctive Therapy 45
6. Biomarkers 46
6.1 Survivin 48
6.2 PTEN 61
6.3 p53 78
7. Principles of Immunocytochemistry 100
7.1 Immunoperoxidase method 104
7.2 Avidin-Biotin-Peroxidase (ABC) Complex Method 105
7.3 Alkaline phosphatase method 107
7.4 One- or two-step method using polymer dextran (EPOS or two- step method- Dako En Vision+ dextran technology) 107
8. Principles of digital analysis of microscopic image 109
PART II 111
9. Purpose of the research study 111
10. Materials and Methods 112
10.1 Origin-Collection-Management of cytological material 112
10.2. Laboratory study algorithm 113
10.3 Epidemiological-Clinical pathological features of study material 113
10.4 Cytochemical process 114
10.5 Immunocytochemistry protocol 114
10.6 Digital Image Analysis Protocol 116
11. Results – Statistical Analysis 118
11.1 Descriptive Analysis 118
11.2 Statistical Tables 118
12. Discussion 129

13. Conclusions....144

14. Abstract....146

15. Περίληψη....147

16. References - Bibliography....149

IMAGES INDEX

Figure 1 Ocular development ... 2

Figure 2 Anatomy of the conjunctiva ... 5

Figure 3 Anatomy of the Cornea ... 8

Figure 4 Voght's limbal girdle ... 14

Figure 5 Terrien's Marginal Degeneration ... 15

Figure 6 Spheroidal degeneration ... 16

Figure 7 Salzmann's nodular degeneration ... 17

Figure 8 Senile plaques ... 18

Figure 9 Pingueculum ... 19

Figure 10 Pseudopterygium ... 20

Figure 11 Nasal pterygium encroaching over the cornea ... 22

Figure 12 Bilateral pterygium ... 23

Figure 13 Atrophic- Flat pterygium ... 25

Figure 14 Elastotic degeneration of collagen in the vascularized subepithelial stroma ... 29

Figure 15 Squamous cell carcinoma ... 40

Figure 16 Apoptosis pathways ... 48

Figure 17 Survivin isoforms ... 52

Figure 18 Localization of Survivin ... 53

Figure 19 Role of Survivin in mitosis ... 54

Figure 20 PTEN loss ... 65

Figure 21 The multiple actions of PTEN ... 69

Figure 22 Role of PTEN in immune response ... 75

Figure 23 p53 structure ... 78

Figure 24 p53 activation responses ... 84

Figure 25 p53 mediated metabolism control ... 88

Figure 26 Computerized image analysis of a case of survivin protein expression objective evaluation. ... 117

TABLE INDEX

Table 1 Immunohistochemical staining criteria........ 116

Table 2 Measurement values of protein expression........ 118

Table 3 Classification according to anatomical location and pterygium morphology 121

Table 4 p53 vs PTEN........ 123

Table 5 p53 vs Control/Lesion........ 124

Table 6 p53 vs Survivin........ 125

Table 7 PTEN vs Survivin........ 126

Table 8 Survivin vs Peripheral-Central........ 127

Table 9 PTEN vs Fleshy / Flat........ 127

Table 10 Total results of the examined markers and also p values........ 128

PREFACE

The purpose of this doctoral thesis was presented first as an idea, in 2013. Two main reasons exist, that are responsible for the formation of this research. The first reason is the extremely common complaint of ocular pterygium in the Greek population, especially in agricultural communities. The second reason, is the recent advent of biological therapy options.

Pterygium, identified as a benign growth of the conjunctiva, has a high prevalence that can rise up to 40% in countries, closer to the equator. So far, it exhibits poor response to conservative treatment with a high recurrence rate, after a surgical approach.

On the other hand, biologic agents have the ability to control tissue growth, with fewer adverse reactions, compared to traditional cancer treatment. Biological drugs are a diverse category of complex molecules produced through biotechnology in a living system, such as a microorganism, plant cell, or animal cell.

Understanding the etiopathogenesis and the various factors involved in this particular lesion, may allow advances on therapeutic strategies to prevent its onset and/or progression and may even result in less surgical manipulation, in the future. Although numerous studies have already been conducted, important genes and proteins have not yet, been fully explored. In this sense, additional research to better understand the mechanisms that lead to the appearance of pterygium, could promote more targeted and effective treatment options. Especially in recurrent cases, that are so far, proven to be extremely challenging, to treat.

The aim therefore, of this study is to investigate possible pathophysiologic mechanisms, that could explain pterygium's behavior, its striking similarities to tumor histopathology and characteristics. Furthermore, this study could identify a possible link between the disease and newer therapeutical approaches, justifying their use for pterygium treatment, aiming for a higher success rate.

PART I

1. Embryology

The eye is formed from ectoderm and mesenchyme, and its formation begins early in the fourth week of intrauterine life from diencephalon. The rudimentary eyeball develops from an ectodermal diverticulum from the lateral aspect of the forebrain. The diverticulum grows out laterally to the side of the head and the end becomes enlarged to form the optic vesicle, and the proximal side becomes constricted to form the optic stalk [1].

The neural tube gives rise to the ectoderm that later forms the retina, optic nerve and smooth muscle of the iris. The surface ectoderm at the side of the head gives rise to the corneal and conjunctival epithelium, the lacrimal and tarsal glands and the lens [1].

A small area of surface ectoderm above the optic vesicle thickens to form the lens placode, which sinks under the surface ectoderm, to form the lens vesicle. The optic vesicle invaginates to produce the double-layered optic cup (Figure 1). The inferior edge of the optic cup is deficient and this notch is continuous with a groove at the inferior margin of the optic stalk, called the optic choroidal fissure [2].

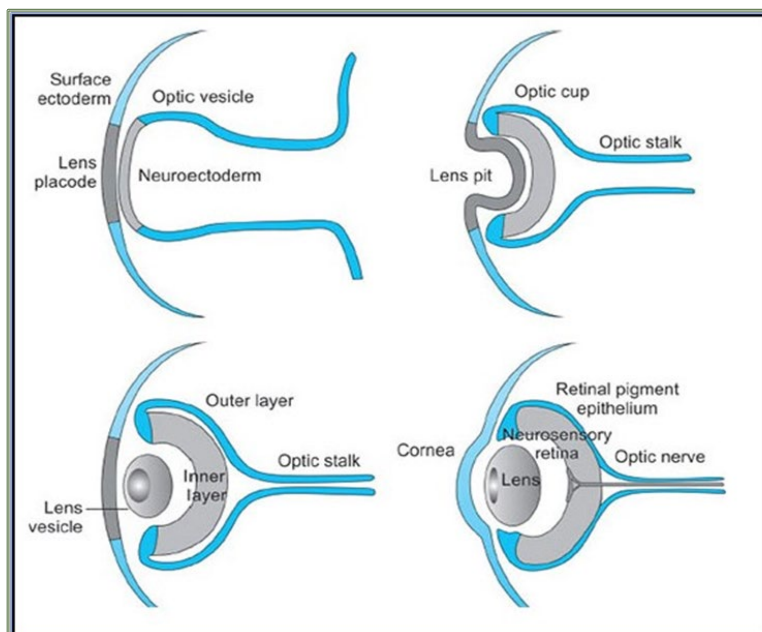


Figure 1 Ocular development

The mesenchyme forms corneal stroma, sclera, choroid, iris, ciliary musculature, the vitreous body. The origin of the corneal endothelium is uncertain and thought to be of the neural crest. Vascular mesenchyme grows into the optic fissure and forms the hyaloid artery. This fissure by the seventh week of embryonic growth closes and forms a narrow tube inside the optic stalk, the optic canal [2].

By the fifth week, the lens vesicle separates from the surface ectoderm and lies within the entrance of the optic cup, the edges of which will form the pupil.

The extraocular muscles and the orbital bones are formed from the mesenchyme in the region of the developing eyeball, the orbicularis muscle from the second pharyngeal arch, while the eyelids form as folds of surface ectoderm above and below the developing cornea with their core connective tissue from mesenchyme, as well. The eyelids remain fused until the seventh month of gestation [1, 2].

2. The conjunctiva

2.1 Anatomy of the conjunctiva

Conjunctiva is a thin mucous membrane that overlays the inner surface of the eyelids and is spread along the superior and inferior fornices onto the anterior surface of the eyeball. The conjunctiva epithelium is continuous with the epidermis of the skin at the lid margin and with the corneal epithelium at the limbus.

The conjunctiva can be divided anatomically to the palpebral conjunctiva, the conjunctival fornices and the bulbar conjunctiva [3].

The palpebral part of the conjunctiva is firmly attached to the posterior surface of the tarsal plates of the eyelids.

The conjunctival fornices form the transition areas between the palpebral and the bulbar conjunctiva. The fornices are loosely attached to the underlying fascial expansions of the extraocular muscles sheath. Contraction of the muscles can pull on the conjunctiva, so that it can move with the eyelids and the eyeball. The ducts of the lacrimal glands open into the lateral part of the superior fornix [3].

The superior fornix is situated about 10mm from the limbus and the inferior about 8mm, from the limbus. Medially, the fornices are absent and replaced by the caruncle and plica semilunaris. On the lateral side of the eye, the fornices are deeper and lie about 14mm from the limbus, extending posterior to the eyeball equator [4].

The bulbar conjunctiva lines the eyeball (Figure 2). It is thin, translucent, and loosely attached by connective tissue to the underlying visible white sclera. The bulbar conjunctiva is also attached to the fascia covering the extraocular muscles. At the corneoscleral junction, the conjunctiva is more firmly attached to the underlying connective tissue and the sclera [4].

The ring formation produced from the fusion of the cornea and conjunctiva is called the conjunctival limbus. This is where the pathological pterygial tissue is formed. It is 1mm anterior to the corneoscleral limbus, where the fusion of the

cornea and sclera occurs. The bulbar sheath of connective tissue fuses with the sclera 1,5 mm posterior to the corneoscleral limbus [2].

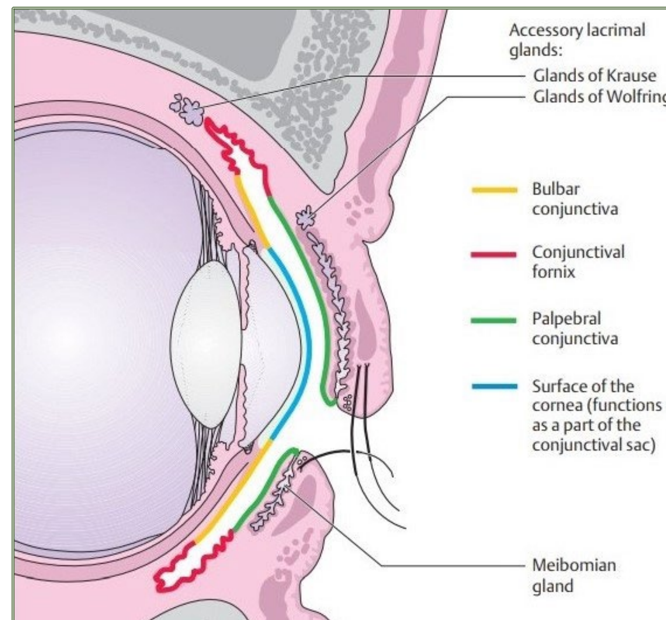


Figure 2 Anatomy of the conjunctiva

2.2 Histology of the conjunctiva

The conjunctiva is composed of two layers, the epithelium and the lamina propria also described, as stroma [4].

Epithelium is a nonkeratinized stratified columnar epithelium with admixed columnar goblet cells. It consists of two to five layers of epithelial cells resting on the substantia propria, the loose underlying connective tissue. At the limbus, the columnar cells convert to squamous cells. Also, at the limbus, the lamina propria displays papillae that are not seen anywhere else on the conjunctiva; this formation is called the palisades of Vogt. The thickness of the conjunctival epithelium varies regionally. The flattened surface cells demonstrate many microvilli and they are coated by a glycocalyx and mucin [4].

Although, conjunctival epithelial cells can slide over to corneal defects, and transdifferentiate to normal corneal epithelial cells, the ability of the corneal epithelium to heal and maintain a normal cycle of corneal epithelial cell turnover depends on the presence of epithelial stem cells at the limbus, represented by areas of thickened epithelium between the palisades of Vogt. Damage to the limbal

epithelium from disease or mechanical injury can significantly alter the healing process [5].

On the posterior border of the lid margin, the conjunctiva fuses with the skin along the posterior margin of the tarsal glands. Here, the nonkeratinized conjunctival squamous epithelium fuses with the keratinized squamous epithelium of the epidermis [6].

Goblet cells constitute approximately 10 percent of the conjunctival epithelial cell population. They are scattered along the surface of the conjunctiva, being more numerous in the fornices, plica semilunaris and caruncle, especially inferonasally. They secrete mucin, a hydrophilic protein, that is dispersed on the surface, thus decreasing the surface tension of the tear film. This allows for the tears to be evenly distributed on the ocular surface [6].

Type 1 goblet cells are found in the forniceal conjunctiva and secretion is by an apocrine method and type 2 goblet cells are found diffusely throughout the conjunctiva and secrete by a merocrine function, with discharge of the product from an intact cell.

The conjunctival epithelium, contains also, nonepithelial cells similar to those in the skin. Melanocytes are present basally and Langerhans cells are scattered throughout. Lymphocytes are also present and cytotoxic/suppressor T cells (CD8 cells) dominate [2].

The substantia propria of the conjunctiva plays a significant role in the ocular immune mechanisms. The lymphocytic population here is predominantly helper T cells. However, the number of suppressor T cells may increase in disease states. Scattered B cells and macrophages are present. Lymphocyte population increases with age. Langerhans cells are not present and mast cells increase significantly in allergy [6].

Situated in the connective tissue, there are accessory lacrimal glands, that are found throughout the conjunctival sac and their ducts open to the free surface of the conjunctiva. In the deeper parts of the submucosa, there are blood vessels, nerves and smooth muscle. The lamina propria projects toward the surface epithelium at the lid margins to form conjunctival papillae [2, 6].

The arterial blood supply of the conjunctiva is from the anterior ciliary arteries and the two palpebral arches, branches of the ophthalmic artery and the inner carotid artery. The conjunctival veins are more numerous, they accompany the arteries and merge into the palpebral veins or directly drain into the superior and inferior ophthalmic veins.

The conjunctival lymph vessels are arranged as a superficial and a deep plexus in the substantia propria. Those from the lateral side, drain into the superficial parotid nodes and those from the medial side pass to the submandibular nodes [4].

3. The cornea

3.1 Anatomy of the cornea

The cornea is the transparent convex fibrous structure that forms the anterior one sixth of the eyeball. It serves as a light refractive medium and a mechanical and chemical barrier between the eye and the environment. Corneal curvature is greater than the rest of the eyeball, for this reason, there is a slight sulcus, where the cornea joins the sclera., the corneoscleral sulcus. When seen from the posterior surface, the cornea appears spherical but the anterior surface is in effect slightly elliptical in shape. Although the dimensions vary from one person to the other, the average anterior horizontal diameter is 11.9mm and the vertical one is about 10.9mm, whereas the posterior diameter of the cornea measures around 11.7mm. The radius of the anterior corneal surface is about 7.7mm and that of the posterior surface is 6.9mm. However, the vertical plane is more curved than the horizontal, giving rise to the phenomenon of astigmatism. The cornea is thinner in the center with a thickness of 0.5 - 0.6mm and is thicker at its periphery, measuring around 0.7mm [6].

The cornea is the main refractive anatomical structure of the eye and its anterior surface provides approximately 48 diopters of power, it consists of five layers (Figure 3). From the front to the back, they are: the Epithelium, the Bowman's layer, the Stroma, the Descemet's membrane and the Endothelium.

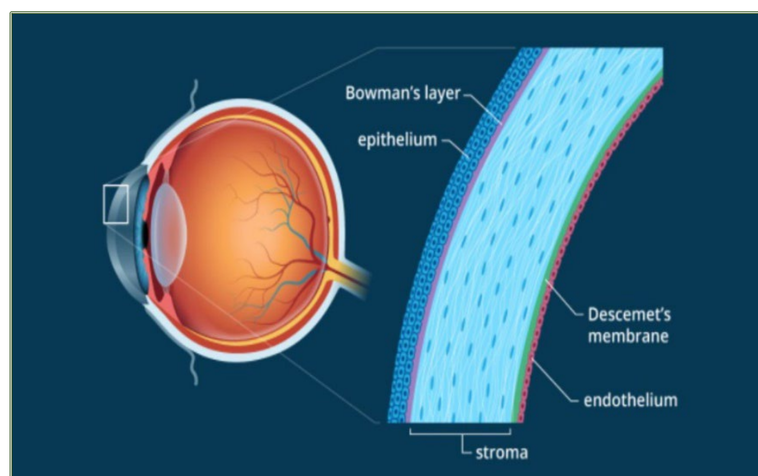


Figure 3 Anatomy of the Cornea

Although not part of the cornea, the preocular tear film is intimately associated with the cornea and the conjunctiva, anatomically and functionally. The surface of the cornea must be kept moist to prevent damage to the epithelium. Moisture and smoothing of the ocular surface are provided by the tear film, in conjunction with the spreading function of the eyelids, during blinking. The tear film consists of two structurally identifiable layers. A thin anterior lipid layer derived from meibomian gland secretions that delays evaporation of the preocular tear film and prevents thus drying, between blinks. And a posterior thicker aqueous layer into which a mucin rich glycocalyx extends. The aqueous component is mainly produced by the lacrimal gland, situated in the superior temporal orbit, at a rate of 1.2μl/min. tears leave the eye by evaporation, conjunctival absorption and flow through the punctae and canaliculi, to the nasolacrimal duct and its opening at the inferior nasal meatus. Although the ocular surface is a good barrier against diffusion of polarized or physically large substances, water can move, within limits, back and forth between the ocular interior and the tears, which can regulate the effects of evaporation to some extent. The rate of tear evaporation is approximately 3μl/hr. at 30 percent relative humidity and is significantly reduced in dry environment. The tear film mucin is secreted by conjunctival goblet cells. Mucin forms links with the epithelial surface glycocalyx, forming a several microns thick hydrophilic coating on the cornea, rather than interacting directly with epithelial surface membranes [1].

3.2 Histology of the cornea

The corneal epithelium is stratified squamous epithelium. It presents four to six layers of cells and comprises the 10 percent of the corneal thickness. It is divided morphologically into three layers. The superficial flattened squamous cell layer, the middle or wing cell layer and the deep or basal columnar cell layer. The basal cells are the only corneal epithelial cells that undergo mitosis. The cells thus formed, push anteriorly and change their shape, conforming to the surrounding wing cells. As the cells continue to move anteriorly, to become the superficial cells and eventually disintegrate and are shed into the tear film, in a process known as desquamation. The cell turnover is approximately every seven days. At the limbus

the corneal epithelium transforms into transitional epithelium and then into conjunctival epithelium [6].

Two layers of superficial cells are usually seen. The cells are polygonal in shape and 40 to 60μm in diameter. Their role is to maintain the tear film and form a barrier between the cornea and the environment. These cells possess microscopic projections that extend into the tear film, termed microvilli, reticulations and microplicae. Their outer surface is thickened and supports an extensive fibrillar glycocalyx layer that increases the adherence of the tear film mucin layer [6].

An important property of the superficial cells is the tight junctions that surround every cell and bind it to the adjacent ones and resist the flow of fluids through the epithelial surface. Therefore, if aqueous humor passes through the endothelium into the stroma, it gets trapped within the epithelium, resulting in oedema [6].

The middle wing cells are polyhedral in shape, with convex anterior surfaces and concave posterior surfaces. They are joined together by desmosomes and at their periphery they are extremely elongated and interdigitated. Numerous gap junctions permit free intercellular communication [2].

The deepest basal cells are tall, columnar and form a single layer resting on a basement membrane. They represent the germinal layer of the epithelium. Their lateral margins interdigitate with one another and are attached by desmosomes and gap junctions. Their posterior surface is flat attached to the basement membrane by hemidesmosomes that tether to the stroma by anchoring fibrils, passing through the basement membrane and the Bowman's layer [6].

Bowman's layer lies immediately beneath the epithelial basement membrane, measures about 8 to 12 μm in thickness, it is acellular and consists of randomly arranged collagen fibrils that merge to the more organized anterior stroma. It functions as a dome shaped structure that is anchored to the limbus. It offers resistance to changes of the corneal shape, hence it is impossible to change the anterior surface curvature of the cornea without first cutting through it [1].

The stroma takes up 90 percent of the corneal thickness, it is transparent, fibrous and compact. It consists of many lamellae of collagen fibrils neatly stacked, that run parallel to the surface. The arrangement of fibrils is more regular in the

posterior stroma. The parallel fibrils are mainly type 1 collagen with smaller amounts of type 3, 5 and 7. It is calculated that there are 250 to 500 flattened lamellae, each of which is about 2μm thick. The direction of the collagen fibrils in any given lamella is the same, but they run at roughly right angles to those of adjacent lamellae. The lamellae are bound together by fibrils that pass between them. Collagen is approximately the 70 percent of the dry weight of the cornea and is the structural macromolecule that provides tissue transparency and mechanical resistance to intraocular pressure. The collagen fibrils are embedded in glycosaminoglycans of the keratan sulfate and the chondroitin sulfate type, which occur in a ratio of 3:1. Lying between the lamellae are flattened fibroblasts called keratocytes, occasional macrophages, lymphocytes and polymorphonuclear leukocytes. Keratocytes occupy 3-5 percent of the stromal volume, their function is to sustain the collagen fibrils and extracellular matrix by a continuous synthetic activity. These cells present extensive processes that span the lamellae, but usually they do not extend out of the lamellae, instead they form tight junctions with their neighboring keratocytes. Keratocytes undergo extensive cellular transformation in response to wounding or acute oedema, entering a fibroblastic state. At this state, their processes become less numerous, but in the vicinity of the wound, new keratocytes extend toward the damaged area [4].

Descemet's membrane is the thick basal lamina formed by the endothelium. It lies just posterior to the stroma. It is strong and homogeneous and measures 10μm in thickness. It is sharply demarcated by the stroma and is thicker than the endothelium. It is composed of two layers. The anterior one is a banded layer that is produced during fetal development and contains banded collagen and the posterior layer that is nonbanded and is produced by the endothelium throughout life. It consists mainly of type 4 collagen arranged in a hexagonal pattern, embedded in matrix. Descemet's membrane increases in thickness during life but this accumulation is limited to the posterior layer. It is also, loosely attached to the stroma and tends to separate easily and curl up if incised. If endothelial cells are stimulated to produce excessive amount of basal lamina material, focal thickenings occur that are called guttae and occur with age at the corneal periphery, known as Hassal-Henle bodies. If these guttae are seen in the central area, they are indicative of endothelial cell dysfunction [1].

The endothelium forms a single layer of flattened cells, that are hexagonal in shape and whose plasma membranes interdigitate with one another. The cell cytoplasm contains numerous mitochondria, prominent endoplasmic reticulum and a Golgi apparatus, indicating that the endothelium is actively involved in the synthesis and transport of fluid. The cells maintain tight apposition with their neighboring cells. They coat each other with elaborate interdigitations, such that the paracellular pathway is at least 10 times the thickness of the cells. Specific tight junctional complexes are located near the apical membrane of the cells, close to the anterior chamber and their free surfaces show a few microvilli. These cells play a major role in controlling normal hydration of the cornea, both by a barrier function that regulates access of water from the aqueous humor to the corneal stroma and by an active transport mechanism. They cover the posterior surface of the Descemet's membrane and are continuous with the endothelial cells that line the iridocorneal angle and the anterior surface of the iris [2].

Corneal endothelium cells are of neural crest origin and like the central nervous tissue, are essentially amitotic after birth. However, they have the remarkable ability to enlarge and maintain normal function in the face of cellular inadequacies or deficiencies. Endothelial cell density at birth ranges from 3.500 to 4.000 cells/mm^2, in adulthood it is between 1.400 to 2.500 cells/mm^2 and they can maintain corneal clarity even with a population of 1.000 cells/mm^2 [6].

When endothelial cells are lost, the remaining cells may lose their hexagonal shape and become irregular in shape (pleomorphism) and size (polymegathism).

The cornea is avascular and devoid of lymphatic drainage. The capillary blood vessels from the anterior ciliary arteries of the conjunctiva and sclera end at the circumference of the cornea. The cornea is nourished by diffusion from the aqueous humor and the capillaries at its edge. The central part receives oxygen indirectly from the air, via oxygen dissolved in the tear film and the peripheral parts of the cornea take up oxygen by diffusion from the anterior ciliary blood vessels [1].

4. Degenerations of the conjunctiva and the corneoscleral coat

A degenerative disorder is one in which progressive and permanent cellular or tissue dysfunction ultimately results in cell death and loss of specialized function. Degenerative changes may be primary or secondary.

Primary degenerations progress in the absence of associated disease processes and are a major cause of morbidity in the elderly. They occur throughout the eye, and the deposition of endogenous substances, as in age- related macular degeneration, is often a feature.

Some primary degenerations have an inherited component in their etiology. Secondary degenerations are associated with other disease processes such as inflammation and neoplasia [7].

Conjunctival and corneal degenerations may be unilateral or bilateral. Conjunctival degenerations occur at any age and usually affect the interpalpebral area, while corneal degenerations often present later in life at the periphery and spread to involve central areas. Scleral degenerations comprise increased thickness and rigidity and are frequently age-related [4].

4.1 Vogt's white limbal girdle

Vogt's white limbal girdle is a common harmless age related peripheral primary corneal degeneration characterized by an opacity consisting of fine crescentic white lines extending irregularly from the medial and temporal limbal regions (Figure 4).

Two types are defined according to the presence (Type I) or absence (Type II) of a clear interval between the girdle and the limbus. Type II is the commonest, and exhibits subepithelial elastotic degenerative changes similar to those of pingueculae and pterygia. Type I limbal girdle is possibly early calcific band keratopathy [7].

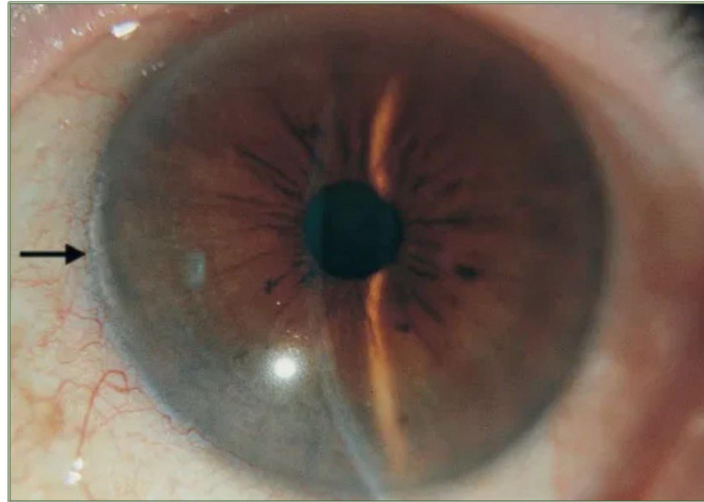


Figure 4 Voght's limbal girdle

4.2 Terrien's marginal degeneration

Terrien's marginal degeneration is an uncommon primary corneal degeneration of unknown etiology. It is usually bilateral but often asymmetric and is seen mostly in middle-aged or elderly males, in whom it slowly progresses over the course of years. Although often asymptomatic, vision may be seriously affected by irregular astigmatism and, rarely, perforation [7].

This condition starts superiorly as a marginal opacification and progresses to stromal thinning, but is separated from the limbus by a clear zone (Figure 5).

The epithelium remains intact, but a yellow border of lipid is present at the advancing edge. Circumferential spread is associated with superficial vascularization and occasionally the formation of pseudopterygia.

The peripheral corneal thinning and the intact epithelium can be confirmed histologically, and an irregular Bowman's layer is associated with a mild inflammatory cell infiltrate of the superficial stroma.

The condition may result from the activity of histiocytes, which appear to phagocytose collagen precursors, stromal ground substances and possibly lipids. Circulating immune complexes have not been demonstrated [6].

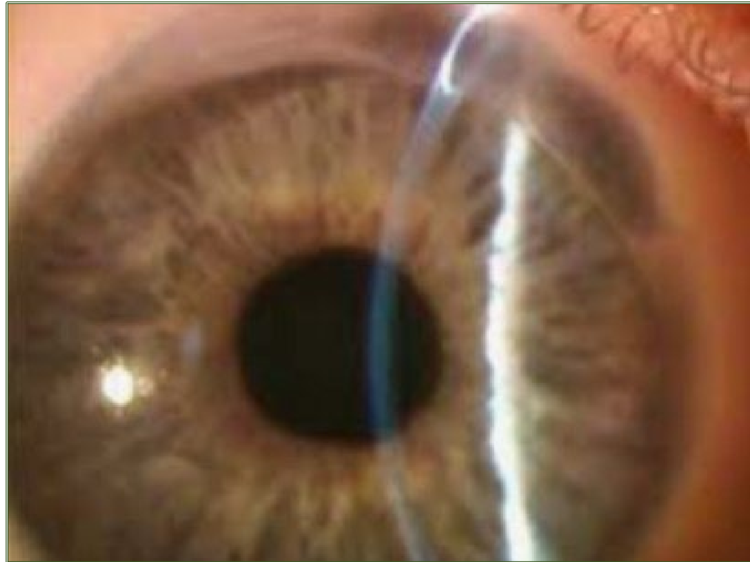


Figure 5 Terrien's Marginal Degeneration

4.3 Spheroidal degeneration (climatic droplet keratopathy, Labrador keratopathy)

Spheroidal degeneration of the cornea is related to climatic conditions, there being a strong association with UV-B light exposure. It occurs usually in men who spend their working life outdoors. Commencing in the periphery and spreading centrally, amber-colored subepithelial oil-like droplets or spheroidal granules appear in the interpalpebral fissure (Figure 6). These can spread to the conjunctiva and may be associated with pingueculae.

Spheroidal degeneration can also occur as a secondary change in absolute glaucoma, phthisis bulbi and in various chronic corneal conditions including post-traumatic scars and lattice dystrophy.

Varying-sized deposits are seen on histological examination to replace Bowman's layer and also lie in the anterior stroma. In unstained sections the deposits auto fluoresce, but the proteinaceous material of which they are composed has not been identified and its source of origin is unclear.

Electron microscopy suggests that the deposits are extracellular and lie in relation to collagen fibrils [7].

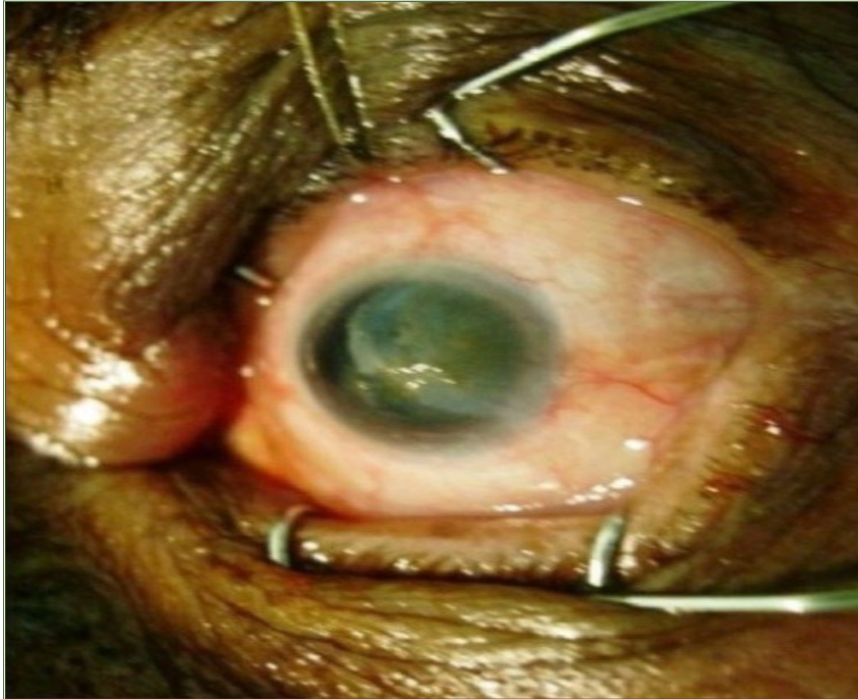


Figure 6 Spheroidal degeneration

4.4 Salzmann's nodular degeneration

Salzmann's nodular degeneration is manifested as multiple bluish-white superficial nodules, usually in the mid- peripheral cornea (Figure 7). Those mostly affected are elderly women and are asymptomatic, although some may develop epithelial erosion or decreased vision.

Predisposing factors include inflammatory diseases, epithelial basement membrane dystrophy, contact lens wear, keratoconus and corneal surgery.

The nodules are areas of hyalinized collagenous tissue lying between the epithelium and Bowman's layer, which is often focally destroyed [7].

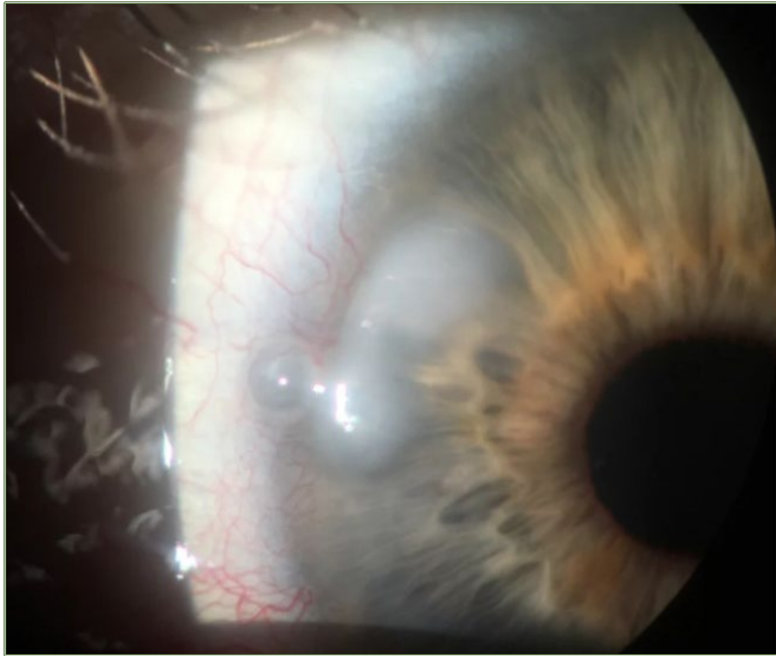


Figure 7 Salzmann's nodular degeneration

4.5 Senile Scleral Plaques

Senile scleral plaques appear on the sclera rather than the cornea or conjunctiva, but they are frequently mistaken for a melting process similar to that of corneal degenerations or as conjunctival depositions (Figure 8).

They appear as yellow, gray, or black vertical plaques, anterior to the insertion of the medial and lateral rectus muscles, in elderly patients. They become more frequent after the age of 60 years and, like pinguecula and pterygium, may be due to ultraviolet light exposure.

Histologically, calcium deposits alongside decreased cellularity and hyalinization are observed. These lesions do not need therapy [6].

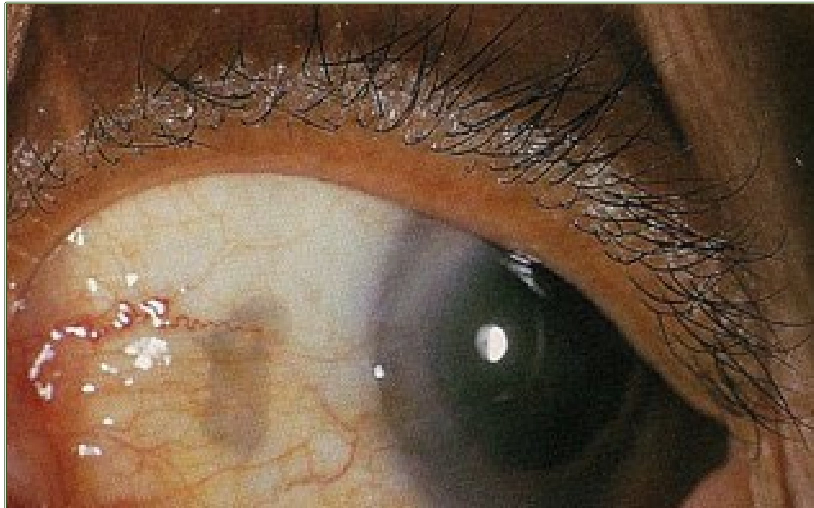


Figure 8 Senile plaques

4.5 Pingueculae

Pingueculae and pterygia are clinically distinct but have similar histological features and pathogenesis.

A pingueculum is a localized elevated yellowish-white lesion of the bulbar conjunctiva in the interpalpebral fissure close to the nasal and/or temporal limbus (Figure 9).

The causes of pingueculae are not yet clearly identified. Good evidence exists, of an association with increasing age and ultraviolet light radiation exposure. Pingueculae are encountered in most eyes by 70 years of age and in almost all by 80 years of age. Chronic sunlight exposure has been documented to be a factor [8]. There is a strong association with equatorial residence and outdoor work. In some studies, the strength of this association is less than that for pterygium.

The connection with light exposure has also been found in welders, for whom a higher rate of pingueculae occurs than for non-welders as well as an increasing rate with increasing welding exposure. It is believed that the predominantly nasal location is related to reflection of light from the nose onto the nasal conjunctiva [7].

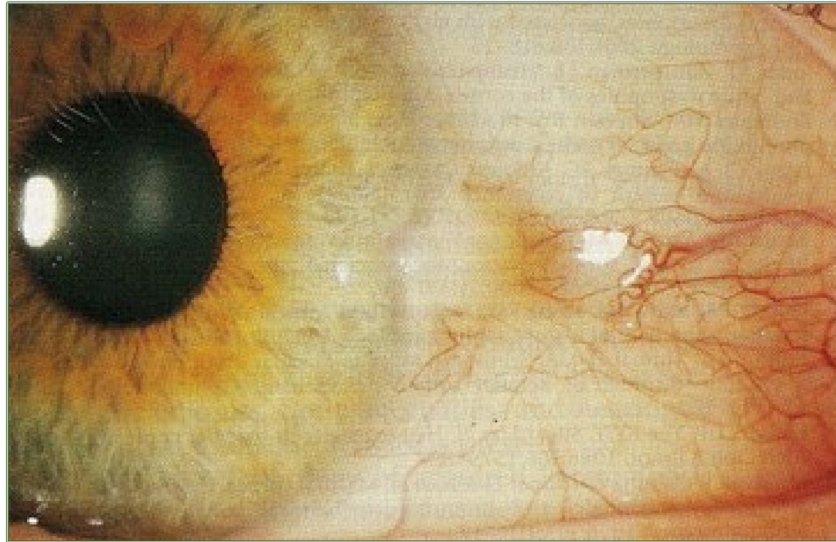


Figure 9 Pingueculum

Pingueculae are associated only rarely with any symptoms other than a minimal cosmetic defect. They may become red with surface keratinization. When inflamed, the diagnosis of pingueculitis may be given.

Distinguishing pingueculae from other lesions is usually not a problem because of the typical appearance. The histopathology of pingueculae is characterized by elastotic degeneration with hyalinization of the conjunctival stroma, collection of basophilic elastotic fibers, and granular deposits [6].

Hyalinization of subepithelial collagen is associated with a variable amount of elastotic degeneration, as evidenced by abnormal curled fibers on light microscopy. Abnormal curled fibers are particularly well demonstrated with elastic stains, although this reaction is not abolished by the enzyme elastase. Some lesions exhibit deposits of amorphous material and calcium. Elastotic degeneration may sometimes be histologically evident in clinically normal conjunctiva [6].

The main histological difference between a pinguecula and a pterygium is that the latter is more vascular. The epithelium overlying pingueculae may become atrophic, hypertrophic or hyperplastic, or may even develop dysplastic and in situ neoplastic changes (intraepithelial neoplasia), but associated infiltrating malignancy (squamous cell carcinoma) is rare. Conjunctival drying, chronic irritation and the effects of radiation may collectively lead to the degenerative changes which resemble actinic degeneration of the dermis of the skin;

pingueculae are associated with a warm dry climate, a dusty environment and exposure to UV light [7].

4.6 Pseudopterygium

Pseudopterygia are not degenerations. They are folds of conjunctiva fixed only by their apices to the cornea, and occur secondary to corneal disease such as peripheral ulceration and degeneration (Figure 10). Subepithelial elastotic degeneration is not a feature [7].

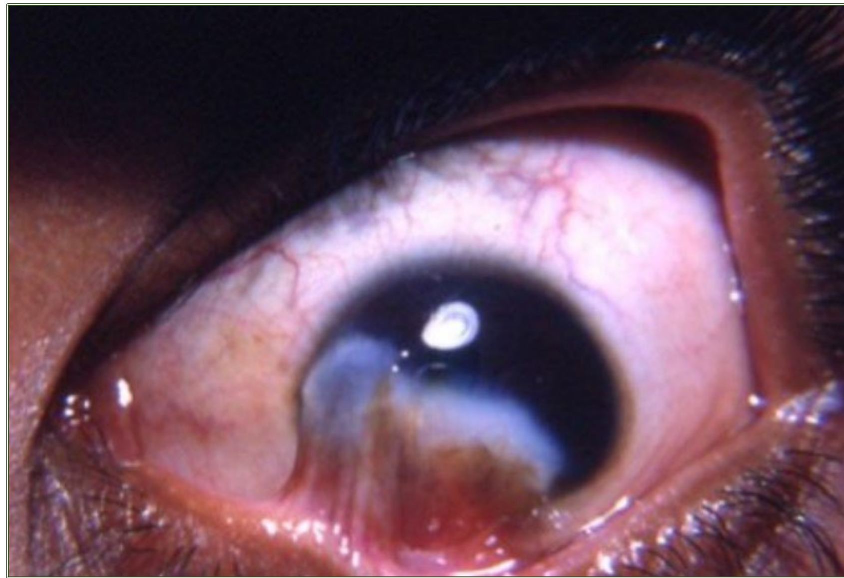


Figure 10 Pseudopterygium

5. Pterygium

Pterygium is an extremely frequent ocular surface disease worldwide, that merits further analysis and attention due to its aggressive nature and poor treatment response.

5.1 Incidence and Prevalence

The prevalence of pterygium has been explored in several population-based studies. Percentages widely vary depending on the studied population, ranging from 2.8% to 38.7%. Cameron's map of world prevalence rates of pterygia demonstrates the direct relationship between prevalence and proximity to the equator. The nearer the population to the equator, the greater the prevalence. Beyond the fortieth parallel, the prevalence of pterygia is negligible (2 percent or less) [6].

A large-scale analysis of the rate of pterygium has not been conducted yet. A metanalysis published in 2013, covering a total of 20 studies involving 12 countries with 900,545 samples, disclosed a combined prevalence rate of 10.2%, with 95% confidence interval, in the general population [6, 9].

Factors other than geographic location also affect the prevalence of pterygia:

1. Pterygia are seen nearly twice as often in men as in women.
2. They are more common in farmers than in city dwellers.
3. They are more common in those who do not wear eyeglasses.
4. Prevalence differs with respect to age.

Although the elderly have the highest prevalence rate, a much younger (20- to 40-year-old) group has the highest incidence rate. Pterygia rarely occur in patients younger than 20 years. Curiously, the discrepancy between incidence and prevalence rates is not seen with pingueculae, which are widely accepted to be the precursors of pterygia. Both the incidence and prevalence of pingueculae increase with age. Another difference between pingueculae and pterygia is that pingueculae occur with similar frequency in both sexes. Also, pingueculae are common even in regions where pterygia rarely occur [10].

5.2 Clinical Features

A pterygium presents as a fleshy triangular band of fibrovascular tissue, originating from the bulbar conjunctiva encroaching over the cornea. It has a broad base on the nasal or temporal epibulbar surface and a blunted apex on the cornea (Figure 11). Rarely, it can affect both the nasal and the temporal side of the cornea (Figure 12). The axis of the triangle is not exactly horizontal; instead, it gently slopes superiorly on the corneal side [6].

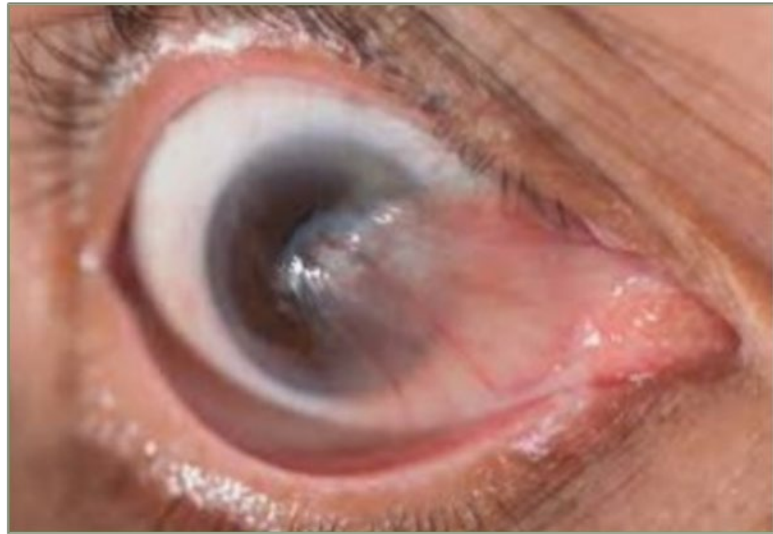


Figure 11 Nasal pterygium encroaching over the cornea

A pterygium has several components:

- A gray zone, or cap, precedes the apex, or head. The cap is a flat, grayish-white avascular zone of variable size located in the subepithelial corneal tissue, surrounding the head of the pterygium like a halo.
- Sometimes, round, gray, coin-like extensions of the cap precede it ("Fuchs spots").
- In some cases, a golden-yellow iron line (Stocker's line) is evident in the corneal epithelium, bordering the corneal side of the head.
- Between the head and the cap are small capillaries that often anastomose with the limbal plexus.
- The head of the pterygium, is slightly elevated and white. It is the only site of firm adhesion of the pterygium to the globe; the body of the pterygium can be readily lifted from the epibulbar surface.

• The body is a fleshy sheet of pink, highly vascularized tissue that is outlined from the physiological conjunctiva superiorly and inferiorly by sharp folds and attaches on a broad base. The body is under horizontal tension, as evidenced by the vessels, which appear stretched and straight. Application of fluorescein frequently reveals punctate staining over the epithelial surface of the body and occasionally on the cornea immediately in front of the head [7].

• Corneal dellen may occur, but are rare, although central dry spots are common.

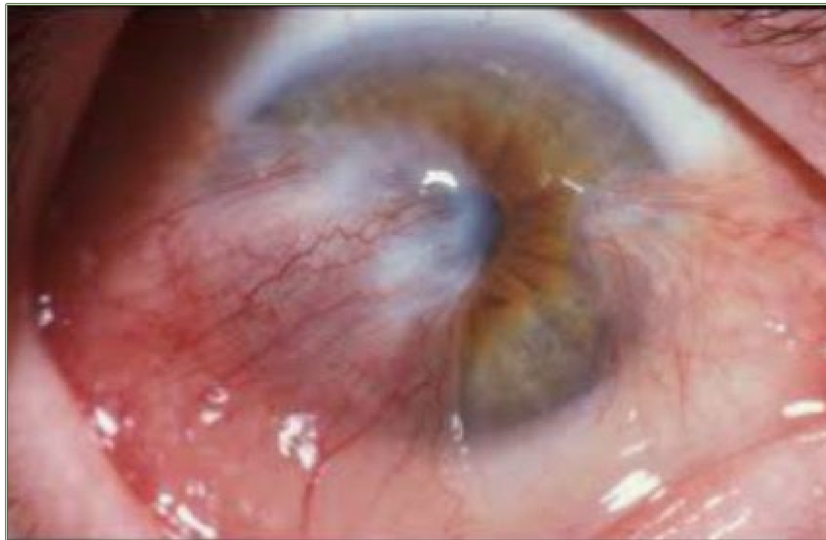


Figure 12 Bilateral pterygium

5.3 Clinical Course

Usually, a pterygium is already fully developed when the patient first seeks help. The history reveals that the lesion grew rapidly over a period not exceeding 2 or 3 months [6]. In some cases, the patient acknowledges that before the onset of rapid growth, the lesion has been a small mound present for many months or even years [7]. The lesion progresses in the following manner:

1. The initial lesion is indistinguishable from a pinguecula.

2. In the weeks or months following, prominent new vessels continue to appear on the conjunctival side of the mound, while on the corneal side many delicate vascular twigs project toward segmentally engorged limbal vessels.

3. With the appearance of superior and inferior folds, the body of the pterygium is defined.

4. The entire lesion becomes a true pterygium when the yellow mound, now the head, encroaches on the cornea. Actual encroachment is heralded by the appearance of a halo-like subepithelial cap in the cornea.

5. In contrast to the relatively rapid formation, growth across the cornea is slow, and the pterygium usually takes several years to reach the rim of the pupil. Often, however, growth abruptly ceases. Inactivation is characterized by the absence of episodic congestion, the disappearance of punctate staining over the body and the shrinkage of the halo-like cap [6].

6. The lesion may remain stationary for several years.

7. Eventually, involutional changes occur. The head progressively, thins and flattens, leaving a scar that blends imperceptibly with adjacent cornea, and the body withers into a veil-like membrane transversed by only a few delicate blood vessels.

Actively growing pterygia typically occur in the young age group (20- to 30-year-old patients), the group with the highest incidence of pterygium [8]. The most exuberant pterygia, that is, extremely fleshy masses with marked corneal extension, are seen in people who are exposed to the elements, such as farmers and construction workers [6]. Pterygia can be classified in relation to their behavior to:

1. Actively growing pterygia
2. Slowly growing pterygia
3. Stationary pterygia

According their morphological features to:

1. Fleshy pterygia
2. Atrophic pterygia (Figure 13)

Diagnosis is established with slit lamp examination, in which the pterygium can be classified as grade 1, when the fibro- vascular tissue extends to the limbus; grade 2, when it slides over the cornea about 2 mm; grade 3, when it approaches the pupil margin; and grade 4, when it exceeds the pupil. Recently, a functional

classification utilizing corneal topographic data, that based on corneal higher order irregularity was proposed, to objectively evaluate pterygium severity [11]. Pterygium was graded according the corneal irregularity within the three zones: 1.0, 3.0- and 5.0-mm diameters from the corneal center. Therefore, increased corneal irregularity within a 1.0-mm diameter was considered to highly result in the risk for visual function impairment, and increased corneal irregularity within a 5.0-mm diameter was graded as mild severity with visual function influences [12].

Figure 13 Atrophic- Flat pterygium

5.4 Associated Findings and Symptoms

During intermittent episodes of inflammation, the pterygium becomes hyperemic and causes photophobia, tearing and foreign body sensation [13].

Visual acuity may decrease as a result of astigmatism, increased glare and decreased contrast sensitivity and encroachment of the pterygium on the visual axis. In severe cases, diplopia may be caused by symblepharon formation and limitation of ocular motility [14].

Astigmatism induced by a pterygium is most common with-the- rule (steep vertical axis) but can be against-the-rule, oblique, or irregular. As much as 9 D of astigmatism can occur. Regular astigmatism induced by a pterygium occurs long before the pterygium encroaches on the entrance pupil and certainly before it reaches the visual axis [15]. Corneal topography has shown that the distortion of the precorneal tear film by the leading edge of a pterygium alters the topographic

mires, thereby effectively flattening the horizontal meridian and creating with-the-rule astigmatism [14].

5.5 Histopathology

Epithelial hyperplasia accompanied by fibrovascular growth thought to originate at the corneo-conjunctival junction, points to the presence of altered stem cells that migrate centripetally to reach the central cornea.

Histomorphologically, all the analyzed pterygia could be defined as fibrovascular proliferations, mostly covered by conjunctival epithelium. However, it is impossible to see topographic division to the cap, head, neck and body in the examined pterygia [7].

Three basic elements characterize the histologic appearance of a pterygium:

1. An epithelial covering of either atrophic, hypertrophic or even hyperplastic conjunctiva extends beyond its normal anatomic confines onto the cornea. The head of pterygium exhibits the morphology of modified stratified squamous epithelium of the cornea [16].

In the epithelium of this region, cellular and nuclear polymorphism is found, especially expressed in the surface layer of the epithelium, where young cells with large nuclei are seen. Previous studies showed that scleral epithelium of progressive pterygium consisted of more layers in comparison to stationary pterygium. It makes up numerous wrinkles, invaginations, as well as partial and isolated epithelial canals. The epithelium of progressive pterygium shows significantly higher hyperplasia and basal germinative layer activity than stationary pterygium. Particularly, clearly defined hyperplasia and activity of the basal layer of the epithelium were found in the progressive pterygium base, where numerous invaginations and epithelial canals can be seen. However, it was proven that these larger invaginations, as seen in longitudinal cross-sections, are in fact a gap between lateral lobe of pterygium and its base. The epithelium is extremely attenuated in certain parts (especially in the lateral parts of the lobe), due to epithelium stretching caused by enlargement of connective tissue mass of pterygium. Measurement (quantification) of the degree of division of pterygium

epithelial cells showed no increase. It is certainly possible that during pterygium development over the years, proliferative activity varies. The recent studies indicate increased telomerase activity, a biomarker of cell proliferation, in nearly two thirds of the examined pterygiums [17]. This epithelium overlies the second element:

2. A bulky mass of thickened, hypertrophied, and degenerated connective tissue, the collagen part of which assumes a coiled, fibrillated shape reminiscent of elastic tissue. Signs of young, newly-formed connective tissue in the avascular part of the progressive pterygium head are present. This tissue strongly resembles the corneal connective tissue, except that the lamellas are thinner and irregularly intertwined. It has significantly more cell elements of connective tissue, roundly shaped, in comparison to normal cornea. Immature character of this newly formed avascular connective tissue is also confirmed by histochemical analyses. Thickening and de-layering of the basement membrane on pterygia specimens is observed when stained with PAS reaction. This membrane sends abundance of filaments towards the underlying connective tissue [18].

In the stationary pterygium head, there is more compact connective tissue than in progressive pterygium, composed of thicker corneal-like lamellas, but not so regularly arranged. This tissue contains less cell elements (thin and hardly seen) in comparison to newly-formed connective tissue in progressive pterygium. These indicate that connective tissue breakdown occurs. The basal epithelial membrane in the avascular part of the stationary pterygium is less thickened and less de-layered than in progressive pterygium [17].

The main part (body) of the pterygium is composed of connective tissue, in the course of pterygium evolution, undergoes pathological changes that are accompanied by the increase of the mass of the pterygium itself. The newly formed structure in this part of the pterygium, is located subepithelially and progresses towards the cornea. In the corneal part of pterygium, this newly formed and well vascularized conjunctiva is positioned between the epithelium and the rough original layers of stroma (including Bowman's layer). In such a way, that changes in extracellular matrix responsible for physical properties of

tissue occur. As a result, mechanical stability of tissue is lost. Mechanical stress cannot be absorbed anymore, so the tissue is bulged above the eyeball surface [17].

According to statistic data, this tissue protrusion then forms lobes laterally to the pterygium basis, with simultaneous modification and expansion of epithelial surface. For these reasons, during the surgical procedures not only this surface loose tissue in the region of cornea and sclera should be removed, but the tissue underneath and around lateral margins as well, to prevent recurrences of residual pathologically changed cells [19].

In regards to characterizing elastotic material within the pterygium, the term “elastotic degeneration” was introduced to describe tissue susceptibility to Weigert’s and Verfhoff's elastic tissue stains, but at the same time the lack of tissue degradation by pancreatic elastase [17].

The abnormal collagen displays basophilia and an affinity for elastic tissue stains, but it is not digested by elastase. Hence, it is not elastic tissue but falls into a category of elastotic degeneration. Elastotic material within the pterygium is possibly formed in three ways: 1) by degenerated collagen, 2) by abnormal fibroblastic activity and 3) by ground substance disorder. Recently, ultrastructural analysis attributed the elastotic degeneration only to abnormal fibroblastic activity with the production of abnormal maturational forms of elastic fibers [18].

Electron-microscopic studies, have revealed elastogenesis in substantia propria in normal conjunctiva, then in deeper episcleral connective tissue, as well as at the level of Tenon’s capsule. Ultrastructural examinations also disclosed evidence of elastogenesis in the body of pterygia, but with distorted morphogenetic sequence of fiber formation (Figure 14).

In substantia propria, elastodysplasia (immature formation of elastic fibers) and elastodystrophy (degenerative changes of elastic fibers and formation of electron-dense inclusions) were verified. The amorphous materials in the epibulbar part of pterygium are composed of large number of hollow-centered microfibrils, precursors of elastic fibers that show the tendency to agglomerate centrally in the form of large aggregated envelopes. These irregular elastic fibers exist in the

substantia propria of the body part of the pterygia, and fibroblasts have a role in their formation [18].

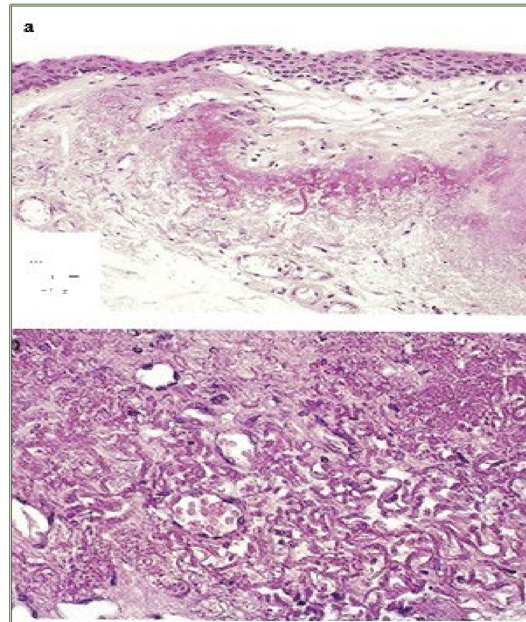


Figure 14 Elastotic degeneration of collagen in the vascularized subepithelial stroma a) low magnification b) high magnification

There is no difference in the cellular proliferation between pterygial and normal conjunctival tissue, and pterygium is not a disorder of overexpressed cellular proliferation. By transmission electron microscopy it was proven, that extracellular matrix is a dominant component of pterygium, and that cellular proliferation of primary and recurrent pterygium does not significantly differ. It is confirmed that the fibroblasts of pterygium, thanks to their adherent ability and higher growth kinetics, are superior to fibroblasts of normal conjunctiva [20]. This might explain the high growth ability in ocular pterygium. But there are significantly higher number of mastocytes in progressive pterygium in comparison to stationary one, with dominating de-granulated forms [19].

3. New blood vessels, usually congested, are dispersed among the hypertrophied collagen fibers. Additionally, the episcleral bed beneath the pterygium is hyperemic.

The number, diameter and type of blood vessels showed extreme variability [6]. Fragile capillary network of multiple blood vessels in edematous stroma of pterygia with discretely exposed fibrinoid change can be seen in evolutive shorter

forms, while complexes with abundant branching of well-developed capillaries with greater lumen are characteristic for other evolutive forms. Pterygia specimens had a greater number of blood vessels positive to vWF (von Willebrand factor) and VEGF (vascular endothelial growth factor) [21]. Generally, there is a greater number of VEGF positive cells, than in normal conjunctiva. Over-expression of VEGF in pterygium tissue, along with abundance of vWF stained blood vessels, can support the idea that angiogenesis plays an important role in formation of pterygium [18]. Vascularization in the body of the progressive pterygium is more abundant, in comparison to the body of stationary one. The patients with Fuchs' spots present, marking the zones of pterygium progression, had higher inflammation in pterygium tissue in comparison to the group of patients without progression zones. This was expected, since Fuchs' patches are a significant clinical indicator of progressiveness or activity of the pterygium tissue [22].

Tenon's capsule is fused with the body of the pterygium and contributes to its vasculature and bulk. Because Tenon's capsule is interposed between the pterygium and the episclera, the body of the pterygium is loose and not adherent to the sclera [18].

On the corneal side, just in front of the head of the pterygium, an advancing row of fibroblasts penetrates the cornea between Bowman's layer and the basement membrane of the overlying epithelium. This plane of invasion corresponds anatomically to that of the subconjunctival connective tissue. These fibroblasts account for the gray zone, or cap, and are thought to prepare a path for fibrovascular tissue to enter the cornea. As this tissue enters the cornea, Bowman's layer is pushed posteriorly and eventually becomes fragmented. The head of the pterygium becomes firmly adherent to the superficial stroma over these sites of focal interruptions in Bowman's layer [6].

5.6 Pathogenesis

Aside from corneal invasiveness, the most impressive characteristic of a pterygium is the exuberant fibrous tissue proliferation that is observed clinically as a thick head and a bulky body. This feature has been likened to keloid

formation and is reminiscent of the subepithelial scar tissue prominent in alkali burns. Indeed, many of the findings suggest that a pterygium is a type of burn produced over several years by the cumulative absorption of infrared and UV radiation from sunlight [23, 24].

Also, the anatomic location in the interpalpebral region implicates sunlight as an etiologic agent. The conjunctiva and cornea absorb most of the infrared and UV radiation from sunlight, which can damage epibulbar tissue. Although the proportion of infrared radiation greatly exceeds that of UV radiation (40 percent vs. 2 percent), the latter is much more biologically active [23].

In one study, those who worked outdoors mainly in a sandy environment had an increased risk of several hundredfold; those who worked in a concrete environment had an increased risk of 20-fold. Persons who spent their first 5 years of life at latitudes less than 30 degrees had almost 40 times the risk of pterygium formation compared with those who spent their first 5 years of life at latitudes greater than 40 degrees [23].

Some have proposed that the iron line (Stocker's line) seen at the head of an advanced pterygium is evidence of drying and exposure from the stagnation of tears and failure of the tear film to spread over the area. Others argue that neither dellen nor micro-ulcerations of the corneal epithelium are consistent findings in active pterygia. In addition, they believe that the presence of an iron line at the head of a pterygium suggests pooling of tears in this area, quite the opposite of a proposed mechanism based on corneal desiccation [22].

UV radiation exposure

UV radiation from the sunlight is subdivided into three categories:

UVA (wavelength, 320-400 nm) exhibits the longest wavelength and the highest penetration power. It penetrates the ozone layer in the atmosphere; thus, it is a significant inducer of pigmentation and results to premature skin aging, immunosuppression and carcinogenesis [25].

UVB (wavelength, 280-320 nm) is obstructed by the ozone layer and constitutes 1%-10% of the total UV radiation that reaches the Earth. It is accountable for

various biological events, including sunburn, immunosuppression, and carcinogenesis [26].

UVC (wavelength, 200-280 nm) possesses the highest energy among the three UV rays and presents strong mutagenic properties. It is almost completely deflected by the ozone layer, thereby imposing negligible effects to human eyes [12].

UVB light exposure has been evaluated as a major cause of pterygium. This type of radiation has the ability to potentially damage and transform cells and tissues. It exerts its actions by direct phototoxicity effects on the cellular DNA and formation of reactive oxygen species, which can damage the cellular DNA. Wavelengths below 300 nm are known as the most biologically active forms and are absorbed by the cornea. Exposure to UVB radiation leads to cellular oxidative stress, which may lead to upregulation of many potential mediators of pterygium growth. It is possibly due to ultraviolet-induced damage to the limbal stem cells that pterygium grows and hence considered as a focal stem cell deficiency [27].

Angiogenesis and lymphangiogenesis are widely accepted but incompletely characterized processes, involved in the pathogenesis of human pterygium. Several mechanisms for blood and lymphatic vessel development in human pterygium have been proposed, including inflammation, growth factor overexpression, hypoxia, and transcription factors. UV light–induced angiogenesis was reported many years ago, that demonstrated increased endothelial cell proliferation within existing blood vessels, leading to telangiectasia and new blood vessel development in mice skin due to a UV radiation–induced imbalance between a positive angiogenic molecule (basic fibroblast growth factor) and a negative angiogenic molecule (interferon-beta). A recent study reported UV light–induced DNA damage in endothelial cells from human pterygium blood vessels and suggested the involvement of UV light–induced angiogenesis in the pathogenesis of the human primary and recurrent pterygium [28].

If UV light–induced DNA damage has already been recognized in the pathogenesis of the skin lesions and now described in human pterygium, data are now available about DNA damage in the pericytes, as the presence of thymine dimer positive reaction in the pericyte nuclei surrounding small blood vessels

from the fibrovascular compartment of primary and recurrent pterygia, suggest [29].

Viral infections

The polymerase chain reaction technology permitted examination of the possible involvement of viral infections in pterygium pathogenesis. Some studies demonstrated the presence of herpes simplex virus and human papilloma virus (HPV) in pterygium samples. Viruses encode proteins that can inactivate p53, leading to chromosomal instability and increasing the likelihood of cell progression to malignancy [30]. HPV is frequently encountered in the pterygium, with variable prevalence rates. Its association as a cofactor in the pterygium pathogenesis is suggested, but remains controversial [31]. If HPV is involved in pterygium pathogenesis or recurrence, anti-viral medications or vaccination may be new options in pterygium therapy [32].

Molecular mechanisms

Many studies have offered possible mechanisms of pterygium development, including oxidative stress, DNA methylation, loss of heterozygosis, extracellular matrix modulators, apoptotic and oncogenic proteins, inflammatory mediators, lymphangiogenesis, transition from mesenchymal to epithelial cells, and cholesterol metabolism alterations [23, 29, 33]. These studies demonstrated that several molecules, such as matrix metalloproteinases (MMPs), growth factors, and interleukins are related to proliferation, inflammation, angiogenesis, and fibrosis [34].

Tumor suppressor genes

Tumor suppressor genes protect cells from converting into cancer cells and regulate cell growth along with proto-oncogenes. One of the tumor suppressor genes that has been extensively studied is p53. A survey showed that >20% of all pterygium samples were positive for p53 expression [35]. Another immunohistochemical study evaluated pterygium samples and normal conjunctiva, which disclosed that 54% of pterygium were positive for p53 aberrant expression and no pathological staining was observed in the normal conjunctiva [36]. These data are supported by the study we conducted, using liquid-based

cytology assay to examine fifty specimens. Among them, 38 specimens were pathologic tissue, from pterygium lesions and 12 were specimens of normal conjunctival epithelia [37].

Therefore, the aberrant expression of p53 is believed to promote cell proliferation and slow down apoptosis, thereby accelerating the development of pterygium [38, 39]. The growth of limbal tumors is also suggested to be influenced by cellular DNA damage that causes mutations in other genes. Additionally to p53, other tumor suppressor genes, such as p63, p16, and p27, were possibly involved in the development of pterygium [40]. P63 is overexpressed in the basal and parabasal layers in primary pterygium and through the total thickness of the epithelium in recurrent pterygium. Increased expression of p16 protein was also detected in pterygium. Both p63 and p16 seem to be rarely expressed in the normal conjunctiva [41]. P27 gene showed low nuclear immunoreactivity in pterygium tissues, differing from other tumor suppressor genes.

Apoptosis-related proteins

The protein survivin is encoded by the BIRC5 gene in the human genome; it is a member of the inhibition of apoptosis family and is expressed in the pterygium epithelium, as our study has also shown [37]. The mechanisms of survivin function are still not well understood; however, survivin regulation seems to be associated with the p53 protein [42, 43]. Oxidative stress has been demonstrated to promote the activation of survivin, leading to pterygium growth. Moreover, survivin has been identified as overexpressed in all pterygium tissues, but not in the normal conjunctiva. Survivin was reported to be closely related with COX-2 in primary pterygium, suggesting an antiapoptotic mechanism [44].

Bcl-2 is the core member of the Bcl-2 family of apoptosis regulatory proteins, which can induce or inhibit apoptosis [45]. It is encoded by the Bcl-2 gene and its expression was noticed in the basal epithelial layer of all pterygium samples, but the normal conjunctiva showed no evidence of the protein. Decreased miR-122 expression in the pterygium could lead to cell apoptosis, due to regulation of Bcl-w expression, which is also, a gene of the Bcl-2 family, with anti-apoptotic properties and subsequently contribute to the development of pterygium [46].

Rapamycin complex 1 (mTORC1) is a key regulator of cell growth, transcription, protein synthesis, proliferation, and autophagy. mTORC1 activates the protein translation and the mTOR signaling is highly activated; therefore, aberrant apoptosis and cell proliferation were observed in pterygium samples [47]. Activation of mTORC1 is believed to inhibit apoptosis by regulating Beclin-1-dependent autophagy and targeting Bcl-2. mTORC1 also, downregulates fibroblast growth factor receptor 3 (FGFR3) by inhibiting p73, that leads to stimulation of cell proliferation in the pterygium. This provides evidence, that mTORC1 signaling is highly activated in pterygium and provides new pathways on its pathogenesis and progression [48].

Cell adhesion molecules

Cell adhesion molecules appear to play a significant role in a variety of physiological and pathological phenomena [49]. These proteins are localized on the cell surface and are intimately involved in cell bridging and other extracellular matrix components, related to cell adhesion, including selectin, and integrin. The positive expression of Intercellular Adhesion Molecule-1 (ICAM-1) is obvious in pterygium and absent in the epithelium of a normal conjunctiva [50]. Beta-1 catenin and E-cadherin and have also been discovered to be abundant in the pathological tissue of pterygium and are possibly involved in the epithelial proliferation and adhesion, that the pterygium exhibits [49].

Proliferation-related proteins

Proliferation related proteins such as cyclin D1, Ki-67 and nuclear proliferation antigen play an important role in the cell cycle [40]. Ki-67 is an important biomarker of cell proliferation and its abnormal expression was demonstrated in pterygium samples when compared to normal conjunctiva [51]. Proliferating Cell Nuclear Antigen (PCNA) is a nonhistone protein that is essential for DNA synthesis and its expression can be utilized, as a marker of cell proliferation. The expression of PCNA is significantly higher in pterygium than that in normal conjunctiva. Cyclin D1 is also, a well-known protein that controls cell cycle and facilitates cell cycle progression. A study presented PCNA and cyclin D1 to be overexpressed across the limbal area of pterygium epithelial cells compared to normal conjunctiva samples, implying that it might lead to hyperproliferation of

epithelial cells [52]. Cyclin D1 protein expression in fleshy pterygium is found significantly higher than in the atrophic pterygium lesions. Another study indicates that β-catenin expressed in the nuclei/cytoplasm could potentially increase cyclin D1 protein expression, that favors the proliferation of pterygium cells [53]. A point mutation of CYP1A2 protein, which is also related to tumorigenesis, may be linked, due to increased enzymatic activity and subsequent proliferation [54].

Heat shock proteins

Heat shock proteins (HSP) are a group of proteins, produced by cells in response to exposure to stressful stimuli. They were originally described in connection to heat shock, but are recently found to be expressed during other stresses, as well, including exposure to cold temperatures, UV light, and during wound healing or tissue remodeling. The expression of HSPs, i.e., Hsp27, Hsp70, and Hsp90, and hypoxia inducible factor-1α (HIF-1α) were found elevated in pterygium. Hsp27 expression was detected in the epithelial, endothelial, and vascular smooth muscle cells in pterygium, but only in the epithelium, in normal conjunctiva [55]. Changes in HIF-1α and HSP levels in pterygium are believed to represent an adaptive process for cell survival under stressful conditions [56].

Tight junction proteins

Tight junction proteins accommodate cell-to-cell adhesion in the endothelial and epithelial cells, conforming continuous seals around the cells, serving as a physical barrier to prevent solutes and water from entering the cell freely, through the paracellular space. Claudin proteins are essential for this functional and structural barrier and dysregulation of their expression may result in various diseases, including cancer. In normal cornea and conjunctiva tissue, claudin-1 and claudin-4 positivity were demonstrated immunohistochemically [57].

Claudins are indispensable for the formation and maintenance of tight junctions. A strong immunohistochemical expression of claudin-1 was identified in normal epithelial conjunctiva samples. In the contrary, its expression in pterygium was low. The significant decrease observed in claudin-1 expression, in the pterygium

compared to normal conjunctiva appears to be implicated in the pathogenesis of pterygium [58].

Extracellular matrix proteins

The extracellular matrix (ECM) is another group of extracellular molecules secreted by resident cells that deliver mechanical and biochemical support to the surrounding cells. The abnormal expression of extracellular matrix proteins is thought to facilitate the proliferative growth of pterygium, because it is a fibrovascular tissue characterized by an excessive deposition of extracellular matrix and vascular growth [59]. The extracellular matrix proteins contain elastin, collagen, keratin and fibrin, among others. K8, K16, K17, K14, and AE3 were shown to be present throughout the whole thickness of the pterygium epithelium but absent in normal conjunctiva. Pterygium samples revealed a higher mRNA level and tropoelastin expression than physiological conjunctival tissue [60, 61]. Type II collagen expression was found positive only in pterygium, whereas the collagen types I, III, and IV were detected in both the normal conjunctiva and pterygium [62].

Matrix metalloproteinases and tissue inhibitors of metalloproteinases

Matrix metalloproteinases (MMPs), also known as matrixins, hydrolyze elements of the extracellular matrix. These enzymes play a key part, in numerous biological procedures, like normal tissue remodeling, wound healing, angiogenesis, and embryogenesis. Also, they are important molecules, in diseases such as atheroma, arthritis, cancer, and tissue ulceration. MMPs are a multiple gene family of >25 secreted and cell surface enzymes that process or degrade various extracellular matrices, which can be divided into five subgroups based on substrates: collagenases (MMP-1, MMP-8, MMP-13), stromelysins (MMP-3, MMP-10), gelatinases (MMP-2, MMP-9), membrane associated MMPs (MT1-MMP, MT2-MMP), and others like MMP-12, MMP-19, MMP-20. Tissue inhibitors of metalloproteinases (TIMPs) bind to and block the action of MMPs. The relation between pterygium and these two groups of proteins in the pathogenesis of pterygium has been studied [63]. MMP-1, MMP-2, MMP-3, TIMP-1, and TIMP-3 were discovered in greater amounts in epithelial cells, and fibroblasts of pterygium tissue, as compared to normal conjunctiva. MMP-3 was positively

found, located in the pterygium epithelium, which may help explain the various pterygium phenotypes. A study proposed that cyclosporin A can reduce MMP-3 and MMP-13 expressions in the pterygium fibroblast culture [64]. MMP and TIMP expressions appear to vary at the different stages of evolution of the pterygium. The balance breach between MMPs and TIMPs may be responsible for the progression or recurrence of pterygium [65].

<u>Interleukins</u>

Interleukins are a group of cytokines and signal molecules originally thought to be secreted and expressed by white blood cells (leukocytes). These cells are intricately involved in the inflammation process; thus, ILs can be closely related to pterygium [66].

The expression of IL-1α, IL-1β precursor proteins were detected, via immunofluorescence, in primary pterygium and normal conjunctival epithelium. High levels of IL-1 group of proteins were present in pterygium, only. Furthermore, IL-1α was found to be highly expressed not only in primary, but also in recurrent pterygium [12].

IL-6 and IL-8 were highly expressed in the pterygium epithelium compared to the normal cornea, conjunctival limbus. Additionally, IL-6 and IL-8 proteins were significantly elevated in pterygium treated with UVB, suggesting that UVB could promote the secretion of these two interleukins. IL-8 can induce corneal vascularization, directly. IL-10 has also, been described to be expressed more in pathological pterygium tissue, than that in the physilogical conjunctiva. Also,recently, IL-17 was found to be upregulated in the ocular surface in inflammatory pathologies, such as pterygium. Another chemokine was also, found overexpressed in pterygial stroma, CXCR4 [67, 68].

<u>Growth factors</u>

A growth factor is a normally occurring substance capable of stimulating cellular growth, proliferation, healing, and cellular differentiation. They are important in the regulation of various cellular processes, such as mitosis.

A large number of growth factors are considered to play a significant role in pterygium pathogenesis, like the vascular endothelial growth factor (VEGF),

transforming growth factor beta (TGF-β), basic fibroblast growth factor (bFGF), insulin like growth factor, nerve growth factor, heparin binding epidermal growth factor-like growth factor and connective tissue growth factor (CTGF) [69-71]. The VEGF family has been in depth investigated in ophthalmology, because of its significant part in pathological angiogenesis and in accelerating the vascular permeability in ocular diseases, like pterygium and choroidal neovascularization diseases [72].

Increased expression of VEGF would ultimately lead to angiogenesis and lymphangiogenesis, which could affect the normal metabolism of connective cells and promote vascular growth [73]. When compared to the normal conjunctiva, pterygium exhibited higher VEGF levels [74]. TGF-β regulates various processes common to tissue repair and disease, including fibroblast proliferation, angiogenesis, synthesis, and degradation of extracellular matrix proteins. TGF-β1 and TGF-β2 were also observed to be positively upregulated, but transforming growth factor beta receptor 1,2 (TGFR-β1, -β2) was found to be negatively regulated in pterygium [75].

5.7 Differential Diagnosis

The differential diagnosis of pterygium includes the following conditions:

1. A pseudopterygium is a fibro vascular tissue that may occur on any quadrant of the cornea. Like pterygia, the majority of these lesions develop at the 3 and 9 o'clock positions because these areas are constantly exposed to dryness and irritation (Figure 10). In contrast to a pterygium, a pseudopterygium lacks organization into different regions (cap, head, and body) and firm adhesion at the limbus [7].

Pseudopterygia may be seen secondary to peripheral corneal disease, such as marginal ulceration, chemical burns, cicatricial conjunctivitis and hard contact lens wear, especially with a lens that rocks on a steep vertical axis in a cornea with significant with-the-rule astigmatism. Chronic mechanical irritation from contact lens movement and abnormal tear wetting may induce a punctate keratopathy that can stimulate peripheral corneal vascular ingrowth. A contact lens-induced pseudopterygium has a broad, ill-defined leading edge on the

corneal surface, which distinguishes it from a true pterygium. Furthermore, a pseudopterygium caused by hard contact lens wear may regress after removal of the lens [76].

2. A conjunctival malignancy (e.g., squamous cell carcinoma) must be differentiated from a pterygium (Figure 15). Rarely, the epithelium overlying a pterygium may undergo malignant degeneration [16]. Therefore, all surgical specimens should be submitted for pathologic examination to rule out neoplasia [77].

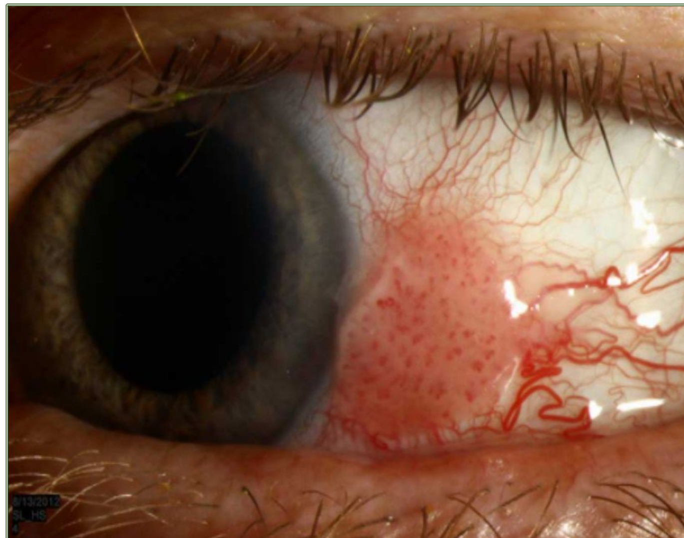


Figure 15 Squamous cell carcinoma

5.8 Treatment

Surgical manipulation of a pterygium should not be undertaken casually. The possibility of a recurrent pterygium is high, and the recurrence is often worse than the primary lesion. In addition, complications may be associated with any surgical technique. Therefore, when possible, medical management should be attempted first [78].

<u>Medical Treatment</u>

A small pterygium that does not involve the visual axis may be amenable to medical management. Such a pterygium can cause signs and symptoms suggestive of local inflammation, including hyperemia, foreign body sensation, and tearing, which may be alleviated with

• Artificial tears

• Lubricants

• Brief courses of topical corticosteroids

Any associated blepharitis should be treated [79]. The eye should be protected from a hostile environment, including exposure to UV light, dust, and dry hot wind. Sunglasses that filter UV light are also recommended [80].

Surgical Treatment

Signs and symptoms of pterygium that are not amenable to medical management and require surgical intervention include

• Significant cosmetic blemish

• Decreased visual acuity from encroachment of the pterygium on the visual axis

• Regular or irregular astigmatism [81]

• Restriction of ocular motility

• Formation of dellen

• Recurrent inflammation unresponsive to medical therapy

Pterygia would certainly arouse less interest, if it were not for their tendency to recur despite various surgical strategies designed to avoid just that. The many therapeutic options available for the treatment of pterygia imply that no single method is universally successful [82]. The successful removal of a pterygium depends on the surgical technique used, as well as on the type of lesion encountered:

• Slow-growing, stationary, and atrophic pterygia can be removed by virtually any technique, with little risk of recurrence.

• Actively growing or fleshy pterygia are more likely to recur.

Avulsion, the earliest technique used to treat pterygia, was practiced by the ancient Greeks and still has its proponents. The method that followed, simple excision, has the same high recurrence rate as avulsion (23 to 75 percent). This complication spurred the development of alternative and adjunctive surgical procedures [6].

The bare sclera technique involved leaving an area of bare sclera adjacent to the limbus to give the raw corneal surface time to heal before the conjunctiva grew up to the limbus again. The proponents of this approach emphasized the importance of excising subconjunctival connective tissue present under the body of the pterygium in deterring recurrence [82].

Evolving excision techniques were coupled with new graft techniques:

Some thought it was crucial to cover the entire exposed epibulbar defect with normal conjunctiva. Various rotating conjunctival flaps have been designed to accomplish this.

Others recommended the use of a free graft to cover the defect, and they introduced the barrier concept, wherein the grafted tissue forms a barrier against the passage of new vessels into the cornea, thus helping to avoid recurrence.

Conjunctival autografts allow coverage of large defects that occur from large excisions, which are often encountered in advanced and recurrent pterygia [83]. The grafts can cover large areas of sclera and exposed extraocular muscle and provide tissue for reconstruction of the fornix [84]. A recurrence rate of 5 percent was reported when this technique was used on advanced and recurrent pterygia; two of the three recurrences were successfully treated with repeat conjunctival autografts. However, a recurrence rate of 21 percent after conjunctival autografting was found in a study in the Caribbean, where the risk of pterygium recurrence is high [85].

In tropical countries, where many patients have trachomatous scarring and xerosis, sufficient conjunctiva for a graft cannot always be obtained. To overcome this obstacle, labial or buccal mucous membrane may be used. No recurrences were reported with oral mucous membrane harvested from the lower lip. However, 6 percent of the patients complained of a cosmetically unsatisfactory result because of the color differences between the graft and ocular tissues [86].

A more recent alternative to autografts, Amniotic Membrane Transplantation, appears to be a safe and efficacious adjunct to the surgical treatment [87]. Although, AMT possibly offers the advantage of better control of pathological neovascularization, scar tissue formation and lower levels of postoperative

inflammation, it does not appear to convey a lower recurrence rate than conjunctival autografts [88].

Excision of Recurrent Pterygia

Most patients with stationary or atrophic pterygia do well with simple excision and sliding conjunctival flaps. However, if the same technique is used for an active or fleshy lesion, the pterygium usually recurs promptly [89]. In a retrospective analysis, regardless of the type of procedure, the following was found:

Surgery	Mean time to recurrence
Excision of a primary pterygium	123 days
Second surgery	97 days
Third surgery	67 days

In terms of surgical treatment, a recurrent pterygium is considerably different from a primary pterygium. In a recurrent pterygium, subconjunctival fibrous tissue is more abundant and is tightly bound to the underlying sclera.

Complications

Intraoperative complications

1. Perforation of the cornea during the keratectomy is rare with microscopic visualization and maintenance of a superficial plane of dissection.

2. Perforation or dissection into the sclera can be avoided by taking care to observe the limbal junction and then shifting to a more superficial plane.

Postoperative complications

1. Some degree of scarring is inevitable at the keratectomy site, but it is usually mild and does not interfere with vision unless the pupillary axis is involved.

2. Minor amounts of astigmatism may occur with excision of lesions having considerable corneal involvement.

3. Damage to the medial rectus muscle after pterygium excision has been reported. Symptoms of injury to the horizontal rectus muscle include diplopia and restriction of ocular movement.

4. Symblepharon formation, due to scarring over the denuded areas.

5. Conjunctival graft edema generally occurs within the first 10 postoperative days; it is usually caused by one of the following, excessive surgical manipulation, inadequate Tenon's capsule excision, poor graft orientation [84].

6. Conjunctival graft retraction can occur when the graft has excessive Tenon's capsule, is of inadequate size, is of poor quality because of cicatricial processes, including trauma, prior surgery, infection, or inflammatory reaction

7. Necrosis of conjunctival autografts has been associated with avascular scleral beds resulting from radiation therapy or the use of antimetabolites.

8. Inadvertent inversion of the graft, such that the epithelium is apposed to the sclera, will cause the graft to fail.

9. Epithelial cysts and Hematomas are another possible complication due to poor apposition of the tissues and excessive manipulation.

10. Recurrence is more common in younger patients and is often associated with a family history. It is the most daunting challenge, with a rate as high as 88%, for the bare sclera technique [85].

Two or 3 weeks after the removal of a pterygium, fine capillaries may be seen in some cases. These vessels appear to arise from the episcleral surface under the conjunctival flap. Many parallel vessels race forward toward the area of bare sclera in a brush-like fashion. If not treated, they often cross the bare area and extend into the cornea [90]. In a matter of weeks, the vascularized region is covered by fibrous tissue similar in appearance to the original pterygium, so that the patient is left with a lesion as unsightly as the original one [82].

Whether this lesion constitutes a true recurrence is unclear. From the patient's point of view, the pterygium has grown back [91]. Therefore, prompt action is required as soon as the brush-like vessels are detected. Various methods of adjunctive therapy may be applied at that time [92].

5.9 Adjunctive Therapy

Antimetabolites decrease the recurrence of pterygia by preventing the proliferation of abnormal tissue [93, 94]. Cyclosporine A, 5 Fluorouracil (5FU) and mitomycin C have been investigated for their ability to prevent pterygium recurrence [95-98].

Anti-Vascular Growth Factors, like bevacizumab and ranibizumab appear to arrest angiogenesis. Anti- VEGF drugs have been widely used for the treatment and control of ocular diseases associated with vascular proliferation [99]. Although some studies suggest the use as an adjuvant therapy for surgery, large scale studies conducted to characterize them for the treatment of pterygium are still lacking [100, 101].

Historically, β-radiation has been used to cause tissue damage by an emission of high -speed electrons that induce ionizing radiation [102]. Severe complications of postoperative irradiation after pterygium excision are known to occur as late as 3 to 20 years after treatment and the method is largely abandoned.

The theoretical use of the excimer laser as an adjunct to pterygium surgery is to polish the cornea after excision of the pterygium to create an ultrasmooth surface, which may re-epithelialize more quickly and uniformly and help to prevent recurrence of the pterygium [103].

Some have reported a high success rate in treating pterygia with the argon laser. It was hypothesized that an angiogenic factor released by the cornea leads to neovascular tufts or membranes similar to those seen in the retina of diabetic patients. Early pterygia that have not encroached on the limbus or cornea that are inflamed and elevated, can be effectively treated with argon laser [104].

6. Biomarkers

A biomarker is defined as an objectively measured characteristic that depicts a physiological or biological state in an organism by analyzing molecules such as DNA, RNA, protein, peptide, and biomolecular chemical modifications. Interestingly, one must bear in mind that the definition of biomarkers has been continuously, evolving over the past years, with one particularly broad definition, by the World Health Organization suggesting that "A biomarker is any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [105].

According to the United States National Cancer Institute, a biomarker is "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease," such as cancer [106].

Biomarkers typically assist in differentiating an affected person from someone without the disease. The variations can be due to a number of factors, including germline or somatic mutations, transcriptional changes, and post- translational modifications. There is an enormous variety of biomarkers, which may include proteins (e.g., a receptor or an enzyme), nucleic acids (e.g., a microRNA or other non-coding RNA), antibodies, and peptides, among other categories [107].

A bio-marker can also be a collection of adaptations, such as proteomic, metabolomic signatures and gene expression. Biomarkers can be detected in the body circulation (whole blood, serum, or plasma), excretions or secretions (stool, urine, sputum, or nipple discharge), and in this way, easily assessed non-invasively and repeatedly. In addition, biomarkers can be tissue-derived, and require either biopsy or special imaging for effective evaluation. Genetic biomarkers can be inheritable, and detected as sequence variations in germ line DNA isolated from sputum, whole blood, or buccal cells, or they can be somatic, and identified as mutations in DNA, extracted from tumor tissue. More importantly, in terms of clinical use, a cancer biomarker can assess the possibility of presenting cancer in a particular tissue or, alternatively, may measure the risk of cancer progression or possible response to therapy [108].

Apart from providing useful information for guidance in clinical decision making, cancer biomarkers are increasingly connected to specific molecular pathway deregulations and cancer pathogenesis to justify application of certain therapeutic/ interventional strategies [108].

Cancer biomarkers can be classified into the following categories based on their applicability. Predictive biomarkers prognosticate response to specific therapeutic interventions such as positivity/activation of HER2 that predicts response to trastuzumab in breast cancer. A prognostic biomarker, on the other hand, does not have to be directly associated to or trigger specific therapeutic decisions, but their purpose is to inform physicians of the risk of clinical conclusions such as disease progression or cancer recurrence, in the future [105].

A suitable example of a prognostic cancer biomarker is the 21-gene recurrence score which is predictive of breast cancer overall survival and recurrence in tamoxifen-treated, node-negative, breast cancer. Another class of biomarkers are the diagnostic biomarkers. They are used to identify whether a patient suffers from a specific disease. Diagnostic biomarkers have recently been utilized for colorectal cancer surveillance by testing for stool cancer DNA [109].

It is important distinction to make the distinction between biomarkers and targets, since in many cases these are not equivalent. A biomarker may act as a target for therapy, or it may not be the actual target of therapy. Instead, it may predict responsiveness to anti-tumor therapies. It is important to bare in mind this distinction, when clinical studies of potential biomarkers, are contemplated [110].

6.1 Survivin

Survivin is a member of the Inhibition of Apoptosis Protein family (IAPs). This is an important group of proteins involved in regulation of apoptosis. Apoptosis is the principal manifestation of programmed cell death and relies on cysteine proteinases, named caspases, to disassemble the cell in a controlled manner [111].

Two apoptotic pathways exist: the intrinsic and extrinsic pathway. Upon sustaining endogenous stress signals or irradiation, mitochondria instigate the intrinsic cascade and release of cytochrome c, through loss of the mitochondrial outer membrane potential. This cascade causes activation of the initiator caspase, caspase 9 (Figure 16). On the contrary, the extrinsic pathway is independent of mitochondria and is generated by the binding of ligands to receptors at the cell surface. For example, the TRAIL-bound TNF receptor (TRAIL, also known as TNFSF10), which activates caspase-8 via FADD, which can be inhibited by cIAP1 and cIAP2. After stimulation of the initiator caspases (caspase 8 or 9), both pathways converge on the effector caspases 3 and 7, which cause cellular demise by cleaving downstream macromolecules [112].

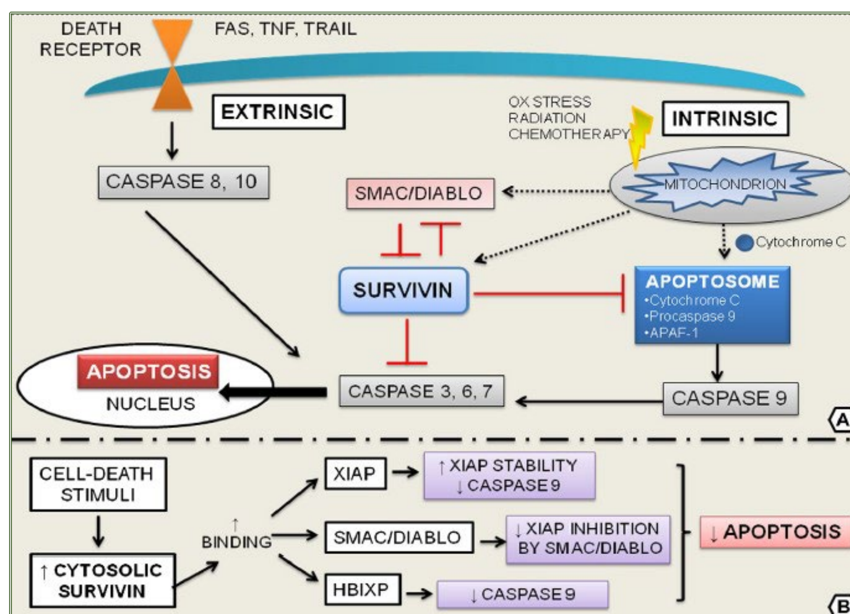


Figure 16 Apoptosis pathways

The IAP family of proteins, as the name suggests, hinder this mechanism of programmed cell death. Inclusion in the IAP family is based on the identification

of, at least one baculovirus inhibitor of apoptosis repeat domain (BIR), which is, a globular fold that has been originally discovered in insect viruses [113]. Inhibitors of apoptosis family of proteins are present in almost all species, from lower to higher vertebrates. First located in baculoviruses, as apoptotic suppressors, eight IAP homologs namely baculoviral IAP repeat-containing protein 2 / human inhibitor of apoptosis protein-2 (c-IAP1/HIAP-2), neuronal apoptosis inhibitory protein (NAIP), baculoviral IAP repeat-containing protein3/ human inhibitor of apoptosis protein-1 (c-IAP2/ HIAP-1), X-linked inhibitor of apoptosis (XIAP), IAP like protein 2 (ILP2), survivin, melanoma inhibitor of apoptosis protein (MLIAP) and BIR repeat-containing ubiquitin enzyme system (BRUCE) have been identified in humans till date [114]. IAPs are functionally and structurally similar. Their function involves the regulation of programmed cell death [115].

Considering the fact, that IAPs are an important protein family, which regulates cell fate in response to stress signals or genomic instability, any dysregulation in IAP function has a direct association with cancer development, induction of oncogenesis or drug resistance [116]. Their structure is similar, all members contain approximately 70 amino acid long Baculovirus IAP Repeats (BIR) domains at the N-terminus, which are important but not sufficient for their anti-apoptotic activity [117]. Even though, the number of BIR domains varies among IAP members, each BIR domain consists of cysteine and histidine residues in a well-defined pattern (Cx2Cx6Wx3Dx5Hx6C), that represents a novel zinc-binding fold [118].

In addition, several insect, viral and mammalian IAPs require a ring finger domain (RING) near the C-terminus for apoptosis suppression [119]. However, C-terminal RING is not the key, for suppression of apoptosis by human c-IAP1, c-IAP2 and XIAP. Human cIAP-1 and cIAP-2 possess a Caspase Recruitment Domain (CARD). The significance of CARD, during apoptosis suppression, is not fully understood. Also, IAPs present additional domains; ubiquitin-associated (UBA) domain (in c-IAP1, c-IAP2, XIAP and hILP2), ubiquitin conjugating (UBC) domain (in BRUCE/APOLLON). These domains serve in inducing ubiquitination and proteasome degradation of particular caspases, resulting in suppression of apoptosis [120]. Of all the IAPs identified so far, survivin is the smallest IAP

protein with a C-terminus Coiled Coil (CC) domain and a single N-terminus BIR domain. When compared to other IAPs, survivin presents a most specific and restricted expression in adult tissues and plays a critical part in modulating both apoptosis and cell division. It is not expressed in normal, terminally differentiated adult tissues, in the contrary it is highly expressed in most human cancers [115].

Humans have eight IAPs, of which survivin is the smallest one. Originally, it was speculated, to bind to and inhibit caspase activity directly, but modern views propose that only the canonical member of this family, XIAP, can efficiently and directly block caspases, in vivo [113]. Nevertheless, XIAP can cooperate with other IAPs, including survivin, as well; this may strengthen its stability and enhance the inhibitory effect of XIAP. Regarding XIAP, both the BIR domains themselves and the linker between two adjacent BIR domains, contribute to the prevention of apoptosis. The former negates the catalytic cysteine residue of the effector caspases, meanwhile the latter can arrest dimerization of the initiator caspases, which is crucial for their activation. Upstream factors such as Smac, that is released from the mitochondria upon apoptotic stimulation, can hinder IAPs by binding to the BIR domain; this impedes Smac from binding to its caspase target, and there is accumulating evidence to propose, that survivin might in fact, inhibit the inhibitor [121].

IAPs also have a significant impact in regulation of T cell responses, in anti-tumor immunity. Survivin occupies a central position because of overexpression in cancer cells [122]. It is hypothesized that survivin overexpression in tumor cells facilitates tumor progression by numerous pathways, such as dysregulation of apoptosis and cell division, promoting survival of cancer stem cells, altering sensitivity to antitumor drugs. Survivin can ideally, serve as a universal tumor antigen because it is expressed in most human malignancies and has the ability to trigger immune effector responses [123]. Therefore, blocking survivin functions by different immunotherapeutic or molecular approaches is emerging as a promising therapeutic strategy, in cancer [124].

Survivin is encoded by BIRC5 gene and is composed of 4 exons and 3 introns covering 14,796 nucleotides on chromosome 17q25. It forms transcripts with multiple functional domains. BIRC5 gene encodes wild type survivin (WT, four

exons; 142 amino acid) additionally, five known splice variants i.e.; ΔEx3 (Survivin with deletion of exon 3; 137 amino acid), 2B (survivin with an additional exon; 165 amino acids), 3B (five exons; 120 amino acid), 2α (2 exons;74 amino acids), 3α (two exons;78 amino acids) [125].

All of survivin's isoforms share complete sequence identity in the N-terminus region, including some or the entire BIR domain, but they are different in the carboxyl end (Figure 17). Survivin isoforms also present variable expression patterns and cellular localization as compared to wild type form. Survivin- ΔEx3 is identified predominantly in the nucleus whereas Survivin- 2B is found in the cytoplasm [126]. Alternative splicing of survivin has been observed to have correlation with disease activity, in different patient studies. Survivin WT, 2B and ΔEx3 variants have all been extensively investigated for clinical and prognostic association and applicability, in cancer. The presence of ΔEx3 variant has been associated with unfavorable clinical outcome and prognosis [127]. The data that exist for clinical and pathological correlation of variant 2B in cancer, remain conflicting; some studies demonstrate association of 2B variant with exacerbated disease and poor survival rates, while other studies suggest that presence of 2B variant is associated with less severe disease [128]. Overall, there is a common consensus that ΔEx3 has anti-apoptotic properties and 2B has pro-apoptotic function and that these variants may be involved in contrasting functions in tumor progression and response to therapy [129]. Presence of survivin isoforms has also been shown to influence angiogenesis [130]. But whether, alternative splicing of survivin is an adaptation used by cancer cells to sustain their proliferation and escape detection by immune surveillance, still remains unclear. The association of splice variants with distinct pathological and survival outcomes, indicates possible role of these variants in disease progression.

Although, a few human adult tissues. express survivin, like vascular endothelial cells, primitive hematopoietic cells, erythroid cells, adult peripheral blood T cells, polymorphonuclear neutrophils, megakaryocyte, cells of colonic and gastrointestinal mucosa, placenta, testes, ovary, neurons, melanocytes etc., the presence is almost minimal, in most terminally differentiated tissues [116]. The more mature and differentiated the tissue, the lesser the expression of the protein.

Instead, survivin appears to interfere with angiogenic regulation, vascular remodeling, cell cycle regulation and proliferation, erythropoiesis, maintenance of normal adult hematopoiesis, megakaryocyte maturation, apoptosis inhibition in neural development hepatocyte proliferation, spermatogenesis, oogenesis and self-renewal and differentiation of cryptic stem cells [131].

Despite the fact, that the majority of normal tissues does not express the splice variants, some tissues do express minimal amounts of certain isoforms. S-ΔEx3 expression is found in vascular endothelial cells. Additionally,S-ΔEx3 is the only isoform expressed in adult cerebrum, whereas fetal brain tissues disclosed the expression of WT and S-2b, as well. Minimal expression of S-2α has also been discovered in normal brain cerebellum and breast tissues. Expression of other splice variants is unknown, till date [132].

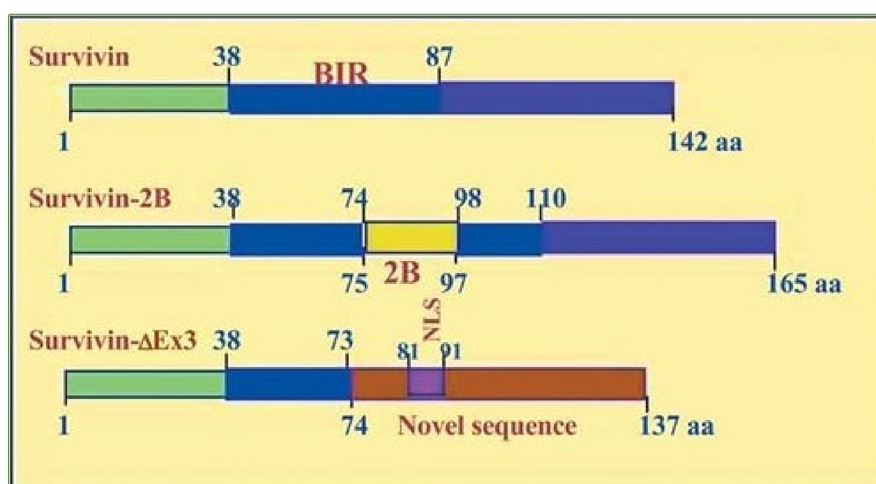


Figure 17 Survivin isoforms

Survivin is predominantly localized in the cytosol of tumor cells (Figure 18). However, a smaller nuclear fragment of survivin localized in kinetochores of metaphase chromosomes has also been reported in tumor and proliferating cells, indicating that these different subcellular pools of survivin may have different functions. Cytosolic survivin is thought to function as apoptotic suppressor while nuclear Survivin is speculated to regulate cell division [133]. However, the pathological significance of nuclear survivin as a favorable prognostic marker for tumor cells, is still debatable. The data from patient studies are so far, equivocal regarding the role of nuclear/cytosolic Survivin expression as an unfavorable or favorable prognostic marker in cancer. Apart from the cytosolic and nuclear pool,

survivin has also been found in mitochondria and was shown to be released in the cytosol, in response to cellular stress stimuli. There, it is believed to suppress caspase activation [134].

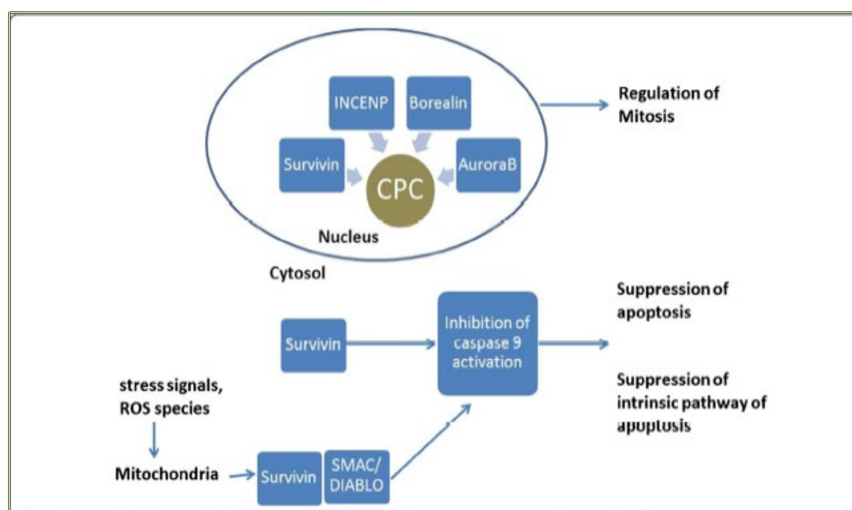


Figure 18 Localization of Survivin

Extracellular pool of survivin has also been found to exist as exosomes in form of 40–100 nm membrane vesicles, that are secreted from tumor cells and taken up by surrounding cells. In fact, many IAPs such ascIAP1, cIAP2 and XIAP including survivin have been shown to exist as tumor exosomes in cancer cell lines. Exosomes carrying survivin, have been shown to re-enter the cells and promote tumor growth. Extracellular pools of survivin have the ability to stimulate neighboring cancer cells and increase their resistance to therapy, rapidly proliferate, and achieve an increased potential for becoming invasive, in vitro [135]. Elevated levels of plasma derived exosomal survivin from prostate cancer patients has been found to correlate with disease severity. Thus, both intracellular and extracellular expression of survivin in cancers, can be responsible for aggravated disease [136].

The Multiple roles of Survivin

Cell proliferation

Regulation of cell division is accepted as prominent function of survivin. Normal cells exhibit cell cycle dependent synthesis, expression and degradation of survivin. Survivin constitutes an integral part of the Chromosomal Passenger Complex (CPC) which ensures correct segregation of chromosomes and

cytokinesis during cell division [137]. Numerous checkpoints ensure proper nuclear division, correct attachment to mitotic spindle and cytokinesis. CPC is a hetero-tetrameric complex which is found in different sites, at different times during mitosis. This helps in regulating key events, in cell division such as proper spindle assembly, chromosome-microtubule attachment and occurrence of cytokinesis [138].

Aurora B kinase is known as the enzymatic component of CPC, whereas, the other three components; inner centromere protein (INCENP), survivin and Borealin (also known as Dasra) have targeting and regulatory functions. Changes in any of the four components can result to a defect in chromosomal segregation and/or cytokinesis and cause genomic instability [139]. Analysis of the separate individual contribution of these proteins to the formation of CPC, implies that the enzymatic part of Aurora B kinase is directed in the mitotic cell, by the other three proteins of CPC, namely INCENP, survivin and Borealin/Dasra. INCENP appears to act as a scaffold protein that stabilizes the complex. Borealin's function is, to facilitate binding of survivin to INCENP and survivin acts as a determining agent, in centromere localization of CPC [140]. Although, survivin protein carries a central role in mediating CPC targeting, other proteinic components of CPC act, to secure a stable structure. Additionally, a specific pool of subcellular survivin is connected to polymerized tubulin and regulates microtubule formation, during cell division [141].

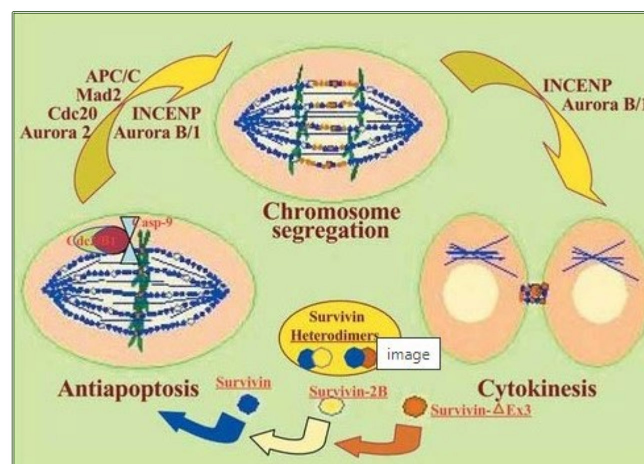


Figure 19 Role of Survivin in mitosis

Metastasis, angiogenesis and chemo-resistance of tumor cells

One of the various pathways responsible for survivin mediated tumor progression is stimulation of angiogenesis, in cancer cells. Survivin upregulates VEGF expression and encourages endothelial cell (ECs) proliferation by mechanisms which are still not clear. Existence of a positive feedback loop that connects survivin expression in tumor cells, to PI3K/Akt enhanced β-catenin-Tcf/Lef-dependent transcription induction of VEGF secretion and angiogenesis, has been proposed [142]. Blocking of survivin in glioma has been shown to inhibit angiogenesis. Small interfering RNA (siRNA) that mediate silencing of survivin, sensitized human breast cancer cells to apoptosis and inhibited tumor formation and angiogenesis in breast or cervical cancer xenograft model, in vivo [143].

Survivin induced VEGF expression also, contributes to chemo-resistance by stimulating the assembly of tubulin into distinctly organised fibers [144]. Survivin is especially overexpressed in tumor vascular ECs as compared to normal tissues, and in this way, it appears to be conferring drug resistance on tumor vascular ECs [145]. It is evident therefore, that targeting survivin in tumors, will promote not only, tumor cell death but also it will sensitize the cells of tumoral vascular network to chemotherapeutic drugs. Survivin may also, cooperate with other IAP protein members to promote metastasis. Survivin upregulation, enhanced migration of human melanocytes and melanoma cells on fibronectin whereas survivin knockdown under sub-apoptotic conditions blocked their migration and invasion [146].

Inter-molecular interaction between XIAP and survivin accelerates tumor cell invasion, in vitro and metastatic propagation, in vivo, in murine model of breast cancer and rat insulinoma [115]. This pathway operates independently of the role of IAPs in cell survival. Signal transduction through this process resulted in transcriptional upregulation of fibronectin, NF-κB activation, autocrine/paracrine signaling by β1 integrins, and consecutive phosphorylation, i.e., activation of cell motility kinases, FAK and Src. This implies direct involvement of IAPs in promoting metastasis. Most importantly, signal transduction via this pathway did not produce the usual Epithelial Mesenchymal Transition (EMT), but rather provoked an adhesion gene signature and many fold increase in expression of

fibronectin gene, in tumor cells [147]. Survivin has also been proven to enhance melanoma cell metastasis through integrin upregulation. Survivin induced breast cancer lymphatic metastasis, through cooperation with vascular endothelial growth factor-C (VEGF-C) [148]. Multiple patient studies have also, indicated that overexpression of survivin corresponds to increased tumor invasion and metastasis, thereby suggesting a wider role for survivin, beyond regulation of apoptosis and cell division of cancer cells [149].

Stem Cells

Increased survivin expression has been reported in early Embryonic Stem cells (ES) and subsequently, it reduces during fetal development [150]. Also, survivin is found overexpressed in some adult stem cells. Survivin expression in apoptosis regulates stem cell layer of stratified epithelia of skin, human fetal tissues including thymic medulla, gastrointestinal tract and endocrine pancreas. In this way, the protein is assisting in the viability of stem cells, in a developmentally conformed fashion, thus helping in tissue homeostasis and differentiation [150]. The measurement of the protein expression in umbilical cord blood cells and adult bone marrow CD34+ cells and umbilical cord blood cells showed that, the protein is regulated by hematopoietic cytokines and depends on hematopoietic growth factors. The protein expression is found in all the stages of cell cycle and showed an inverse correlation with apoptosis, in a caspase dependent manner [151].

Survivin upregulation is a mandatory procedure for entering to the cell cycle, maintenance and self-renewal in normal cord blood CD34+ cells, hematopoietic stem cells and progenitor cells (HSPCs) [152]. In human embryonic stem cells (HES) exposed to ionizing radiation, the expression of survivin along with checkpoint kinase 2 (Chk2) helps to amend DNA damage without the dependency on p21, to support cell cycle arrest [153]. High survivin levels correlate to stability of the state of pluripotency in HES and induction of pluripotent stem cells (iPSCs) [154]. It has been shown that the protein is involved in the regulation of cell proliferation and signaling pathways in stem cells.

A study conducted to clarify the role of survivin in pluripotency, disclosed a significantly higher expression of the protein in human embryonic stem cells, (nearly 9-fold) in comparison to that in differentiated cell types [155]. This IAP is

involved in regulating basal and growth factor dependent survival, differentiation, proliferation, and migration of mouse and human mesenchymal stem cells (MSCs). The expression is shown, in all stages of interphase with extremely high expression in G2/M phase. Increased survivin expression protects MSCs that produce HSCs, when HSCs-supportive niche is depleted post irradiation exposure [156]. In addition, the protein has been linked to increased survival in these cells subjected to stress, produced by heat shock, UV or etoposide, as well [157]. CD44+/CD105+human amniotic fluid MScs, that are ideal for stem cell transplantation, because they proliferate rapidly also, express high levels of Survivin. The participation of hematopoietic adipocyte-derived stem cells (ASCs) in apoptotic resistance demostrated by adipocyte tissue in obese subjects, was correlated to upregulated survivin expression. At the same time, Keratinocyte Stem Cells (KSCs) show increased expression of survivin, that plays a critical part in preventing abnormal mitosis in these cells [158]. The availability of survivin, as a marker for KSCs, that helps in maintaining skin homeostasis, is under investigation [159].

Also, the role of survivin in the regulation of stem cell functions has been demonstrated in neuronal stem cells and intestinal stem cells [160]. HES expresses S-ΔEx3, S-2b, S-2α, S-3b along with WT in various subcellular areas. The expression is consistently elevated in HES, when compared to further differentiated cell types, like MSCs. Survivin isoform, S-ΔEx3 exhibits the highest expression levels followed by S-2b. The other two isoforms are expressed in negligable quantities. S-3γ and S-3γV isoforms with expression higher than WT are responsible for imparting growth factor independent growth in HSPCs. KSCs as well express S-ΔEx3 and S-2b at higher levels than S-2α, S-3b [161].

Cancer stem cells

The presence of cancer stem cells has been proposed as the primary reason for occurrence of cancer and disease relapse. It is hypothesized that tumors are maintained by a subset of tumor stem cells possessing a self-renewal capacity similar to that of stem cells. The role of survivin has been demonstrated in the regulation of adult stem cell physiology such as in hematopoietic stem cells, neuronal stem cells or intestinal stem cells [162]. Survivin is also crucial for

embryonic stem cell and totipotent stem cell function. Since normal stem cells and cancer stem cell share these common features, it is acceptable to assume that like, in normal stem cells, survivin expression on cancer stem cells can also, be involved in regulating cancer stem cell behavior [132].

An investigational study of co-expression of survivin and stem cell specific proteins in esophageal squamous cell carcinoma (ESCC) patients disclosed that those patients that exhibit overexpression of both the stem cell specific protein Oct-4 and survivin, revealed worst prognosis. Survivin expression correlates with Oct-4 expression in ESCC cells, suggesting a probable regulation-interaction between Oct-4 and survivin [163]. The molecular mechanisms that underly the regulation-interactions between survivin and stem cell specific proteins are still not entirely understood. Survivin appears also, to play a role in regulating specifically, the genes involved in Leukemia Cancer Stem Cell (LCSC) fate but not in normal hematopoietic stem cell (HSC). This difference in survivin signaling in LCSC vs HSC, provides new avenues for specific therapeutic targeting and elimination of cancer stem cells [164].

The upregulation of survivin in the majority of tumors as well as in the early embryonic stages, when combined with the protein's relative absence in most normal adult tissues, classifies survivin as a classic oncofetal gene. A deregulation in the usual expression pattern seems to result in survivin overexpression, through the cell cycle. This aberrant expression is detected in transformed cells and is found to be mediated by oncogenes or tumor suppressor genes, thus explaining the selective expression of survivin gene in cancers [165]. Molecular analyses studies compared increased survivin expression to more aggressive and invasive tumor behavior, poor prognosis, reduced response to drugs, abbreviated survival and increased recurrence [166].

A definite majority of malignancies, including breast, blood, colon, lung, ovarian, uterus, liver, uterus, astrocytoma, glioblastoma, meningioma, bladder, prostate, bladder, gastrointestinal, melanoma, nonmelanoma skin cancer, soft tissue sarcoma and viral induced neoplasms, demonstrate overexpression of survivin at different stages in tumor progression [167-170]. Additionally, overexpression of the protein acts as a critical factor for radio-resistance and chemoresistance, in variable

cancers [171, 172]. The presence of survivin may be detected in body fluids of cancer patients, by using survivin antibodies circulating in blood, an action that renders survivin an effective diagnostic marker [173].

The detection of survivin from the urine samples of bladder cancer patients, proves to be a simple and sensitive diagnostic tool, for determining new or recurrent bladder cancer [174]. A study conducted, to identify survivin positive circulating breast cancer cells in peripheral blood, identified its appearance in more than half of breast cancer patients, while being absent in healthy controls [175]. Several studies involving survivin levels in serum, also demonstrated survivin, to be a reliable and sensitive diagnostic biomarker for tumors. The protein's increased expression, in hematological malignancies, has suggested the possibility of using this molecule as a regular biomarker for detection of these types of diseases [176].

Isoform S-ΔEx3 has been shown to be expressed in a wide variety of cancers and is often found in association with malignant tumors. This isoform appearance has been demonstrated as indicative of tumor grade and invasiveness [177]. Studies conducted on S-2b reported, as mentioned earlier, conflicting results regarding the expression of the isoform [127, 177]. Some reports proposed that the expression can be positively correlated with adverse clinical outcome while a few other reports revealed an inverse correlation. The expression was reported to be more in benign or early-stage tumors. Certain cases of breast cancer documented S-2α, S-3α and S-3b expression along with WT, S-ΔEx3 and S-2b. S-2α expression has also been reported in medulloblastoma. The expression of variants ΔptEx2/3 and ΔptEx1/2 is observed in acute myeloid leukemia (AML). The potential use of S-3α and S-ΔEx3 as diagnostic markers for breast and papillary thyroid cancers, respectively, is currently under investigation [125].

Survivin materializes, as well, as a core regulator of clonal expansion of Teff cells, activated mature B cells, proliferation of early B cell progenitors and activated mature B cells, erythroid and megakaryocyte differentiation [178].

Due to the critical role of survivin in tumor cell apoptosis, division, chemo-resistance and cancer stem cell survival; therapeutic arrest of survivin in tumor cells may very likely yield significant, cumulative benefits. Numerous strategies

have been envisioned to block the expression or function of survivin in tumor cells: a) immunotherapeutic approaches to produce immune responses against survivin, b) small molecule inhibitors-antagonists to block the actions of survivin, c) nucleic acid-based treatments that interfere with survivin gene expression or d) gene ablation of survivin to down regulate cell cycle division and induce apoptosis.

6.2 PTEN

Phosphatase and tensin homolog (PTEN) is a fundamental tumor suppressor that arrests cell growth and augments cellular sensitivity to apoptosis. Since, it was first discovery in 1997, the role of PTEN as a biomarker in cancer pathology, has become increasingly significant [179]. In contemporary studies, any alterations in this gene and/or its protein expression are regarded as applicable molecular hallmarks, meaning that their presence has bearing on clinical decision-making [180].

Loss or altered PTEN function has been discovered in a wide spectrum of neoplasms, thus being considered an instigator genetic event for tumorigenesis and tumor evolution. PTEN function can be either dependent upon or independent of the down-deregulation of the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) pathway [181]. Accumulating data propose that PTEN is also involved in the overall DNA damage response and the designing of the adaptive component of the anti-tumor immune response [182]. The practicality of identifying PTEN alterations in clinical practice remains a matter of controversy, given the lack of additional diagnostic tests for their investigation. Furthermore, due to the three-dimensional level of alterations (i.e., DNA, mRNA, and protein expression) involved in PTEN function, the development and validation of a single test approach, that would cover all the clinically significant variations are extremely intricate. In spite of the insights offered by several studies over the past two decades, the multifaceted biology of PTEN remains, not entirely understood [183].

The PTEN gene (10q23.31) consists of 9 exons and a variable additional exon (i.e., exon 5b) that is omitted in the principal transcript [184]. A highly conserved sequence upstream of the promoter involves a canonic E-box sequence, which is responsible for PTEN transcriptional activation. PTEN is a dual-specificity protein phosphatase that is formed of 403 amino acids, that span across five functional domains and has a major enzymatic activity on phosphatidylinositol (3,4,5)-trisphosphate (PIP3). The first 14 residues at the N-terminal region comprise the phosphatidylinositol 4,5-bisphosphate (PIP2) binding domain (PBD). Different

oncogenic mutations can target this region, resulting in a reduced affinity of PTEN for the cell membrane [184].

The protein tyrosine phosphatase (PTPase) domain constitutes the active site of PTEN. This wide and positively charged pocket area is capable to integrate the negatively charged, large PIP3 substrate. Located at the C-terminal region, the PTEN type II calcium-independent (C2) domain appears to interact nonspecifically with the cell membrane. The elaborate interaction between the C2 domains and PTPase, and their critical involvement in PTEN regulation, have resulted in their characterization as a “super-domain” [185]. Numerous driver mutations lead to the breakdown of the PTPase-C2 boundary, with subsequent PTEN inactivation. The PTEN C-terminal extremity consists of a carboxyl-terminal tail (C-tail) and a PDZ-binding domain (PDZ-BD) that acts as a protein-protein interaction repeated pattern [181]. Premature stop codons that can remove the C-tail are driver genetic events for tumorigenesis and tumor expansion [186].

The spectrum of potential post-translational modifications of the PTEN C-terminal region is exceedingly heterogeneous. Phosphorylation in C-tail and C2 domains of the C-terminal region facilitates their interaction, that results in the “closed” configuration of the phosphorylated form of PTEN.

As a consequence of this biochemical modification, the interactions between PDZ-BD and PDZ domain-containing proteins in the cell membrane are hindered [187]. Auto-dephosphorylation reverses this conformational alteration, to “open” status, allowing PTEN to bind to the membrane and PDZ domain-containing proteins. As a general rule, phosphorylation events reduce PTEN activity by increasing its chemical stability. Other post-transcriptional modifications include oxidation, ubiquitination, acetylation, and small ubiquitin-like modifier (SUMO)ylation [188].

Downregulation of PI3K/Akt/mTOR Pathway

The phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) is the most frequently upregulated pathway in human cancers [189]. It is profoundly interwined in many aspects of cell processes, i.e., growth, proliferation, survival, metabolism, and immune response regulation [190]. Abnormal activating events targeting PI3K/Akt lead to a deep disruption of these processes, which

will ultimately lead to tumorigenesis, metastasis, tumor progression, and therapy resistance [191]. PI3Ks are grouped into three categories, based on their substrate specificity and their structure. In specific, class I PI3Ks are a group of signal transducers of tyrosine kinases, small GTPases and G protein-coupled receptors (GPCRs), while class II and class III PI3Ks influence signaling indirectly by mainly influencing membrane trafficking [192]. Activation of G protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs) and chemokine receptors (CKRs), on the cell membrane by specific ligands results in autophosphorylation on tyrosine residues [190].

The three classes of PI3K are, as a consequence, recruited and activated, leading to the production of the second messenger PIP3 from the substrate PIP2. This mechanism results in the recruitment of signaling proteins, including Akt, protein serine/threonine kinase-3 and phosphoinositide-dependent kinase 1 (PDK1) [190]. This process is the core element of cell survival and the control of cell cycle progression. Following the phosphorylation event, the serine-threonine kinases are fully activated, either by the mTOR complexes, the PDK1 or other kinases. Akt is directly blocked by the dephosphorylation from phosphatase domain and leucine-rich repeat protein phosphatase (PHLPP) [193].

In contrast, the negative regulation of mTORC1 and PIP3 function by PTEN, liver kinase B1 (LKB1), tuberous sclerosis 1 (TSC1) and tuberous sclerosis 2 (TSC2) lead to the indirect inactivation of Akt [194]. A significant part of the PTEN tumor suppressor role is expressed by the negative regulation of the PI3K/Akt/mTOR pathway [195].

Loss of PTEN influence leads to the stable activation of the PI3K/Akt signaling, with subsequent abnormal cell survival, growth and proliferation (Figure 20). It is important to stress, however, that another important part of the PTEN tumor-suppressor activity is outside the PI3K/Akt axis [196].

Biochemical Functions in Cancer Metabolism

PTEN, as lipid–phosphatase acts as down-regulator of the class I phosphatidylinositol 3-kinases (PI3Ks) which phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to conform the second messenger phosphatidylinositol-

3,4,5-trisphosphate (PIP3) [197]. The PIP3 promotes molecular signaling, involving the activation of AKT kinases, which act on molecular targets important for different biological roles, like cell proliferation and regulation of cell growth, vesicle trafficking, angiogenesis, anabolic metabolism, angiogenesis and cancer [198]. Therefore, PTEN is critical for the control of the nutrient-responsive signaling process involved in protein synthesis and transcription [196].

Activated AKT is a key regulator of oncogenic metabolism. It is proposed that AKT stimulation advances the glycolytic metabolism of tumor cells. The activation of AKT, that results from PTEN loss, stabilizes the enzyme phosphofructokinase-1 (PFK1), leading to promotion of glycolysis, cellular proliferation, and tumor growth [199]. Phosphorylation of AKT augments cellular glucose uptake and is crucial in inducing glucose transporter 1 (Glut1) and Glut4 translocation to the cell membrane of adipocytes [200].

In addition, AKT activates the mTOR, a serine/threonine kinase that an important part of two distinct complexes, the mTOR Complex (TORC) 1 and TORC2, which directly regulate cell metabolism and growth in response to environmental signals [201].

The mTOR pathway is one of the most disrupted signaling mechanisms in human cancer, and constitutive activation of mTORC1 is very commonly observed in various tumors [202]. Indeed, mTOR exerts its action on many anabolic pathways preserving cell proliferation, such as glycolysis and the pentose phosphate pathway (PPP) and glycolysis, through regulation of hypoxia-inducible factor (HIF)1.

It also activates lipid synthesis by stimulating the transcription factor sterol regulatory element-binding protein (SREBP)1 and nucleotide synthesis. through regulation of the PPP and by activation of an enzyme of pyrimidine composition [203]. The PI3K–AKT–mTOR pathways afford a critical role in cancer development.

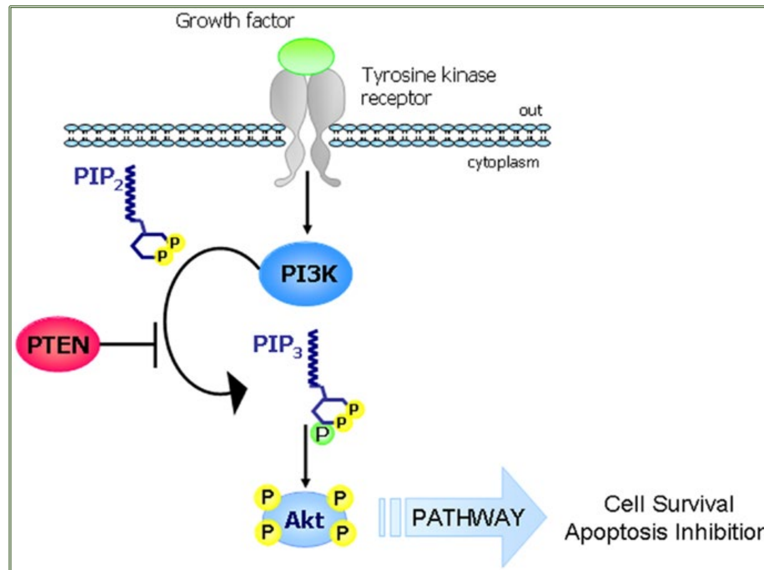


Figure 20 PTEN loss

This role is facilitated, through an increased number of elements within the cascade, whose level or activity is found altered.

Recent studies propose that tumor suppression activities for PTEN are exerted at the biochemical metabolic pathways [204]. The phosphatase function of PTEN controls phosphoglycerate kinase 1 (PGK1) glycolytic enzyme, which is capable of autophosphorylation.

Loss of PTEN function in cancer cells results in increased PGK1 autophosphorylation, ATP production and glycolysis, induction of cancer cell proliferation and tumorigenesis. PTEN presents multiple functions that occur in various cellular compartments for which the phosphatase domain is essential to inhibit cancer development. Consequently, PTEN may serve as one of the main targets for cancer therapy [205].

The Diverse Role of PTEN on Cell Metabolism

Cancer cells can reprogram their metabolism to preserve abnormal cell proliferation, their survival, and long-term maintenance. The most common characteristic of this altered metabolism is increased glucose uptake and fermentation of glucose to lactate.

This phenomenon is discovered even in the presence of completely normal functioning mitochondria and is known as the Warburg effect [206]. This is the most important metabolic hallmark of cancer cells which proves that cancer cells

prefer the glycolysis pathway even in the presence of normal or high oxygen tension.

Despite the fact that aerobic glycolysis is an inefficient mean of generating ATP compared to the amount obtained by mitochondrial respiration, cancer cells assume this metabolic reprogramming as their approach to energy compensation [207]. The reason being, that the rate of glucose metabolism through aerobic glycolysis is accentuated, such that the production of lactate from glucose occurs 10–100 times faster, when compared to the complete oxidation of glucose in the mitochondria. The amount of ATP synthesized over any specific period of time, in cancer cells, is comparable when either pathway of glucose metabolism is utilized [208].

In support of this concept, microarray analysis shows that genes relevant to the glycolysis pathway exhibit increased expression, in the majority of clinically relevant cancers. In addition, the upregulation of plasma membrane glucose transporters and alterations in important enzymes involved in glucose utilization have been found in many tumor types [208].

It is possible that this procedure emphasizes in the importance of avid uptake of glucose, even when its availability is becoming depleted, because of the continuous expansion of the tumor. Some tumors present increased expression and activity levels of PFK1, PFK2, hexokinase (HK) isoforms, aldolase (ADO), enolase (ENO), phosphoglycerate kinase (PGK) and pyruvate kinase (PK) which assist pyruvate production from glucose breakdown [209].

Inhibition of glycolysis in tumor cells is now, considered an alternative treatment strategy for cancer patients; meaning that, drugs targeting the abovementioned regulating enzymes in tumor glycolysis could have new promising applications [210].

Various oncogenic pathways, like Myc, PI3K, or Ras-dependent promote glycolysis instead of oxidative phosphorylation, while multiple tumor suppressors such as Von Hippel–Lindau (VHL), p53 or liver kinase B1 (LKB1) negate the "Warburg effect" [211].

Accordingly, in vivo experiments with tumor suppressor PTEN overexpressed to different levels, have shown mouse embryonic fibroblast (MEF) metabolic changes, where less glucose is utilized, but it is more efficiently absorbed in the mitochondrial Krebs cycle, consistent with an "anti-Warburg effect". More precisely, PTEN tg MEFs reveal higher levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), which is a transcriptional coactivator that controls mitochondrial biogenesis and energy metabolism. These mice present increased oxygen consumption and energy expenditure [212].

Moreover, MEFs exhibit an increased number of mitochondria, together with increased ATP production and oxygen expenditure and decreased levels of lactate secretion. All these characteristics indicate that PTEN reduces the glycolytic rate and promotes the oxidative phosphorylation.

In summary, PTEN tg mice develop an unexpected cancer-proof and very unique metabolic state which is the result of PTEN capability, to control metabolism at different levels both in the cytosol and in the nucleus [213].

Glucose consumption is limited in cancer cells, by PTEN, which prevents the expression of Glut1 on cell membrane. At the molecular level, PTEN obstructs AKT activation which regulates the localization of Glut1 in the cell membrane [208]. The regulator of glycolysis HK2, is increased by the simultaneous loss of PTEN and p53. The process of PTEN deletion dependents on the activation of the AKT–mTORC1 and HK2 protein synthesis. In prostate cancer experimental models with PTEN/p53-deficiency, aerobic glycolysis dependent on HK2 promotes tumor growth [214]. Recently, it has been proposed that the regulation of PTEN/AKT/HK2 could be targeted to overcome cancer resistance to cisplatin treatment [215].

Additionally, PTEN reduces the levels of pyruvate kinase muscle isozyme (PKM) 2 which catalyzes the last step of glycolysis and its expression is known to be associated to the "Warburg effect" of cancer cells [216]. The transcription of PKM2 is induced by mTOR, so that decreased levels of PKM2 are discovered in PTEN transgenic cells. Interestingly, PTEN counteracts the glyoxalase dependent PI3K/AKT/mTOR/p-PKM2(Y105) pathway that induces an increased glycolytic rate and cell proliferation, in prostate cancer [217].

Loss of PTEN, via suppression effects on anaphase-promoting complex (APC) and its coactivator Cdh1-mediated ubiquitination, could achieve stability to the 6-phosphofructo-1-kinase/fructose-2,6-biphosphatase isoform 3 (PFKFB3) family member. PFKFB3 is critical for the first commitment step of glycolysis and its activity has been implicated in cancer [206].

Glutaminolysis, as well as glycolysis, is another mainstay for energy production and anabolism in cancer cells. PTEN loss results in a hyper glycolytic phenotype which would render T-cell acute lymphoblastic leukemia (T-ALL) cells resistant to Notch signaling pathway inhibition, with the fact that the aberrant activation of Notch is present, in over 60% of T-ALL cases [218].

Moreover, the same T-cells appear less sensitive to inhibition of glutaminolysis as a result of elevated glucose-derived carbon input to the Krebs cycle. Notably, PTEN can control glutaminolysis as a critical point in metabolic reprogramming, affected by Notch.

The glutaminases (GLS1 and GLS2) produce glutamate from glutamine and activate the first step, in the glutaminolytic pathway. Glutamine absorption is reduced by PTEN due to the concomitant degradation of GLS1, which is pro-oncogenic; however, GLS2 is anti-oncogenic [219],[220]. The oncogene Myc upregulates GLS1 while the onco-suppressor p53 stimulates GLS2 [221]. In keeping with these data, PTEN blocks the glutaminase GLS1, further sustaining the tumor-suppressive function of PTEN, in cancer metabolism.

In particular, studies performed on the effects of suppression of PTEN expression by a specific miRNA, such as miR-181a, reveal increased AKT phosphorylation and lactate production, resulting in cell proliferation [222]. Notably, miR-181a via PTEN, is a key determining factor of metabolic reprogramming in colon cancer, while no notable changes in the main components of mTORC2 are identified.

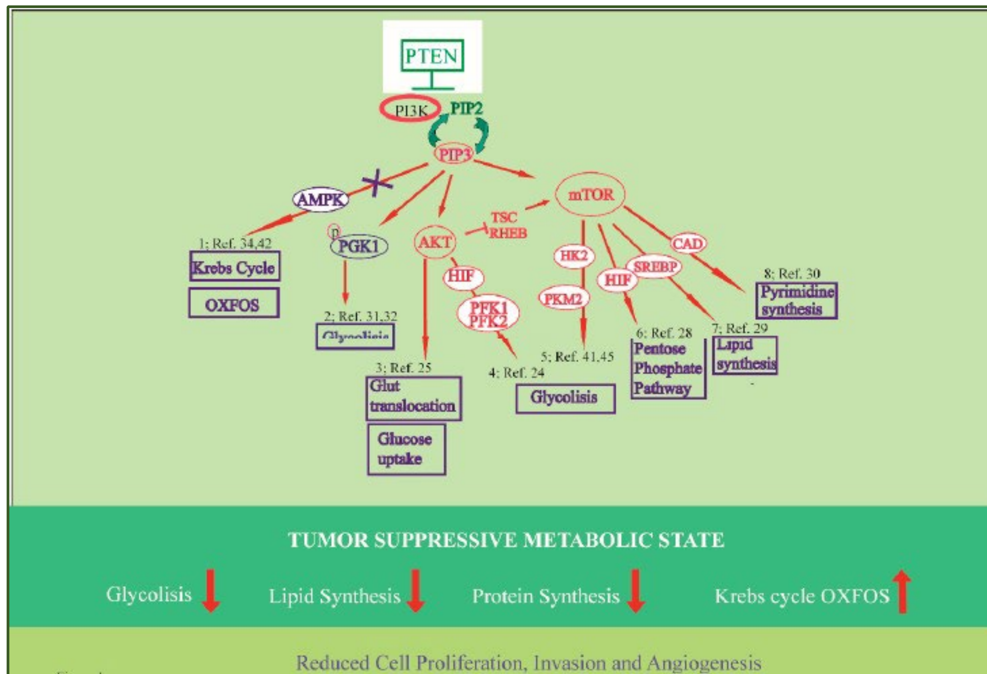


Figure 21 The multiple actions of PTEN

The Krebs cycle takes a central position in metabolism and fulfills most of cell energy requirements by complete oxidation of acetyl-CoA, an important product in the catabolism of carbohydrates, amino acids and fatty acids, to CO2. Recent studies in non-transformed thyrocytes of a PTEN-deficient mouse model, revealed that constitutive PTEN loss influences Krebs cycle and oxidative phosphorylation, leading defective mitochondria to compensatory metabolic switch and glycolysis. In addition, impairment of the Krebs cycle is connected to pathological conditions such as cancer. Genetic and epigenetic modifications of Krebs cycle enzymes encourage the shift of cancer cells from oxidative phosphorylation to anaerobic glycolysis [223].

Moreover, recent information from transgenic mice models, that carry additional copies of PTEN (PTEN tg) suggest that upregulation of this gene produces a tumor suppressive metabolic state [224]. The PTEN tg mice displayed homogeneous and systemic and homogeneous PTEN overexpression (2–3 fold higher than normal mice) and shared remarkably overlapping phenotypes. Elevation of PTEN results in normal metabolism indicated by elevated energy consumption and diminished body fat accumulation (Figure 21). Cells derived from these mice prove resistant to oncogenic alteration and exhibit reduced glucose and glutamine uptake and elevated mitochondrial oxidative phosphorylation [224]. These data demonstrate

that PTEN is a key nodal element for the regulation of tumorigenesis, related to dysregulation of cell metabolism. Interestingly, PTEN plays also, a central role in insulin-mediated oxidative stress and DNA damage in human hepatocyte cell lines and in vivo animal models [225]. Increased levels of reactive oxygen species (ROS), stress-proteins, and genomic damage in the liver of PTEN haplo-deficient mice produced by a high fat diet, is advocated to further support a causative role of PTEN in hepatic and extrahepatic tumorigenesis, observed in obese patients [226].

Notably, in PTEN tg mice, oxidative phosphorylation is accentuated together with the elevated amount of ROS [224]. Since, the increased expression of PTEN is associated with cancer resistance, this increase in ROS levels can not sufficiently produce relevant effects on DNA. Besides, it should be considered, that PTEN promotes the transcription of genes that mediate antioxidant functions through the Forkhead box O (FOXO)3 transcription factors [227].

PTEN modulation of DNA damage response

PTEN although, once considered a strictly cytoplasmic protein, is now proven to be present and functional in the mitochondria, nucleus, endoplasmic reticulum, and the extracellular space [228]. Specifically, PTEN action in the cellular nucleus is crucial for tumor suppression, separately of PTEN phosphatase function. However, there is gap in understanding how the dysregulation of PTEN nuclear functions influence tumorigenesis [229].

PTEN is located within the centromere, where it normally interacts with proteins that are important for centromere conformation and stabilization, such as centromere-specific binding protein C (CENP-C) [230]. The stable condition of the centromere is the result of RAD51 expression by PTEN modification. RAD51 is considered to be a key component of the double-strand breaks (DSB) homologous recombination (HR) DNA repair systems, so PTEN is currently viewed as a DNA-damage response control [231]. Especially, PTEN loss can lead to DSB, through increased Akt-mediated cytoplasmic sequestration of the checkpoint kinase 1 (CHK1). So that, in response to genome damage, PTEN loss will result in altered G2/S arrest [232]. Additionally, it has a key role in controlling and maintaining the integrity of various checkpoints during the G1-S and G2-M cell cycle transitions.

Specifically, the intra-S checkpoint is a critical DNA damage stage which blocks cell cycle progression to favor repair of DNA damage [233]. Accordingly, radiation-induced DNA damage combined with an impaired PTEN activity, has been linked to an accelerated transition from G2/M to G1, while PTEN phosphorylation leads to expedited G2-M transition [234]. Furthermore, an additional nuclear function of PTEN participates in its interaction with p53, which eventually leads to G1 arrest [235]. Another mediator of PTEN-related cell cycle regulation is represented by histone acetyltransferase (HAT). The interaction that exists between PTEN and HAT is particularly noticeable in the management of chromatin dynamics and global gene expression. Moreover, with its C-terminal domain, PTEN is able to bind histone H1 and to preserve a physiological chromatin condensation [236].

The variable functions of PTEN, the input across different cellular procedures and the interactions with various cellular components emphasize the important role that allows scientists to address it, as a novel guardian of the genome [237].

Another important observation that establishes the function of PTEN, in controlling DNA damage response is exhibited by its expression in endometrial tumors. Notably, loss of PTEN expression and microsatellite instability (MSI) are found to be two of the most common molecular alterations in endometrial carcinoma [238]. Indeed, in the MSI mutated molecular subgroup, PTEN reveals high mutational rates and reduced protein expression [239]. These modifications coincide with those in phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit, alpha isoform (PIK3CA), both in the mismatch repair (MMR) deficient-MSI and the copy number-low subgroups. It has been proposed that the 5'-polyadenosine tracts in PTEN might be a possible target for mutations in MMR-deficient tumors[240]. Subsequently, numerous studies have disclosed that many PTEN mutations associated with MSI-high (MSI-H) status appear with increased frequency in the poly(A6) regions, compared to PTEN mutations that are found in Microsatellite Stable Tumors (MSS) , particularly in endometrial cancer and colorectal cancer [241]. In non-endometrioid endometrial carcinomas, PTEN-retained and/or wild-type status co-occur significantly, with a preserved positive expression of the MMR proteins [242]. Additionally, significant correlation

between MMR proficiency, PTEN wild-type expression, and a better outcome have been reported, in breast cancers [240]. These data can be of clinical importance, not only for Lynch syndrome screening (e.g., endometrial cancer, colorectal cancer) but at the same time, for prognosticating and immunotherapy prediction (e.g., breast cancer) [243]. Genomic alterations in the MMR system are relatively rare in breast cancer, being reported in ~2% of cases [244]. However, this subject is ambiguous in literature, given the scarcity of diagnostics and/or tumor-specific guidelines for MMR analysis [240]. Unlike, in endometrial and colorectal cancers, MSI is confined to a minority of breast cancers showing MMR protein loss [245]. Of note, MMR protein and not PTEN protein expression present a remarkable degree of intra-tumor heterogeneity, by immunohistochemistry (IHC), resulting in a possible diagnostic algorithm to overcome this issue [183].

Recent additional data demonstrate that cell metabolism is tightly related to the autophagic pathways. Specific enzymes such as protein kinase A (PK) A, AMP-activated protein kinase (AMPK), and mTOR play a role in cellular energy homeostasis and regulate autophagy process together with respiration amending energy requests for cellular metabolism [246]. Cell proliferation is possible in specific energy conditions affected by cellular ATP demands. Autophagy, which is, in effect, a catabolic process, produces glucogenic and ketogenic amino acids and is capable of fueling the Krebs cycle, at multiple entry points. Thus, autophagy contributes to the ATP supply. During autophagy, lysosomes degrade damaged cell elements and, in this way, precursor molecules, metabolic requests and energy for neo-synthesis are generated. Consequently, autophagy maintains an adaptive response through which cells tolerate unfavorable and adverse conditions. Furthermore, autophagy appears to be a protective mechanism capable to avoid hazardous situations in the cell (e.g., increasing ROS or DNA damage), thus preventing cancer initiation and progression [183].

Notably, genetic manipulation that causes impaired autophagy in mice demonstrates that tumor formation can be prevented by autophagy process. For example, mice with allelic loss of BECLIN 1, the master autophagic gene, exhibit increased susceptibility to tumor progression compared to wild-type mice [247].

Although autophagy preserves tumor metabolism and expansion during Ras-induced transformation and tumorigenesis, compelling evidence suggests that onco-suppressors mediate autophagy process particularly, by targeting mTOR. Specifically, AMPK, LKB1, tuberous sclerosis proteins (TSC) and PTEN induce autophagy, equally, oncogenes that activate mTOR block autophagy [248].

PTEN is frequently mutated and ectopic expression of functional PTEN in U87MG glioma cells, induces the autophagic flux, in the lysosomal mass. Nevertheless, proteasome activity and ubiquitination of proteins, are inhibited. Notably, the effects are independent of PTEN lipid phosphatase activity on the PI3K/AKT/mTOR signaling pathway [249]. These results suggest a novel signaling pathway, that is mTOR-independent and by which PTEN may possibly, act on intracellular protein degradation affecting autophagy. The molecular constituents by which the tumor suppressor PTEN controls proteolytic systems, related to cancer evolution could represent innovative therapeutic targets for patient treatment [205].

Blocking autophagy, in connection with PTEN hemizygosity, permit tumor development and an early death related to pancreatic cancer compared to autophagy-competent mice [250]. Especially, autophagy-deficient tumors are also PTEN-deficient but interestingly, wild-type for p53 [251]. This condition further strengthens the crucial protective role of PTEN.

Published data show that PTEN can determine autophagy's contribution to tumor development by oxidative stress increase, increased inflammation DNA damage, and metabolic reprogramming. For example, molecular pathways controlling autophagy are affected by the cellular messenger nitric oxide (NO) produced by distinct isoforms of nitric oxide synthase (NOS). In detail, NOS1 activates the survival of nasopharyngeal carcinoma cells through S-nitrosylation of PTEN proteins, introduction of AKT/mTOR, and blockage of the autophagic flux [252].

Normally, autophagy effects are context-dependent to produce a tumor suppressive or oncogenic action; there is an association of casein kinase 1 alpha 1 (CK1α)-dependent autophagic pathway and the tumor-suppressor action exerted by PTEN/Atg7 signal, in xenograft models as well as, in lung cancers [253].

Furthermore, CK1α increases PTEN stability and activity counteracting PTEN polyubiquitination and abolishing of PTEN phosphorylation. These events are responsible for AKT inhibition and FOXO3a-induced transcription of Atg7. The results of PTEN deficiency are also, investigated in hepatocyte of PTEN-deficient mice. A decrease of autophagosome formation and maturation, inhibition of Atg conjugation reaction and activation of insulin pathway, imply that hepatic PTEN loss causes accordingly, relevant action on the whole mice metabolism [254]. However more detailed characterization of Atg proteins is important to improve the understanding of autophagy defects, in the PTEN loss and cancer appearance [255].

Loss of PTEN is crucially involved in immune resistance in cancer disease. For example, in cancer cells and in mouse models of melanoma, PTEN knockout negates T cells' action on tumor cells and reduces T cell trafficking in cancer tissue [256]. Loss of PTEN prompts a mechanism that is controlled by immunosuppressive cytokines, dictating a reduction of T cells infiltration into tumor tissue and suppression of autophagy and eventually T cell–mediated cell death. In patients, PTEN loss correlates with worse outcomes with PD-1 inhibitor therapy [256].

In humans, cancer evolution is commonly associated with long term immunosuppression and chronic inflammatory diseases [257]. For example, head and neck squamous cancers are characterized by neutrophils in inflammatory infiltrates [258].

Neutrophils identified in mouse tumors antagonize effector T cell function, assist in the generation of immunosuppressive T cell populations. Neutrophils also, inhibit the lysis of tumor cells by natural killer cells (NK) or cytotoxic T cells [259]. In mice, biallelic inactivation of LKB1, involved in starvation-induced autophagy and PTEN, causes lung squamous cell carcinoma, displaying histologic pattern and gene expression similar to human disease [260]. These tumors are typical of tumor-associated neutrophils and of epithelial cell populations expressing increased levels of the CXC chemokines. These chemokines regulate the agility and adhesion competence of neutrophils. In addition, cancer stem cells population exhibit enhanced tumor-propagating ability as well as increased

expression of the immune evasion marker (PD-L1), suggesting that these cells possess immune evasion capacity [260].

The results are also indicative of the inefficacy of checkpoint inhibitors in these models, a strong indicator for non-responder tumors. Moreover, a meta-analysis of the data of non-small-cell lung cancer (NSCLC) patients treated by immunotherapy revealed that while epidermal growth factor receptor (EGFR), p53 and LKB mutations are not correlated with the immune response, PTEN was found to be associated to resistance to anti-PD-1 [261].

Furthermore, studies established that PD-1 may be employed as an haploinsufficient tumor suppressive effector, in T cell lymphoma. This molecular pathway appears to be related to the PD-1 activity which elevates PTEN levels and decreases AKT and protein kinase C signals in premalignant cells (Figure 22). Therefore, enhancing PTEN function could be a possible treatment strategy for therapy of these types of cancers; for instance, checkpoint inhibitors could reactivate T cell in lymphoma patients [262].

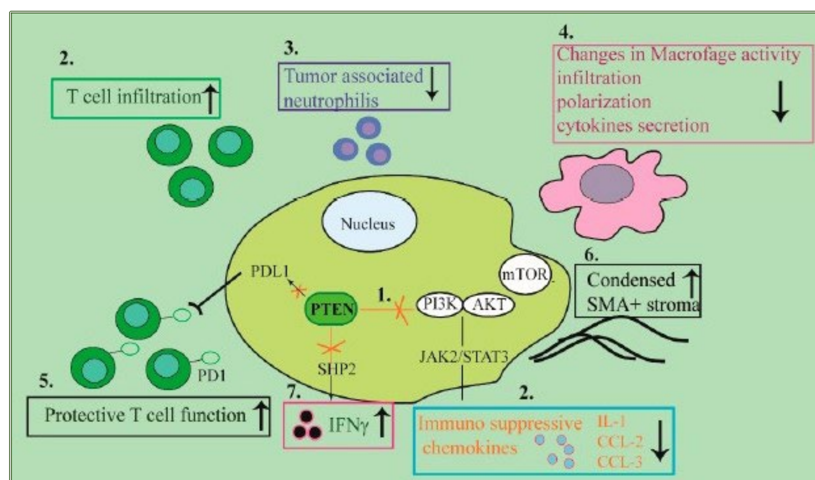


Figure 22 Role of PTEN in immune response

Systemic immunosuppression was indicated in patients with glioblastoma and in glioma animal models [263]. Particularly, glioblastomas had a decreased amount of infiltrating T cells and harbored quite a small number of somatic mutations compared to other tumors. Nevertheless, a considerable augmentation of PTEN mutations, correlated with the immunosuppressive expression model, in glioblastomas patients resistant to anti-PD-1 immunotherapy [263]. At the same time, another study in glioblastoma reveals that expression of PD-L1 leads to an

increase in human glioma, when accompanied by PTEN deletion as well as, dysregulation of PI3K signal [264].

Role in Macrophages Function

Additionally, the immune response in cancer cells, involves macrophages. These cell types become activated and initiate immune responses, after pathogens and other noxious stimuli. The different stages of macrophage activation are defined as M1 and M2 polarization and characterized by specific phenotypes, that are triggered by inflammatory stimuli and affected by the cellular context [265]. Macrophages isolated from metastatic human cancers commonly present an M2-like phenotype, consistent with the cancer-related inflammation [266]. PI3K/AKT pathway and its downstream targets play a critical role in the activation of macrophages [267].

Furthermore, PTEN action on various converging pathways, regulates macrophage behavior. It is advocated, that PTEN controls macrophages' activation by increase of Arginase I release, that results in a hypo-inflammatory environment [268]. Further evidence suggests, that a potent inhibitor of PTEN, VO-OHpic, blocks adverse cardiac remodeling due to the macrophage's polarization [269]. In animal models treated with doxorubicin, which can trigger cardiomyopathy, pro-inflammatory M1 macrophages are decreased, while anti-inflammatory M2 macrophages are increased [269].

Tumor Microenvironment

Tumor proliferation and metastatic process depend on intrinsic characteristics, namely, the response of host tissue and signals arising from Tumor Micro Environment (TME). The TME consists the extracellular matrix (ECM), blood vessels close to cancer cells, and other non-cancer cells [270]. These stromal non-cancer cells, comprise fibroblasts (CAFs), immune response cells such as T and B lymphocytes, natural killer cells and tumor-associated macrophages (TAM) but, only rarely adipocytes. Tumor cells propagate the infiltration of immune cells inside the TME [271]. Tumor cells and tumor-infiltrating immune cells interact with each other, subsequently immune responses proceed to inhibit tumor growth. To this purpose, different stromal cells, CAFs and immune cells, that closely

interact with tumor cells can produce an inflammatory response, secrete chemokines and growth factors, and eventually promote tumor development, progression, and metastasis [272].

6.3 p53

p53 was discovered, during the peak of tumors linked to viral infection research studies, as a 53 kD host protein bound to simian virus 40 large T antigen in virally-transformed cells [273]. Initially classified as an oncogene, subsequent data established that wild-type p53, encoded by the TP53 gene, suppresses oncogenic transformation and growth in cell culture. Inactivating TP53 mutations are common in tumors affecting humans and prevail in many cancers, linked to poor patient prognosis [274].

p53 is a sequence-specific DNA binding protein that controls transcription [275]. The p53 protein comprises of two N-terminal transactivation domains followed by a central DNA binding domain, a conserved proline rich domain, a C-terminus, encoding its nuclear localization signals and an oligomerization domain necessary for transcriptional activity (Figure 23). In keeping with, the importance of p53-mediated transcription in tumor suppression, the majority of tumor-derived TP53 mutations appear in the region encoding p53's DNA binding domain [276].

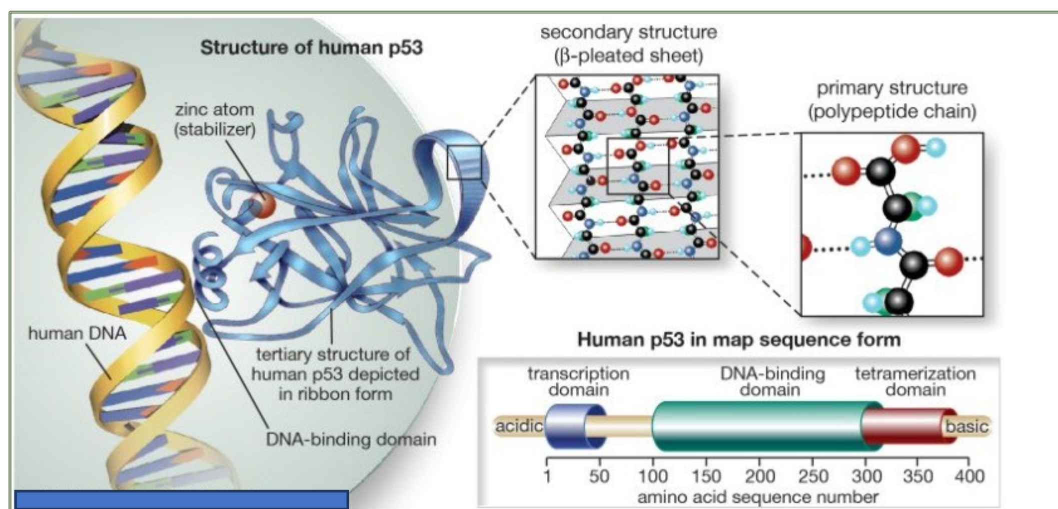


Figure 23 p53 structure

In physiological cells, p53 protein is limited at low levels by a number of regulators including MDM2. MDM2 functions as a p53 ubiquitin ligase to expedite its degradation [277]. Nevertheless, p53 is stabilized in response to numerous cellular stresses, including replication stress and DNA damage produced by deregulated oncogenes. Processes leading to p53 activation can be exogenous stimulus-dependent: for example, DNA damage promotes p53

phosphorylation, blocking MDM2-mediated degradation, while oncogenic signaling activates the ARF tumor suppressor to inhibit MDM2 [278, 279].

Initial studies from the early 1990s disclosed that p53 is critical for a reversible DNA damage-induced G1 phase checkpoint that is mediated, in part, by its ability to transcriptionally activate the p21 cyclin-dependent kinase inhibitor gene, presumably facilitating DNA repair prior to further cell division [280]. In certain circumstances, p53 activates cellular senescence, a long lasting, if not permanent cell cycle arrest mechanism, that also requires the retinoblastoma (RB) gene product [281].

p53 can also trigger apoptosis, relying on the induction of pro-apoptotic BCL-2 family members whose action facilitates caspase activation and cell death [282]. Why p53 activates cell cycle arrest in some cell types and apoptosis in others is not completely understood. The conditions under which, p53 can be activated to arrest or eliminate pre-malignant cells, have guided current investigations, as to why p53 is such a potent tumor suppressor. Its ability to arrest or discard cells following DNA damage suggests that it might prevent cancer initiation by blocking the accumulation of oncogenic mutations [283]. In line with this idea, p53 loss indirectly favors tumorigenesis by increasing the number of mutations in surviving daughter cells. At the same time, the ability of p53 to arrest proliferation in response to aberrant oncogene expression implies a role in containing the consequences of oncogenic mutations.

Therefore, p53 loss directly enhances cancer development by allowing oncogene-expressing cells to proliferate undisturbed, explaining why TP53 mutations cooperate with oncogenes in cell transformation [284]. In both models, p53 functions as the “guardian of the genome” to limit the harmful consequences of mutation [285]. Although, this long-established view delivers the basic conceptual framework, as to why TP53 mutations are so frequent in human tumors, more recent work delivers a far more detailed picture of p53 action that underline its context-dependent regulation and the broadly diverse ramifications of its activation.

Upon DNA damage, p53 is activated to either promote the disintegration or repair of damaged cells, ultimately eliminating their risk of propagating mutations. DNA

Damage Response (DDR) kinases phosphorylate p53 thus, driving cell-cycle arrest, senescence, cell-cycle arrest, or apoptosis [286]. In addition, p53 regulates DNA repair by activating target genes that encode components of the DNA repair mechanism, as demonstrated by p53-null cells, that are defective in certain DNA repair activities in vitro [286]. While TP53 mutation can correspond with patterns of specific co-mutated genes and single nucleotide variants, what is interesting, is that the association between TP53 mutation and copy number variation (CNVs) is strong and universal in a pan-cancer analysis [287].

Also, cancers possessing TP53 mutations are typically aneuploid, with substantial alterations in the numbers of whole chromosomes [287]. Various biological explanations for this correlation have been proposed, but one mechanism contributing to this relationship is the ability of p53 to control processes in G2/M transitions [288]. For example, p53 loss disassociates the spindle assembly checkpoint by derepressing MAD2, resulting in an increased rate of chromosome missegregation and tetraploidization [289].

Regarding tetraploid cells, p53 loss results to an increased rate of multipolar mitoses and subsequent chromosome missegregation [290]. An alternative explanation of how p53 works, is the ability of p53 to restrict chromosomal instability through destroying cells at risk of aberrant mitoses, especially following centrosome amplification and/or telomere dysfunction [291]. Extra centrosomes lead to Hippo signaling pathway upregulation that, in turn, activates p53 by inhibiting MDM2 [292]. Accordingly, TP53 mutations are correlated to whole genome doubling events in human tumors [293]. More studies suggest that p53 deficient cells are better at tolerating proteomic stress produced by aberrant gene dosage, yet others suggest that p53-mediated destruction of aneuploid cells is more productive against structural aneuploidy than it is against whole chromosome imbalances, implicating the role of DDR in response to chromosome shearing [294, 295]. Therefore, it appears that the loss of p53 both facilitates the accumulation and permits the survival of aneuploid cells.

p53 also, appears to restrict a specific type of chromosome shattering, a massive genomic rearrangement event, known as chromothripsis. After p53 and RB gene inactivation, cells that bypass replicative senescence can proliferate despite

telomere erosion [296] . Failure at this checkpoint, telomere dysfunction initializes chromosome breakage-fusion-bridge cycles that lead to chromothripsis [297]. Although, the extent to which chromothripsis endorses tumorigenesis remains an unanswered question. This phenomenon is significantly more prevalent in tumors harboring TP53 mutations [298].

An unexpected way in which p53 assists in maintaining genomic integrity is by suppressing retrotransposons. Retrotransposons are latent virus-derived genetic products, whose aberrant expression can promote mutagenesis, through their mobilization and re-insertion across the genome [299]. Experimental studies in activation of mobile elements in drosophila induces DNA double strand breaks and p53-mediated apoptosis that could, theoretically, reduce their mutagenic effects [300]. However, recent evidence suggest that the association between p53 mutation and retrotransposon expression is more than simply an elimination effect. Indeed, p53 binding to target sites within Long Interspersed Nuclear Elements (LINE) and other transposon sequences are associated with their downregulation [301]. p53 mediated repression is dependent upon epigenetic silencing of retrotransposon loci and not apoptosis, and derepressed retrotransposons are capable of reintegration into the genome, promoting mutagenesis [302].

Genomic analyses have disclosed that retrotransposon mobilization is frequent in human cancers [303]. While the precise impact is still undetermined, there is a significant correlation between repeated element expression and p53 condition in mouse and human tumors [304].

The immediacy with which p53 loss collaborates with oncogenes to transform cells is indicating that genomic instability is not absolutely necessary, for tumor initiation [281]. However, the genomic instability that is fueled by p53 loss, enables acquisition of additional instigating events with the potential to enhance transformation, metastasis and drug resistance [305]. The same way, species' diversity in an ecosystem, is related to its robustness, subclonal diversity and not the total number of mutations in a tumor, determines the resilience of a cancer cell population under changing, adverse conditions and challenges. Therefore, p53 inactivation may be unique in its ability to both accelerate genomic instability (by

increasing the rate of new variants) and allow survival of a wider pool of genetic configurations (decreasing the likelihood of extinction of variants) [306]. These observations underline the possibility that p53 inactivation contributes to intratumoral heterogeneity.

As if regulating genome integrity, apoptosis and cell cycle arrest were not enough functions for a single gene, a continuously growing body of evidence proposes that p53 also regulates additional "non–canonical" programs that contribute to its effects [307]. As examples, p53 can modulate metabolism, autophagy, repress pluripotency and cellular plasticity, and initiate an iron-dependent form of cell death, that is known as ferroptosis [308]. Even low, basal levels of p53 can reinforce various other tumor suppressive networks [309]. Hence, a take-home message is that the p53 response is remarkably flexible and depends on the cell type, its differentiation state, collaborating environmental signals and stress conditions.

The numerous functions of p53 are dependent in its ability to regulate distinct sets of its many target genes [310]. For example, observations that apoptosis and cell cycle arrest are associated with upregulation of p21 or pro-apoptotic Bcl-2 proteins, respectively, conceal the fact that the global transcriptional response to p53 activation involves many other potential modifiers of the metabolic outcome [311]. Historically, genes have been implicated as p53 targets if p53 binds their locus and the mRNA is induced [312]. The nature of p53 targets identified, provides strong confirmation that non-canonical processes including ROS control, tissue remodeling, autophagy, and metabolism are valid processes controlled by p53 [313].

Attempts to identify a universal set of p53 target genes have failed. Meta-analyses from 16 genome-wide datasets revealed that about 60 genes were implicated as common targets [314]. It is noteworthy, that the surveys explored a restricted number of different cell types and applied specific methods for p53 induction. However, a key point is that cellular environment and variable stimuli induce transcription of qualitatively separate sets of genes and not just different levels of the same set of genes. It appears simplistic to expect that oncogene expression in

different tissues (for example, KRAS activation in pancreas, colon, and lung) would provoke an identical p53 transcriptional response [315, 316].

Furthermore, one would not hypothesize, that the p53 output produced by DNA damage would exactly reflect the gene expression signature induced by oncogene activation, even in a single cell type [317]. In spite of existing data, indicating that p53 can, in principle, regulate a wide variety of biological processes, the physiological conditions, in which one or more procedures predominate, are not completely understood and merit more systematic study [313]. Cellular metabolism is a non-canonical p53-controlled process, that has received a lot of attention. The group of metabolic target genes regulated by p53 affects many individual processes. p53 is noted, to increase anti-oxidant activity, glutamine catabolism, promote fatty acid oxidation, downregulate lipid synthesis, or stimulate gluconeogenesis [318]. p53 can also have contrasting actions, on the same metabolic processes, depending on the cell type. For instance, in breast and lung cancer cells, p53 negates glycolysis by attenuating glucose uptake or repressing the expression of glycolytic enzymes [319, 320]. On the contrary, in muscle cells, p53 induces glycolytic enzymes [318]. At the same time, p53 typically increases but can also decrease flux across the tricarboxylic acid (TCA) cycle [321, 322]. Taken into account, these results propose that p53 can control variable aspects of metabolism that produce specific, unique and even factors have yet to be identified [323].

While it is frequently considered that each p53 effector function is a standalone procedure, there is increasing evidence that collaboration between separate input and output pathways is more crucial, than previously recognized. For example, cellular senescence, driven by p53, may be supported by activation of autophagy [324]. Modifications in p53 regulation of metabolism undoubtedly contribute to autophagy, apoptosis and ferroptosis [325]. Under specific conditions, p53-mediated processes can evidently be antagonistic. For example, autophagy has the ability to delay apoptosis by reducing levels of p53 Upregulated Modulator of Apoptosis (PUMA) [326]. However, in situations, where p53 fails to repress glycolysis, autophagy is not effectively engaged and apoptosis is then favored. In

these examples, interaction between unique biochemical processes controlled by p53 elicits different biological results [327] .

The basic mechanism underlying the ability of p53 to induce different biological outputs remains elusive. It is certain that, p53 can control qualitatively different programs that result to different biological outcomes, depending on cell type and stimulus. A possible mechanism, for qualitatively moderating biological p53's responses, involves stimulus-dependent, post-translational alterations (PTMs) that can modify p53 affinity for different target genes; for example, acetyl-p53 (K120) or phospho-p53 (S46) stimulates apoptosis, whereas PRMT5-methylated p53 activates p21 more efficiently than apoptotic genes [328]. A broad array of other PTMs at many different sites in the p53 protein have been described to not only modify protein stability, but also influence target gene bias, such as SUMOylation, glycosylation, and prolyl isomerization. Furthermore, one post-translational alteration can increase acquisition of another, thus unlocking additional layers of regulation of protein to protein interaction, protein stability and biasing DNA-binding toward select target genes [328].

p53 induction can produce either a steady signaling output, or a signaling that can oscillate in subtle waves; remarkably, the kinetics of its expression, independent of maximal p53 protein levels, can determine cell fate in response to genotoxic stress [329].

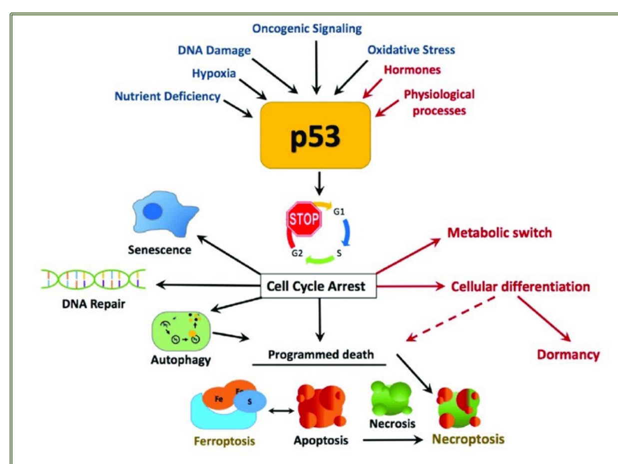


Figure 24 p53 activation responses

p53 activation kinetics can be interpreted as target gene bias, due to differences in p53 binding and dissociation rates at specific target loci. Therefore, the p21

promoter appears sensitive to short pulses of p53 activity whereas the pro-apoptotic p53 target FAS ligand, is not [330]; consequently, a short pulse drives proliferative arrest but a sustained signal induces apoptosis (Figure 24). Perhaps, some p53-driven stress responses initiate a short-term repair and salvage program that, if necessary, reaches a tipping point that converts to cellular self-destruction [317].

Several factors determine how the cell utilizes p53 actions. For example, cell lineage can be a large determinant in the event of a hypothetical tipping point between alternate cell fates. First, cell type and state specific chromatin modifications may make particular genes more or less accessible to p53 transactivation [331]. Transcriptional repressor CTCF insulates the PUMA locus from repressive histone modifications under certain conditions, governing whether PUMA is expressed and apoptosis occurs [332].

In Embryonic Stem Cells (ESCs), p53 can be stimulated to bind to the p21 promoter, but p21 is not efficiently activated, on account of repressive histone H3K27me3 marks, dependent on cell-type-specific, at the locus [333]. The p53 target range can be modified by cooperation or antagonism with other transcription factors, such as NF-κB and FOXO, whose levels and occupancy are also context-dependent [334]. The same transcriptional output may have multiple effects depending on the state of the cells. ATM serine/threonine kinase signaling protects cells from p53 mediated apoptosis. This action is accomplished, not by changing p53-driven transcriptional output, but by blocking autophagy and in this way, maintaining mitochondrial homeostasis and suppressing ROS levels [335].

Together, these considerations suggest that p53 response is not just an "on-off" switch. In the contrary, cell destiny is the result of a rich collection of p53-driven stress responses. Obviously, p53 is rooted in a densely populated and interconnected network of regulators and effectors that allow for a flexible p53 response. The result is, a response coordinated to match cell type and microenvironment, at the time of activation [306]. In conclusion, cellular context (activating signal cell type, tissue microenvironment, epigenetic state) is critical to both the biochemical aspects of p53 actionability and the biological outcome of its response, as well.

Various elements of the p53 pathway regulate the production-utilization of several classes of metabolites including lipids, amino-acids and nucleotides. Increasing evidence indicates that it contributes to many adaptive responses to changes in nutrients and oxygen availability [336].

Mitochondrial Functions of p53 Pathway

Mitochondria, the power producing factory of the cell, serves as a metabolic hub that integrates multiple signals, originating from the p53 pathway. The p53 protein controls various mitochondrial functions, including integrity and replication of the mitochondrial DNA, activity of the electron transport chain (ETC), mitochondrial architecture and dynamics, as well as the flow of numerous metabolic pathways, that take place in mitochondria [337]. Different research studies have confirmed the localization of p53 in mitochondria, where it directly regulates mitochondrial respiration as well as cell death, independently of its transcriptional functions [338]. More recently, mitochondrial pools of MDM2, MDM4, ADP ribosylation factor (ARF) and polycomb complex protein BMI1 have also been described [339-342]. Mitochondrial MDM2 regulates the function of the ETC and controls mitochondria network dynamics, independently of p53. Therefore, in response to hypoxia or oxidative stress, MDM2 translocates to the mitochondrial matrix, where it preferentially binds to the Light Strand Promoter (LSP), causing transcriptional repression of NADH-Dehydrogenase 6 (MT-ND6), a gene from the mitochondrial genome, encoding a necessary complex I subunit, of the ETC. This mitochondrial function of MDM2 is present, both in p53-proficient and in p53-deficient cells and does not involve its ubiquitin E3 ligase activity [340].

The recycling of damaged mitochondria, is activated, when mitochondrial membrane potential is altered and respiration is inefficiently conducted. This physiological process, called mitophagy, is regulated, at least in part, by PARKIN, a ubiquitin E3 ligase that ubiquitinates MITO- FUSIN1 on the mitochondrial outer membrane. MDM2 and PARKIN directly interact to accelerate PARKIN enzymatic activity (self-ubiquitination and MITOFUSIN1 ubiquitination), thereby promoting mitophagy [343]. Notably, the connection between the p53 pathway

and PARKIN extend to p53-mediated regulation of PARKIN transcription and to p53-PARKIN protein-protein interaction [344, 345].

Finally, p53 also controls SPATA18 (known as MIEAP) transcription. MIEAP gene product is involved in intramitochondrial lysosome-like structures that eliminate oxidized mitochondrial proteins and thus, improving mitochondrial functions [346]. These facts reinforce the belief that the p53 network plays a key role in quality control and mitochondria turnover. Other important regulators of the p53 pathway control mitochondrial functions, beyond their effects on p53. For instance, BMI1 represses the transcription of genes (Alox5, Alox15, Cyp24a1, Cyp26a1, Bnip3l, Pmaip1, Duox1, Duox2, Cdo1), encoded by the nuclear genome [336]. This particular array of genes influences mitochondrial function and redox homeostasis. In accordance, cells isolated from Bmi1 knock-out (KO) mice exhibit impaired mitochondrial respiration and a significant increase in the intracellular levels of reactive oxygen species (ROS), resulting in the engagement of the CHK2-dependent DNA damage checkpoint [347]. BMI1 involvement in metabolism also includes its mitochondrial localization where it directly regulates polynucleotide phosphorylase, a ribonuclease that is responsible for mitochondrial RNA (mtRNA) transcripts degradation. Thus, mtRNA homeostasis and bioenergetics are regulated [342]. Furthermore, the versatile protein E4F1, with its intrinsic transcriptional activity, controls the gene expression responsible for encoding a complex I subunit (Ndufs5) or an element of the mitochondrial import machinery (Tomm7), as well as gene groups involved in cardiolipin (a mitochondria-specific phospholipid) biosynthesis and maturation or in pyruvate oxidation, independently of its actions on p53 [348].

Genetic inactivation of E4f1 transcription factor, in murine transformed fibroblasts impacts on oxygen consumption and other metabolic pathways that are compartmentalized in mitochondria, confirming that this E4F1-controlled transcriptional program strongly influences cellular metabolism [348].

Lastly, the MDM4 oncoprotein and the N-terminally truncated isoform of the ARF tumor suppressor that is produced upon translation induction, at an internal in-frame AUG codon, at position 45 (called small mitochondrial Arf or p15smArf), have both been identified, in mitochondria [339, 349]. Despite the fact that, these

mitochondrial sources of MDM4 and ARF were originally associated to the control of cell death, it could be that, similarly to other components of the p53 pathway displaying mitochondrial localization, they also contribute to various mitochondrial activities, involved in metabolism. Collectively, these studies propose that the p53 pathway is firmly connected to mitochondria functions to fine tune metabolism [336].

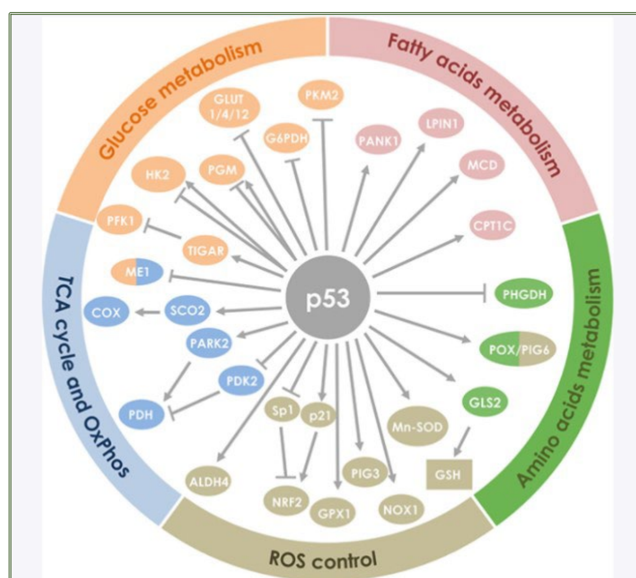


Figure 25 p53 mediated metabolism control

Pyruvate Metabolism

Pyruvate is a central metabolite involved in both glycolysis and Oxidative phosphorylation (Oxphos) [350]. Pyruvate is transported in mitochondria to facilitate the tri-carboxylic acid (TCA) cycle and sustain mitochondrial respiration. Also, Pyruvate contributes to various anabolic pathways supporting the de novo fatty acid and cholesterol synthesis, nucleotide metabolism and gluconeogenesis (Figure 25). One critical enzyme of pyruvate metabolic pathway, is the pyruvate dehydrogenase (PDH) complex (PDC), a large multi-subunit metabolic enzyme that converts pyruvate into Acetyl-Coenzyme A (AcCoA) in the mitochondrial matrix [351]. Reduced PDC activity reroutes pyruvate metabolism towards lactate or alanine production by the lactate dehydrogenases (LDH) and alanine amino-transferases (ALAT), respectively [350]. The p53 pathway is connected to the PDC, at many levels [352]. While, p53 was shown to suppress the transcription of PDK2, a gene encoding an inhibitory kinase of the PDC, MDM2 repression

preserves the protein levels of dihydrolipoamide dehydrogenase (DLD), the E3 subunit of the PDC [353].

Furthermore, the MDM2-DLD complex shuttling, between the cytosol and the nucleus is controlled by the pharmacological inhibitor Nutlin3A, which regulates p53-MDM2 interaction [354]. The tight connection between the p53 pathway and pyruvate metabolism are demonstrated by studies, disclosing that p53 and MDM2 are part of a regulatory network in pancreatic beta cells, controlling the activity of pyruvate carboxylase (PC) and thus, influencing glucose-stimulated insulin secretion and glucose homeostasis [355]. In summary, these data suggest that the p53 pathway is closely linked to pyruvate metabolism in many cell types and that disturbance of this complex network contribute to various human diseases, including inborn metabolic disorders, type-II diabetes, and cancer [338].

Amino-Acid Metabolism

p53 has been linked in many aspects of serine/glycine, glutamine and proline, metabolism. It appears p53-deficient cells are more sensitive to serine/glycine or to glutamine deprivation [356]. The regulation of MDM2 subcellular localization is also involved in amino-acid metabolism, as described by data, showing that MDM2 is engaged to chromatin, independently of p53 in order to control genes, involved in glutamine/glutamate, as well as serine/glycine metabolism [357, 358]. Chromatin-bound MDM2 by binding to the ATF4 transcription factor, activates a transcriptional program involving several genes, encoding transporters that regulate serine uptake (SLC1A4). or its intracellular processing (SERINC1) [357]. The same transporters regulate as well, enzymes involved in de novo serine synthesis, an anabolic reaction that transforms the glycolytic intermediate 3-phosphoglycerate (3PG) into serine through a multi-step enzymatic cascade implicating phosphoserine aminotransferase 1 (PSAT1), phosphoglycerate dehydrogenase (PHGDH) and phosphoserine phosphatase (PSPH) [359]. MDM2 recruitment to chromatin is initiated by oxidative stress, serine and glycine deprivation, or upon inhibition of the M2 isoform of pyruvate kinase (PKM2), a glycolytic enzyme for which serine is an allosteric activator [360]. MDM2 recruitment to chromatin, involving its central acidic domain, most likely with conformational changes, occurs independently of its E3 ligase function. It is

inhibited by its phosphorylation on serine 166 and threonine 351. An event regulated by the glycolytic enzyme PKM2 [357]. Surprisingly, chromatin-bound MDM2 and p53 exhibit antagonistic actions on the transcription of genes involved in serine metabolism [361]. Therefore, whereas MDM2 initiates the transcription of SERINC1, SLC1A4, PSAT1, , PHGDH and PSPH, p53 is found to deactivate the PHGDH promoter. Thus, illustrating the complex interactions between the p53 pathway and serine metabolism and the p53 pathway [361]. Serine/glycine and glutamine/glutamate metabolism influence numerous anabolic pathways, including glutathione (GSH) and nucleotide biosynthesis [362]. Chromatin-bound MDM2, according to its role in these metabolic pathways, was shown to affect the redox status of both cancer and cells, through the modulation of glutathione recycling and recycling.

The idea that MDM2 is a key regulator of serine metabolism was recently confirmed in liposarcomas (LPS), a sarcoma subtype typical of a systematic amplification of MDM2 [363]. Notably, LPS, but not other sarcoma subtypes, exhibit elevated levels of chromatin-bound MDM2. These data confirm, that the selective pressure for MDM2 amplification in LPS is powerful and most likely mirrors its central role in serine metabolism to maintain nucleotide synthesis, in these highly proliferating cancer cells [363].

Lipid metabolism

An important element of p53 metabolic activities is revealed, through its multiple roles in lipid transport and storage, in cholesterol and sphingolipid metabolism, in fatty acids biosynthesis and their desaturation, as well as in fatty-acid oxidation (FAO). Furthermore, different groups have reported that p53 interferes with adipocyte differentiation [364]. It is intriguing that, MDM2, MDM4, and BMI1 are also, associated with lipid metabolism and adipocyte differentiation, supporting the idea that the entire p53 metabolic pathway is vital for lipid homeostasis in distressed and cancer cells, as well as in normal tissues [365](Figure 25). Confirmation of p53-mediated control of lipid metabolism was demonstrated by using genetically engineered mouse models displaying altered MDM2 or MDM4 activity. In detail, mice harboring the Mdm2C305F allele, which encodes a mutant manifesting impaired binding to the RPL11 and RPL5 ribosomal proteins,

demonstrate defective response to nutritional distress, because of their inability to control p53-mediated modulation of FAO [366, 367]. A particular analysis of Mdm4 Knock-Out mice displaying a p53 acetylation mutant, harboring lysine to arginine (K to R) substitutions on the key lysines 117, 161, and 162 of its DNA binding domain (also called p533KR) is another example of the links between the p53 pathway and fatty acid synthesis. This p53 mutant is unable to induce apoptosis, cell cycle arrest, and senescence, but remains competent for its metabolic activities [368, 369]. Interestingly, these Mdm4KO; p533KR compound mice are resistant to high-fat diet (HFD)-induction of obesity, a phenotype that is considered to be influenced by enhanced FAO.

At a molecular level, this result was associated to the transcriptional regulation of Long-chain fatty acid elongase 3 (Elovl3), a p53-target gene affecting adipocyte cell fate and energy expenditure [369]. Notably, MDM2 and BMI1 were also suggested to regulate adipocyte cell fate, independently of p53. This MDM2 function was linked to its ability to control cAMP-mediated initiation of CCAAT/enhancer-binding protein delta (C/EBPΔ) expression, by promoting the recruitment of the cAMP regulatory element-binding protein (CREB)-regulated transcription coactivator (CRTC2) to the c/EBPΔ promoter [370]. In addition, the polycomb member BMI1 blocks adipogenesis of bone marrow stromal progenitors in the hematopoietic stem cell niche, an action accomplished through the epigenetic control of a PAX3-regulated developmental program. This is clarifying the Cdkn2a-independent cell-extrinsic results of BMI1 deficiency on hematopoietic stem cell maintenance [371]. Therefore, these effects suggest that the p53 pathway exerts multiple functions affecting lipid homeostasis and adipocyte cell fate. These p53 attributes have a major impact on tissue homeostasis, metabolic diseases, aging, and cancer development.

Iron Metabolism

Regulating iron levels is crucial for cell survival, and iron overload facilitates carcinogenesis. p53 and MDM2 are important in a complex network affecting iron uptake, storage, and usage, both at the systemic and the cellular levels [372]. p53 controls the transcription of several key iron regulators including Hepcidin (HAMP), iron-sulfur cluster assembly enzyme (ISCU), Ferredoxin reductase

(FDXR), and Frataxin (FXN) [373-377]. Furthermore, its activities are directly regulated by intracellular iron levels [378, 379]. Changes in free iron levels also control MDM2 mRNA levels and protein expression, partly, through the binding of IRP2 to the 3 untranslated region (3 UTR) of MDM2 mRNA, thus interfering indirectly with p53 protein stability [380].

The connection between high iron levels and enhanced MDM2-mediated degradation of p53 in hepatocytes may result in the increased risk of hepatocellular carcinoma in patients suffering from chronic liver disease [380]. Ferroptosis is an important biological process, linked to the regulation of iron metabolism by the p53 pathway. This iron-dependent cell death mechanism is connected to lipid peroxidation [381]. The transcriptional and the non-transcriptional control on inducers and regulators of ferroptosis is a well-recognized role of p53. Recent work revealed that MDM2 and MDM4 interfere with the ability of cells to strengthen their defenses against lipid peroxidation [382]. Inhibition of MDM2 and/or MDM4 permits cells to accumulate endogenous lipophilic antioxidants such as Coenzyme Q10 (CoQ), an effect mediated by PPARα and FSP1 [383]. Of note, p53-mediated regulation of the mevalonate pathway has also been identified to contribute to CoQ biosynthesis, indicating that p53, MDM2, and MDM4 control synergistic metabolic functions, converging on this key metabolite [384].

p53 and Hypoxia pathway

Cells modify their metabolism according to changes in oxygen and nutrient availability. Reduced oxygen concentration activates a complex cellular response, coordinated by the Hypoxia-Inducible-Factor (HIF) transcription factors, as well as other components of the p53 pathway [385]. This biological procedure involves numerous metabolic alterations during which p53 and MDM2 and MDM4, regulate the activity of the Electron Transport Chain (ETC) and the accumulation of ROS, as well as the amount of anti-oxidant molecules to influence cell survival. Initially, HIF1α, the regulating partner of this heterodimeric transcription factor, was shown to stabilize p53, by directly binding to it. [386]. Other pathways leading to p53 activation have been proposed, as well, such as HIF1α interaction with MDM2, a process that inhibits MDM2-mediated degradation of p53 and

which bridges p53 to HIF1α [387]. MDM2 is downregulated, upon its phosphorylation by the p38 mitogen-activated protein kinase. contributing to p53 activation in hypoxic neuronal cells [388].

Additionally, a proteomic study identifying new partners of the von Hippel Lindau (VHL) tumor suppressor, which is an important component of the ubiquitin E3-ligase complex that mediates proteasomal-mediated degradation of HIFs, characterized ARF as a partner of the long isoform of VHL. ARF appears to be interfering with the E3 ligase complex containing VHL, thus enhancing its interaction with the arginine methyl- transferase PRMT3 which methylates p53 [389]. Several other essential factors of the p53 pathway may also mediate the cellular response to hypoxia, independently of p53. As previously reported, MDM2 translocation to the mitochondrial matrix can lead to repression of the MT-ND6 transcription and thereby, specifically impacting on complex I activity [340]. There is substantial evidence, that associates uncoupling of the ETC to the production of mitochondrial ROS by complex I and/or complex III [390]. In accordance with its role in the regulation of complex I activity in hypoxic cells, enhanced recruitment of MDM2 to the mitochondria results in increased mitochondrial ROS levels. Furthermore, mitochondrial MDM2 displays a physiological action in muscular cells in response, to low oxygen levels [391]. In experimental studies, mice MDM2 and p53 deficient in their skeletal muscles, exhibit high ND6 levels that correspond to higher complex I activity, and subsequently, MDM2. p53 double KnockOut (KO) animal models display increased muscular endurance in mild hypoxic conditions when compared to p53 KO mice [340]. Of note, increased mitochondrial-MDM2 levels result in enhancement of the migratory and invasive potential of cancer cells [340]. These data suggest, that mitochondrial-MDM2 could also, increase cancer cell aggressiveness, in tumoral hypoxic areas. Whereas, ARF counteracts hypoxia-induced migration of cancer cells, through its direct binding to the COOH-terminal binding protein (CtBP) family of metabolically-regulated transcriptional, co-repressors [392].

In addition, MDM4 appears also, to play an important part in modulating p53 functions, in hypoxic conditions. A process that involves MDM4 phosphorylation

by the CHK1 kinase and its subsequent sequestration in the cytoplasm by the 14.3.3 protein. Hence, these data suggest that many components of the p53 pathway contribute to coordinate the cellular response to hypoxia [393].

It is intriguing to consider, how and why did the p53 network evolved. The majority of tumors appear after reproductive age, suggesting that the TP53 gene did not originally, evolve to prevent cancer [394]. Furthermore, although it seems surprising, the neonatal p53-null mice seem initially normal, given the diverse outputs of the p53 network. Genes that share resemblance to TP53 by sequence and induction, following DNA damage are also, discovered in simple invertebrates (including worms sea anemone and choanoflagellates), that are not susceptible to cancer [395].

These genes, like mammalian p53, initiate apoptosis in response to stress but, in contrast to mammalian p53, they are expressed mainly, in germline stem cells. It appears that, protection of the germline is crucial and evolved further to suppress emerging tumors, at adult life [300]. A closer look at p53, beyond the germline, focusing on the consequences of its disruption, suggests that it has a significant part in embryonic development. In essence, multicellularity is a necessary compromise between the cells of complex organisms. The most thriving individual cell organisms prevail over populations of single cell organisms, whereas multicellular organisms mandate cellular collaboration, at the expense of competition and antagonism, to maintain coordinated and specialized functions [396]. The necessity for cooperation starts early, in embryonic development, where p53 blocks proliferation of individual "cheater" cells, noted in chimeric blastocysts upon p53 knockdown and following positive selection of spontaneous TP53 mutations detected in commonly used human Embryonic Stem Cell lines (ESC) [397]. Tight control of DNA methylation by DNMT and TET family enzymes involves p53 and it seems that epigenetic influence, leads to clonal heterogeneity in p53-deficient ESC colonies. Downregulation of p53 protects cultured cells from apoptosis, caused by DMNT1 deficiency and subsequent genomic demethylation, supporting the notion of p53 can sense and respond to perturbations in the epigenome [398].

Besides, restricting abnormal clonal outgrowth, p53 also controls target genes that accomplish specific biological requirements in embryonic development, such as LIF, which is important for effective mammalian embryo implantation [399]. Trp53 knockout mice display a variety of low-penetrance and tissue-specific developmental anomalies in the eyes, neural tube and testes [400]. p53 orthologs in more primitive species appear to exhibit conserved non-canonical p53 actions. For instance, promoting redox control and survival are regulated by p53, in simple organisms [308]. Similarly, the ortholog Lvp53 is expressed in the somatic cells of the shrimp, where cross-talk with NF-kB eradicates virally infected cells and activates innate immunity [401]. These observations support the notion that the p53 family is critical, in promoting cell survival or resisting infection. Therefore, it appears that, the p53 network developed diverse physiological roles before its implementation for tumor suppression.

Despite the fact that, p53 protein sequence itself is fairly conserved in higher eukaryotic organisms, the domains involved in p53 regulation on the N- and C-termini and the onward p53 response are subject to continuous evolutionary pressure [402]. Indeed, many p53 response elements display remarkably low conservation compared to other transcription factor recognition sites [403]. Another way that illustrates the p53 network evolution, is its ability to increase gene dosage. The fact that, elephants have acquired up to 20 TP53 retrogenes may provide an explanation, at least in part, as to how an organism with such a large body size and considerable longevity, is not prone to high cancer risk [404]. It seems only suitable, that a more detailed investigation of the elements that have been selected and counter-selected in p53 biology during evolutionary continuity, will be able to provide more detailed insight into the biological processes that are important, for tumor suppression.

There is considerable evidence that p53 has complementary roles in non-pathological tissue homeostasis. It has been illustrated, that p53 function seems to be connected to stem cell biology and their differentiation in the cells of higher organisms. For example, in stem cells and progenitor cells of the hematopoietic system, liver, brain, and breast, p53 restricts cellular self-renewal [405-407]. It evident therefore, that p53 is governing transition between cell states, limiting

cellular plasticity and at its most extreme, it restricts the ability somatic cells possess, to undergo epigenetic reprogramming, if required, into induced pluripotent stem cells [313]. The induced Pluripotency promoting (iPS) factors Oct4 and KLF4 repress p53, and inversely, p53 activity opposes the efficiency of iPS cell reprogramming [408].

A practical application of the previously mentioned principles, can be observed in tissue regeneration and the wound-healing response, a complex process that involves waves of inflammation, angiogenesis, extracellular matrix (ECM) remodeling, tissue regeneration and fibrosis to prevent infection and repair tissue damage. During the initial proliferative phase of tissue regeneration, mitogens are activated and p53 must be downregulated to permit tissue remodeling [409]. By initiating cellular senescence, p53 enhances the release of secretory factors that enable resolution of fibrosis and organize ECM remodeling [410]. Notably, the necessity for p53 to control plasticity seems to be evolutionarily conserved, demanding the synchronized suppression and unsuppression of p53, as evidenced during salamander limb regeneration [411].

It is interesting, that the physiological and developmental functions of p53 are tightly connected to the cancer-associated phenotype of p53 loss [412]. Avoiding terminal differentiation is a pivotal step in malignant transformation and p53 loss may be one available pathway to debilitate this innate barrier to tumorigenesis. In support of this observation, an embryonic stem cell-like gene signature is noted in p53-mutant breast cancer [413]. By interfering with differentiation, incipient TP53 mutations enhance the expansion of hematopoietic stem cell (HSC) clones in otherwise healthy individuals, occasionally occupying the entire hematopoietic system [414]. Additionally, p53 function in wound healing also, regulates the tumor microenvironment. As an example, the p53-driven senescence associated secretory phenotype (SASP) in tumor stroma can produce a tumor suppressive immune condition, that alters the incidence of cancer [415]. In another context, the SASP can be assisting tumor progression, by promoting epithelial–mesenchymal transition (EMT) [410, 416].

The heterogeneity of TP53 mutational events results in distinct functional consequences. New developments, in our understanding of p53 biology have

perplexed, rather than clarified, our views on how TP53 mutations facilitate cancer initiation and propagation. Similarly, our appreciation of the intriguing range of ways in which the TP53 locus is altered in tumors, remains puzzling. The most common and well-identified TP53 mutations are missense mutations in the DNA binding domain, suggesting that this feature of p53 is critical for tumor suppression [417]. Current views, propose p53 classification, as either wild-type or mutant, but TP53 mutations can appear with diverse patterns, specific co-mutations, and in various allelic configurations that result in surprisingly compelling functional and phenotypic consequences [418].

DNA sequencing of thousands of tumors has proved that approximately half of all cancers retain a TP53 mutation, but the distribution and the frequency of mutations can extremely diverse between tumor types. Most of the single nucleotide variants (SNVs) observed across cancers are missense mutations, with 25% of those falling into 5 “hotspot” mutations [419]. Surprisingly, nearly 25% of all TP53 mutations are frameshift or nonsense mutations anticipated to encode truncated proteins, while the remaining 75%, consists of in-frame insertions-deletions and splice site SNVs, of unclear biological significance [419]. Although, several ways to disable the second TP53 allele are feasible, this typically materializes, through “loss of heterozygosity” (LOH) by segmental deletion [420]. These deletions appear to be significantly variable in size and occur at a frequency rate, that is similar to p53 SNVs. Nearly all conceivable allelic combinations can be observed, such that, practically, only 25% of tumors carry the canonical p53 missense mutation/deletion combination [420].

Cancer genome studies have also, disclosed remarkable insights into the range of TP53 mutation and its connection with other somatic events. TP53 mutations often co-occur, in some cancers, with MYC amplification or activating KRAS mutations [421, 422]. This observation is in accordance, with previous functional studies that demonstrated the propensity of p53 loss to cooperate with oncogenes in transforming primary cells [284]. While, epidemiology studies and genome sequencing experiments can suggest variable environmental or endogenous mutagens as accountable for particular TP53 mutations, it is very difficult, if not impossible, to evaluate individual alleles without specific functional studies.

Practically, experimental data emerging over the last 25 years have implied, that particular mutant TP53 alleles exhibit "gain of function" properties that result in phenotypes, different from the null [423]. The most prominent phenotype generated by such mutant proteins is their ability to increase invasion and metastasis, while in other distinct settings, specific mutants support epigenetic reprogramming, drug resistance, or angiogenesis [424].

While the proposed functions are diverse, an emerging general rule, is that tumor-derived p53 mutants oppose wild-type p53 activities or, more accurately, aggravate the consequences of p53 loss [276]. Either way, the idea that not all p53 mutations are functionally equal is further supported, by the evidence, that the tumor onset and pathology, in genetically engineered mouse models, varies with the type of mutant allele [425]. A p53 mutant that has distinct phenotypes from the p53 null is not sufficient to define a mutant as "neomorphic." In theory, p53 mutant alleles may mirror separation of function, attenuation of function, or neomorphic function. Loss of function is a common characteristic in all cancer-associated p53 mutants, since most mutants fail to induce apoptosis [426]. Separation of function, whereby a p53 mutant can maintain some but not all interactions, is also possible [427]. Finally, neomorphic mutant activities have also been identified [428].

In effect, the mutations observed in cancer, acquire a combination of these independent characteristics. Although p53 mutants are in general classified by their impact on structure –i.e., "contact" mutants that disturb DNA binding and "conformation" mutants that lose proper folding – it is currently not possible to anticipate accurately, how a distinct mutation affects function [424]. Some p53 mutant proteins maintain residual transactivation abilities, thus activating novel targets. For example, mutant p53 is proposed to impact chromatin state [429]. Some unstructured p53 mutants sequester other proteins that, in specific settings, facilitate mutant p53 to bind p63 or p73, resulting to changes in transcriptional profiles, that alter receptor tyrosine kinase signaling to enhance invasion and metastasis [430].

Additionally, in an instance of gain-of-function protein-protein interaction, mutant p53 can upregulate the angiogenesis regulator VEGFR2 [431]. It remains difficult

to comprehend, how so many distinct yet specific protein to protein interactions can present for diverse mutant proteins [426]. It is generally accepted that TP53 truncating mutations are null alleles, but there are emerging data that even these alleles can exhibit neomorphic activity. Suggesting some selective advantage, the frequency of TP53 nonsense mutations, particularly targeting exon 6, is greater than expected by chance [419]. Some of these mutation products are not subject to nonsense-mediated degradation, allowing for certain truncated p53 mutants to promote invasion, metastasis, and sustain tumor maintenance in a manner that mirrors established gain of function missense mutants [419]. Provocatively, exon 6 truncated proteins mimic the structure and function of a naturally-occurring p53 splice variant (p53psi) that enhances cell invasion and is transiently expressed during particular wound healing responses, advocating that these mutant genes may represent "separation of function" alleles [432]. It appears that, mimicry and/or aberrant expression of alternative splice variants may contribute to the phenotype of other frequent mutations, as well [433].

Further from the heterogeneity exhibited by different p53 SNVs, the variable extent of human chromosome 17p deletions can result in heterogeneity, both in the nature and in the number of p53- linked genes, during tumorigenesis [306]. Loss of these neighboring genes could well represent a "passenger" event of no functional consequence. Nevertheless,, 17p deletions observed in human cancer often include other genes that are now, functionally validated as tumor suppressors [420, 434].

7. Principles of Immunocytochemistry

The need to find new methods in order to promote the diagnostic process in Pathological Anatomy and Cytology led to the discovery of immunohistochemistry. This is a field of histopathology, that deals with the identification of normal or pathological components of cells and tissues [435]. The immunohistochemistry technique is employed in the search of cell or tissue antigens that range from amino acids and proteins to specific cellular populations and infectious agents [436]. The technique consists of two phases: (1) slide preparation (specimen fixation and tissue processing) and stages evolved in the antigen reaction (in the following order: antigen retrieval, non-specific site block, endogenous peroxidase block, primary antibody incubation, and the implementation of systems of detection, revealing and counterstaining and finally, slide mounting and storage) (2) interpretation and quantification of the obtained expression [437].

Immunohistochemistry is an umbrella term that envelopes many methods used to identify tissue components (the antigens) with the use of specific antibodies that can be visualized through staining. When it is used in cell preparations it is called immunocytochemistry, a term that some scientists apply for all methods entailing the immunological search of cell antigens, even when this involves tissue slices [438].

Immunostaining for cell markers serves as a way to “converse with cells”, because it allows, not only to decipher the histological origin of a cell, but it also indicates its function in vivo, when thoroughly investigated against the correct antibodies [435].

The history of immunostaining methods began when reagents were produced against typhus and cholera microorganisms, using a red stain conjugated to benzidine tetraedro [439]. However, antigen detection provided by red color in tissue slices had very low sensitivity under optical microscopy and, in the early nineteen forties, the localizing of antigens, especially microorganisms, was made possible in tissue slices by using antibodies against Streptococcus pneumoniae, that was stained with fluorescein, and visualized by ultra-violet light (fluorescence microscopy) [437].

Subsequently, the utilization of enzymes as marked antibodies, signaled a new and important era for immunohistochemistry [440]. It was, for the first time, possible to observe these reactions through optical microscopy. These results had great impact and were very much awaited. This innovation improved immunohistochemistry beyond the secluded space of laboratories equipped with fluorescence microscopes, and the technique expanded to a broad group of pathologists and researchers [441].

The following discoveries of the unlabeled antibody peroxidase-antiperoxidase (PAP) method and the alkaline phosphatase-antialkaline phosphatase (APAAP) method significantly expanded the application of immunohistochemistry technique [442, 443]. The diaminobenzidine molecule (DAB) was also available and utilized, during the same period, as a conjugate to antibodies. DAB, currently represents the most common chromogen for peroxidase. Also, it produces an electrodense precipitate which is used in electronic microscopy, substituting ferritin [444, 445]. Subsequently, gold colloidal particles were also, introduced as immunohistochemical colorations and this finding rapidly led to an important method of subcellular immunostaining [446, 447].

The introduction of retrieval methods for antigens (exposing of antigen epitopes in the study tissue, facilitating the antigen-antibody reactions for the next steps of the technique) and also the system of secondary antibody detection (like the avidin-biotin-peroxidase complex—ABC and the labeled streptavidin-biotin complex—LSAB), permitted immunohistochemistry to be applied in fresh specimens as well as in fixed tissues, that further spread the application of the technique in pathology diagnostic routines [448-450]. However, immunohistochemistry became truly incorporated into the diagnostic routine of pathological anatomy, only after the identification of tissue antigens could be demonstrated by immunoperoxidase technique, in tissues fixed in formalin and embedded in paraffin [451].

Cancer cells have different biological behavior resulting in the secretion of mutated biological products of a specific or non-specific type for the tumor type of tumor cells (biological or biochemical tumor markers) [452]. This recognition is achieved by the use of specific antibodies against the target antigens, which are

bound to specific dyes, thus making the localization sites of these antigens visible. Although immunofluorescence - a pioneering technique for detecting proteins through an antibody fluorescent-dye binding process, isothiocyanate fluorescein - has long been the only method of identifying tissue-specific antigens, immunoglobulins and various fractions of the complement [453]; more reliable and specialized techniques developed for immediate and visible by conventional microscopy assessment of the result.

Immunoenzymatic techniques are based on the use of enzymes or enzyme systems applied to tissues or cell smears after antigen-antibody binding, brightly staining their localization sites thus making them visible to the common microscope. The methods of immunoperoxidase, avidin-biotin, streptavidin are the most important, as well as new methods of bypassing biotin using the polymer dextran [454]. A key role in increasing the sensitivity and specificity of the aforementioned methods was the production of specific antisera, i.e., specific binding antibodies to the respective antigens. Two antibody categories were thus developed, which, depending on the criterion of their targeted specificity, are distinguished in polyclonal and monoclonal antibodies [455]. Polyclonal antibodies are less specific, because they target more than one antigenic epitope, whereas monoclonal antibodies target specific antigenic sites, giving greater specificity than the former [456].

Structurally, monoclonal antibodies are immunoglobulins (Ig), which consists of two heavy (Heavy-H) and two light (Light-L) chains based on their molecular weight. Heavy chains are divided into five different subtypes (a, m, c, d, e), while light chains are divided into two types [457]. The isotype of an immunoglobulin is characterized by the type of immunoglobulin heavy chain e.g., IgA, IgG, IgM. The four chains of immunoglobulins are covalently linked together by disulfide bonds. Each light chain consists of a fixed (fragment Fc) and a variable region, while each heavy chain consists of a variable and 3-4 fixed regions. The variable regions are where the antigens bind to the immunoglobulins and are called Fab fragments [458].

The production of antibodies is associated with the body's immune response. The latter in each antigen introduced into the body is polyclonal, meaning that many

different B-cell clones are stimulated to produce antibodies. These antibodies have different molecular structures and respectively recognize different antigen epitopes [456]. Pure antigen is used to immunize an organism; however, this organism produces a variety of antibodies against very small amounts of foreign substances, which are inevitably contained in the antigen. The most specific antisera contain a mixture of antibodies, which differ from each other in biological behavior. For these reasons, immunologists try to produce large amounts of antibodies that are homogeneous in their behavior [459]. Multiple myeloma proteins are known to be homogeneous immunoglobulins (monoclonal), which appear in the γ-globin region during electrophoresis. This is because cancer mutation occurs only in one B-cell clone, which produces large amounts of immunoglobulins of the same biochemical composition [460].

Biologists have developed the fusion of experimental animal cells into tissue cultures. These cells with special genetic characteristics form a new cell, the so-called hybrid [461]. Monoclonal production is accomplished by sensitizing the experimental animal to a specific antigen. After a specified time, the experimental animal is killed and then spleen cells are taken from which the B-lymphocytes are separated and fused with other multiple myeloma cells [462]. Their fusion results in the production of cells with the capacity of continuous production of cells with the same features (hybridomas). Amounts of hybrid cells can be stored in deep freezing, while other cells sometimes are introduced in the peritoneal cavity of experimental animals, where they produce ascites fluid, that contains a high-titer antibody [461, 462].

Monoclonal antibodies and polyclonal antisera are used in histopathology for the differential diagnosis of diseases and mainly neoplasms. The advantages of monoclonal antibodies are that they are produced in large quantities and with great purity, their application is easy and is done by the combination of immunoenzymatic methods or immunofluorescence and their sensitivity is extremely high [455]. The problem of cross-reactions, although much smaller than that of polyclonal antibodies, exists and is due to different antigens having the same or similar antigenic properties [455, 463].

Immunohistochemistry, unlike conventional histochemical methods, is based on reactions specializing in the recognition of the antigenic target. Unlabeled techniques (enzymatic) are based on the immunoactivity of antibodies and the chemical properties of enzymes or enzyme complexes [437]. These react with undyed substrates of chromogenic compounds to form a dyed final product. The enzymes used are mainly hydrogen peroxidase, alkaline phosphatase and secondarily glucose oxidase [464]. The main methods are summarized as follows:

7.1 Immunoperoxidase method

This method is characterized by the binding of antibodies to the peroxidase enzyme. The application of the method is carried out either directly or indirectly, following a series of unbound antibodies against the target antigen for the indirect method [465]. The identification of the deposits (antigens) with the common microscope is finally achieved with the use of chromogens, most commonly with the addition of diaminobenzidine (DAB). DAB polymerizes in the presence of peroxidase and hydrogen peroxide to form an insoluble polymer in the antigen-antibody complexes giving a brown tint to the common optical microscope. These molecular changes of DAB lead to the formation of a solid color that is not soluble in organic solvents. The sections are then placed in hematoxylin solution (Harris, Mayer's) to determine the remaining morphological or histological features of cells [466]. In detail:

a) The direct method is simpler, but at the same time it is quite sensitive for the detection of mainly immunoglobulins, complement fractions and several antigens using polyclonal and monoclonal antibodies. The antibodies are directly bound to peroxidase [455].

b) The indirect method (Sandwich technique) is more sensitive than the one mentioned above and two series of antibodies are used. The first antiserum, which is also the primary, is usually derived from rabbits (polyclonal antibodies) or mice (monoclonal antibodies) and is not peroxidase-bound. The second antiserum is peroxidase-bound and comes from another species of animal (pig) that has already been sensitized to the IgG immunoglobulin of the former [465]. Thus, these

antibodies target those of first series which in this case act as antigen and reveal the primary antigenic site.

c) The "bridge method" (immunoperoxidase bridge method) is an extension of the indirect method. Three series of antisera are used and an unbound antibody is inserted between the first and last antibody [455]. It is also unbound and targets the peroxidase of the animal species from which the primary antibody is derived. The slices are then incubated with an antiserum containing free peroxidase, which binds to the latter antibody by an antigen-antibody reaction [467].

d) The peroxidase-antiperoxidase method (PAP method) is a modification of the enzyme bridge method. The peroxidase binds to its antibody (antiperoxidase) before being applied, to form a cyclic complex in a ratio of 3 molecules of peroxidase to 2 molecules of antiperoxidase. The first and second antiserum are not linked, while the peroxidase-antiperoxidase complex is the third sequence. The great advantage of the method is the possibility of using the primary antibody in high dilution, thus reducing the degree of its binding to unwanted tissue antigens [468]. This method reduces the likelihood of antibodies being cleaved from the antigens because all of their antigen binding sites (Fab fragments) are saturated and reduces the degree of non-specific staining (background) [469].

7.2 Avidin-Biotin-Peroxidase (ABC) Complex Method

This is an immunoenzymatic method, similar to PAP and is considered a more sensitive method than that, due to the strong binding property between biotin and avidin [470]. Avidin is a glycoprotein derived from the egg white and tissue of birds, reptiles and amphibia [471]. It is formed by four identical subunits, with a combined mass of 67 to 68 kDa. Each one of the subunits consists of 128 amino acids and binds one molecule of biotin. Therefore, a total of four biotin molecules bind to a single avidin molecule [472]. The extent of glycosylation on avidin is very high and carbohydrates account for about 10% of the total body of the tetramer [473]. Avidin presents a basic isoelectric point (pI) of 10 to 10.5 and is remarkably stable through a wide range of temperatures and variable pH [474]. Extensive chemical alterations have little effect on the activity of avidin, which makes the protein especially useful for protein purification. In addition, avidin,

due to its carbohydrate content and basic pI, exhibits considerable, nonspecific binding properties [450].

Biotin is a small molecule (MW 244.3), also known as vitamin H. It is present in small amounts in all living cells and is crucial for a variety of biological processes [475]. The valeric acid side chain of the biotin molecule is derivatized, so that it can incorporate different reactive groups that can be used to bind biotin to other molecules. In regards to ImmunoHistoCytochemistry, biotin is conjugated to antibodies or to enzyme reporters, in order to detect target antigens [476].

The remarkable affinity of avidin (AV) for biotin, allows biotin-containing molecules, that are in a complex mixture to be specifically bound to avidin. Avidin–biotin binding appears to be the strongest known non-covalent interaction between a protein and ligand [470]. The bond between avidin and biotin is formed very rapidly, and once it is formed, remains unaffected by extremities in temperature, pH, organic solvents and other denaturing agents [477, 478]. These properties of avidin in detecting or purifying biotin-labeled proteins or other molecules are particularly useful in a number of biomedical applications [479]. The third antiserum then consists of a complex of avidin and biotin-binding enzyme, which has some free avidin binding sites to the biotin of the second antibody [480]. A variation of the method is the replacement of avidin by streptavidin in the aforementioned ABC trinity.

Streptavidin, is a biotin-binding protein, isolated from the bacterium Streptomyces avidinii. It is a 60 KD molecular weight protein and its isoelectric point is near neutral. Streptavidin contains four identical subunits, each of which has a biotin binding site [481]. Its use over avidin is preferred because it is not glycosylated, which makes the protein less prone to nonspecific binding. Also, streptavidin is much less water-soluble than avidin. Guanidinium chloride at pH 1.5 will segregate avidin and streptavidin into subunits, but streptavidin is more resistant to dissociation [482]. Streptavidin is used either as a complex with biotin or as an enzyme labeled (labeled streptavidin).

7.3 Alkaline phosphatase method

Alkaline phosphatase is applied either as an avidin-biotin-alkaline phosphatase complex or as an alkaline phosphatase-anti-alkaline phosphatase complex. The latter complex consists of two antigen molecules bound to one antibody molecule. This arrangement resembles the normal binding reaction of a divalent antibody and therefore the complex is stable for a long time [483].

7.4 One- or two- step method using polymer dextran (EPOS or two-step method-Dako En Vision^{+} dextran technology)

The main virtue of the method is based on the bypass of biotin (pancreas belongs to the organs that produce moderate levels of endogenous biotin) [484]. The system is based on an enzyme-labeled (HRP-Horseradish Peroxidase or AP-Alkaline Phosphatase) water-soluble dextran polymer, to which the secondary antibodies bind after the primary antibody is added. In contrast to this classic standard immunohistochemical treatment, the one-step method system is based on the binding of the primary antibody to the dextran polymer, after endogenous peroxidase binding has taken place [485]. The whole technique allows the fastest and most sensitive final immunostaining, as the required stages of immunohistochemistry are reduced and due to the non-cross-reaction with the endogenously produced biotin no staining substrate is formed [486]. This technique was applied with an antibody-modified protocol to the protein analysis of cancer tissues [485, 487].

Immunocytochemistry is usually performed in four consecutive steps. First, the cells are placed on a solid support, which usually can be either a glass slide or a glass-bottom plate. According to the type of cells and seeding technique, the following incubation time may be necessary before proceeding with immunostaining [488]. When seeding adherent cells, the cells will attach to the solid surface support, during the incubation, which can vary from half an hour to 24 h, for the different cell types. In the second step, the cells are exposed to immunostaining, which involves fixation, permeabilization, and antibody incubation [489]. Fixation ensures that the proteins are retained at their location in the cell and that they preserve their chemical and structural state, at the time

of fixation. This can be achieved by crosslinking or by precipitating the proteins, with the use of organic solvents. Then, membranes are punctured with the use of solvents or detergents, allowing the relatively large antibodies to cross the cellular membranes, this process is called permeabilization. For efficient permeabilization, fixation is required, hence limiting the technique to studying dead cells. During antibody incubation, the antibodies are allowed to bind to target antigens within the cells, after which unbound antibodies are removed by washing. In the third step, the cells and the locations of antibodies bound to target antigens are visualized using microscopy [490, 491]. Images are taken using a camera or other detector, and finally, the images are analyzed and cellular structures annotated [492].

Liquid-Based Cytology

In the early 1980s, work began on improving cytology preparations (CP) by distributing cell collections in a liquid-based preservative before placing them on the slide [493]. Much of the original impetus for this research came from the early attempts to develop automated computer image analysis systems that are suitable for the evaluation of Pap smears. A number of problems with computer image analysis of CP, such as inadequate visualization of cells because of cell overlap and obscuring by mucus, blood and other debris, greatly complicated the development of image algorithms [494]. Liquid-based preparations were soon shown to overcome most of these problems, facilitating the single-cell analysis favored by automated systems. These innovations, along with improved sampling devices, revolutionized cytologic screening that had essentially remained unchanged since the introduction of the Pap smear nearly 50 years, ago [495].

This dramatic acceptance is secondary to a number of theoretical advantages LBC has over traditional CP, including capturing more of the exfoliated cells from the collection device, random and presumably more representative transfer of exfoliated cells to the slide, and improved microscopic visualization attributable to reduced overlapping, obscuring blood, and inflammation [496, 497].

8. Principles of digital analysis of microscopic image

The traditional optical microscope has been the primary tool in aid of pathological examinations [492]. The contemporary digital pathology, combines the power of microscopy with electronic detection and computerized analysis. It enables cellular, molecular, and genetic imaging with extreme efficiency and accuracy, thus facilitating clinical screening and diagnosis [498].

Image analysis systems emerged as a result of the insurmountable need for higher quality controls on high-tech products (transistors, microchips) in the early 1980s in the field of Electronics. They offer the possibility of quantifying the quality through a quick and accurate estimation of the measured parameters [499].

Morphometric analyzes at the level of pathological anatomy and cytology refer to measurements of absolute number of cell areas (e.g., nuclei) or geometric characteristics such as diameter of nuclei or cells, ratio of diameters or areas (N/C ratio), as well as densitometry studies, in which the extent and optical density (color depth) of nuclear or cytoplasmic histochemical or immunohistochemical stains [500].

Two types of image analysis systems are identified, based on the independence of their activity: fully automated, which by using robotic mechanisms and based on modified macros, perform the management and measurement of the desired features in a fully automated way and those which are characterized as semi-automated, in which the physician-operator intervenes by cutting or integrating regions of interest [501-503].

In each histological or cytological plate, representative fields are selected at the desired magnification and under constant light intensity and the absolute number of nuclei and the optical density or the intensity of the immunochemical staining (staining intensity) are measured in the selected cell subsystems (e.g. membrane, cytoplasm),after the corresponding macros are structured in Basic language to determine the color range (in this case shades of brown due to DAB as per RGB standard (Red/Green/Blue) [504]. The digitized image of the microscope occupies a fixed measuring box on the computer screen (active window) of 16848 μm^2 area at 40x magnification, i.e., ¼ the field of view of the microscope. Each pixel receives

a value of the color spectrum, which is the component of the aforementioned basic colors [505]. The optical density range in this system covers the range of values from 0 (absolute black) to 255 (absolute white). In this way, the correlation of staining intensity (decreasing values-color depth) with the biological behavior of the tumor (differentiation-staging) is translated at a clinical level [498].

During the measurement process the analogue image of the microscope is digitally filtered (formation of a temporary or saved digital file of jpeg or TIFF type) and after its immobilization, the pre-made macro captures the measurement areas marked with a color (e.g., red), so that in the final stage, i.e., before the final scorecard (Excel sheet type) is formed, the operator is allowed to intervene by integrating or cutting out regions of interest, which the system ignored or overestimated [506]. The combination of correct macro, stained plate quality (purity-specificity of immunohistochemical staining) and user experience dramatically reduces the statistical error (<5%) [437, 507].

PART II

9. Purpose of the research study

The pterygium is a common eye disease, with a recurrent nature, that mainly affects people who are exposed to intense sunlight. In Greece, the incidence rate of this disease has not yet been estimated, but it is an extremely common complaint especially in rural populations, a fact that is in line with the prevailing view today that sunlight is implicated [65]. It is considered a degenerative disease of the conjunctiva that can cause chronic inflammation with pain, photophobia, redness and if left untreated, it can potentially result to gradual deterioration of vision, for the patients. Most of the research papers that have been published so far, suggest a disorder at the level of cell proliferation, with degeneration of the cell layers of the conjunctiva and development of hypertrophic fibrous connective tissue [80].

The primary aim of this study, was to detect in the pterygial tissue the protein expression of the PTEN tumor suppressor gene-apoptotic factor, the mitotic regulator and inhibitor of cell death survivin, in combination with the expression of p53 tumor suppressor. The current trend in the international literature indicates as a possible pathogenetic mechanism of the occurrence of pterygium, the effect of ultraviolet rays on the mechanism of control of cell division of the layers of the conjunctiva [508]. By investigating survivin and PTEN, factors involved in the oncogenesis process, we intend to study the possibility that the pterygium is a precancerous lesion of the conjunctiva, rather than a simple degenerative process. Such a case would probably change the therapeutic direction. The treatment applied to date, which is mainly the surgical removal of the lesion, has a high recurrence rate, exceeding 60%. We hope that a new approach to pathophysiology and consequently to pterygium treatment will add new, more effective means of treatment.

Our secondary aim, was to identify different expression patterns of these biomarkers, between different morphological features and anatomical location of the pterygium.

10. Materials and Methods

10.1 Origin-Collection-Management of cytological material

Patients from a Tertiary General Hospital were recruited. They were informed to the purpose of our study and taken through the relative procedures, before signing the consent form.

Pathological cells from the pterygium area and healthy cells were brushed off, from 38 patients with pterygium and 12 patients with healthy conjunctiva, respectively. This was done under local anesthesia, in the form of topical proxymetacaine 0.5% eye drops.

Then, this material was placed in a special vial with fixative liquid (alcohol) and the corresponding plates were prepared by liquid phase cytology. P53, survivin and PTEN factors were detected in this material. Each sample was given a serial identification number.

Method of data collection - material identification - ensuring confidentiality

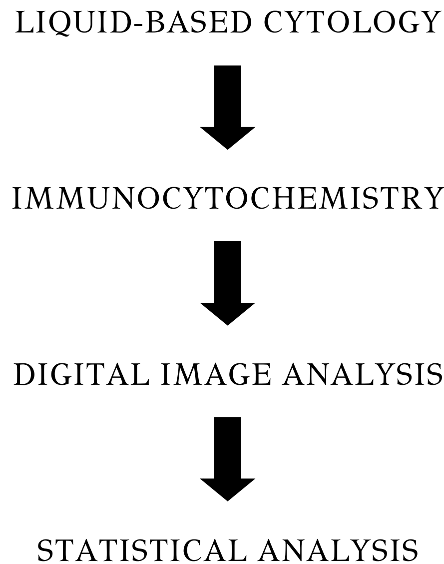
Patients, after signing the consent form, received the relevant serial number, with which the samples of material from the conjunctiva were identified and stored. No other patient data were collected, relating to their personal details, other than the concerning medical history, which would allow their identification at any stage of the research.

Method of file storage

The relevant information was collected in Excell sheets, in a computer, so that the statistical analysis of the findings could be possible and at the same time, archives were kept in hard copies, with the data and information that was collected during the research.

10.2. Laboratory study algorithm

Briefly, the **Laboratory Algorithm** is formulated as follows:



10.3 Epidemiological-Clinical pathological features of study material

The study of protein expression of the three biomarkers, survivin, PTEN and p 53, included thirty-eight patients. Twenty male patients and eighteen female patients, that visited our outpatient ophthalmology department, with a mean age of 63 years, were recruited.

Our secondary aim, was to identify differences in protein expression between different morphological appearances and anatomical distribution.

An initial letter was allocated to each specimen, regarding the anatomical location of the lesion. Eighteen were characterized as central (encroaching towards the pupillary axis, on the cornea) and the rest as peripheral (affecting the peripheral cornea). Furthermore, sixteen specimens were classified according to their morphology as swollen-fleshy and the remaining twenty-two, as superficial-flat (atrophic). The characteristics of the pterygia are reported in Table 1.

10.4 Cytochemical process

A specialized cytology brush, designed to optimize cellular yield was used, to collect the samples. With a rubbing motion, cells were gathered from the area of pterygium and healthy tissue, respectively. Then, the brush was placed in a special vial with fixative fluid (alcohol), the vials were sealed and subsequently the corresponding plates were prepared by liquid-based cytology.

Cytological smear sampling resulted in the embedding of the obtained cells in a fixative fluid for liquid-based cytology analysis (liquid-based cytology: LiquiPrep, FL, USA). This was followed by analysis and coating of the samples on special plates which were stained with immunocytochemical staining. The whole process was performed by a specialized technologist-manufacturer.

10.5 Immunocytochemistry protocol

For the immunocytochemical analysis of the selected **p53/PTEN/survivin** protein markers plates were made from the liquid-phase sample. The antibodies applied during the automated immunocytochemistry process are listed below:

anti-**p53** mouse monoclonal (clone DO-7, Dako NA Inc, Ca, USA; dilution at 1:40)

anti-**PTEN** mouse monoclonal (clone 6H2.1, Dako NA Inc, Ca, USA; dilution at 1:100)

anti-**Survivin** mouse monoclonal (clone 12C4, Dako NA Inc, Ca, USA; dilution at 1:100)

The modified protocol applied during the immunocytochemical analysis of the specific type of plates (liquid-based cytology) is listed below:

Immunohistochemistry Protocol - IHC EnVision two step method (Dako)

Deparaffinization Not applied (cellular substrate)

Inactivation of endogenous peroxidase (Peroxidase blocking)

-Incubation of the plates in 0.03% H_2O_2 solution for 5 min

-Rinse with distilled H_2O for 5 min x 2 times

-Immersion in isotonic TBS solution for 5 min x 2 times

Detection of antigen sites (Antigen Retrieval)

-Enzymatic digestion for 10 minutes at 37° C

-Incubation with the primary antibody for 40-50 min, respectively.

-Rinse with distilled H2O for 5 min x 2 times

-Immersion in isotonic TBS solution for 5 min x 2 times

Binding to the Peroxidase Labeled Polymer Complex

-Incubation with Horseradish peroxidase-labeled polymer-HRP LP

-Rinse with distilled H2O for 5 min x 2 times

-Immersion in isotonic TBS solution for 5 min x 2 times

-Addition of 3 drops of DAB chromogen solution for 30 min

-Rinse with distilled H2O for 5 min x 2 times

-Immersion in isotonic TBS solution for 5 min x 2 times

Antistain and Coating

Immersion in Hematoxylin solution (Hematoxylin MAYRES-water based) for 5-10 seconds

Rinse the sections with tap water for 2-3 min

Progressive dehydration (Dehydration with graded alcohol)

70% Ethanol	immersion for 3 min at room temperature
85% Ethanol	immersion for 3 min at room temperature
96% Ethanol	immersion for 3 min x 2 times at room temperature
100% Ethanol	immersion for 3 min x 2 times at room temperature

Immersion in pure Xylol for 2-3 min x 3 times and affixation of coverslips (previous coating with Histomount just before Xylol evaporates).

The whole procedure, which is based on bypassing the biotin binding to give a cleaner substrate, was performed with the Biogenex I 6000 automatic immunochemistry device.

Immunocytochemical staining was judged as acceptable per marker as follows:

Table 1 Immunohistochemical staining criteria

MARKER	STAINING STANDARD
P53	NUCLEAR/PERINUCLEAR
PTEN	CYTOPLASMIC
SURVIVIN	NUCLEAR/CYTOPLASMIC

10.6 Digital Image Analysis Protocol

The objective evaluation of the protein expression of the examined markers **P53, PTEN, Survivin** was performed by application of digital image analysis protocol with structure and software from Intel Pentium Dual-Core, Digital Camera Sony Cyber-shot (5mPx digital analysis), Microscope Olympus CX-31, Windows XP / NIS – elements image analysis software, Nikon, Jp, 2009. Progressive measurement of the intensity of immunocytochemical staining was performed in cell spots and 5 optical fields.

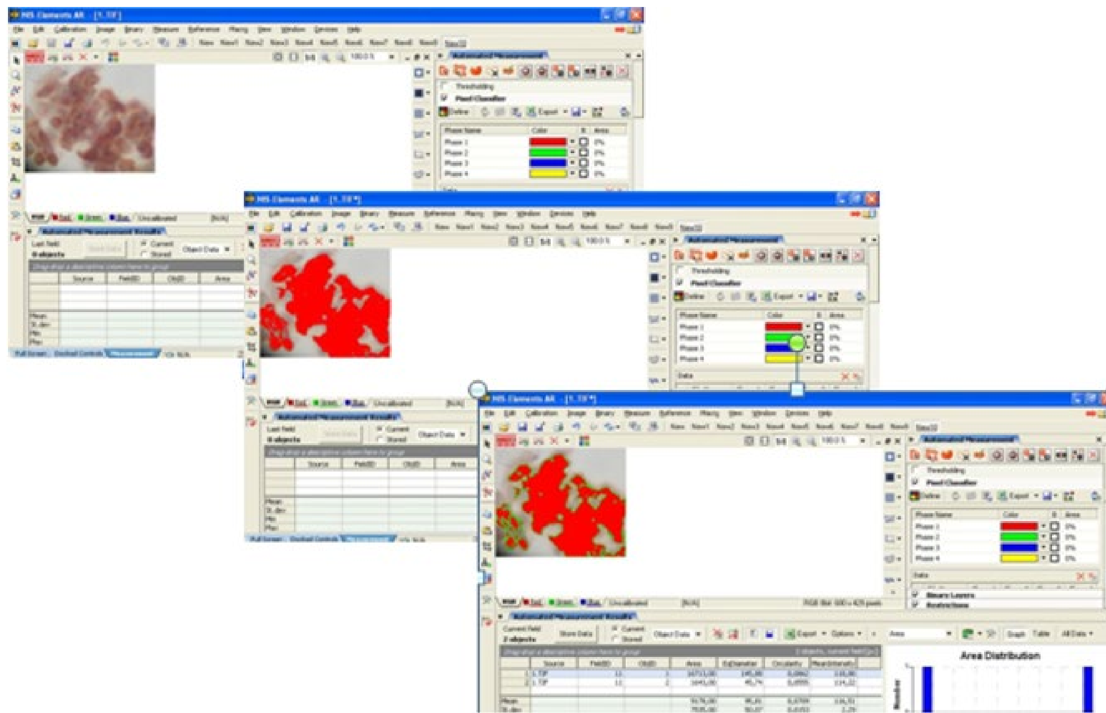


***Figure 26** Computerized image analysis of a case of survivin protein expression objective evaluation.*

Reddish areas demonstrate different expression values (range: 0-255 grey scale immunostaining intensity levels).

Progressive measurement of the intensity of immunocytochemical staining was performed in cell spots and 5 optical fields.

The digital database in excel was forwarded for statistical analysis. The measurement is based on a range of 256 RGB (combined red, green, blue colors per pixel) of continuous values (values leading to 0 correspond to protein overexpression, while leading to 255 to gradual loss of stain). Normal cell substrates from healthy conjunctiva and vaginal cervical smears were set as a control group, to ascertain the level of staining. Based on the measurement values, the cases were categorized as high/medium and low protein expression (low).

11. Results – Statistical Analysis

11.1 Descriptive Analysis

Pathological specimens from thirty-eight patients presenting with conjunctival lesions, identified as pterygia and twelve specimens from normal conjunctiva, were collected by smooth scraping of the conjunctiva.

The majority of the examined pterygia cases overexpressed the markers (p53: 22/38-57.9%. survivin: 30/38-78.9%. and PTEN: 25/38-65.7%. respectively). Interestingly, overall p53/PTEN co-expression was found to be statistically significant (p=0.022). Overexpression of survivin and PTEN is remarkably strong, whereas p53 appears to be expressed to a lesser extent, in the pathological epithelial cells. The association of survivin, PTEN and p53, to abnormal conjunctiva epithelia was with a statistical probability of p= 0.019, p= 0.456 and p= 0.03, respectively.

No statistical significance was observed regarding the anatomical localization of the pterygia (central/peripheral) or their morphological type (fleshy/flat).

11.2 Statistical Tables

Table 2 Measurement values of protein expression

A/A	age	p53	survivin	PTEN	control/pathological
1	74	141.4	142.1	98.91	C
2	67	127.3	133.84	129.15	C
3	77	131.2	140.97	114.31	C
4	72	129.9	102.98	121.58	C
5	56	154.9	136.08	135.19	C
6	78	144.9	135.86	122.74	C

7	71	139.3	135.53	132.11	C
8	81	143.6	137.1	118.16	C
9	79	148.7	122.86	124.37	C
10	84	121.9	130.57	126.76	P
11	72	117.3	123.86	104.69	P
12	69	110.1	86.4	121.15	P
13	75	121	103.01	130.76	P
14	89	117.7	106.43	125.14	P
15	73	131.5	115.47	118.82	P
16	45	137.7	101.76	132.91	P
17	57	121.6	83.08	125.05	P
18	72	100.9	93.64	113.57	P
19	62	115.3	127.68	93.57	P
20	82	120	114.38	124.08	P
21	51	120.1	140.68	92.77	P
22	49	131.8	98	141.79	P
23	58	116.1	101.5	139.04	P
24	66	144.8	92.29	137.8	P
25	53	130.7	138.33	143.84	P
26	60	117.9	108.47	107.2	P
27	55	134.2	101.04	144.65	P
28	59	108.4	113.25	82.29	P

29	68	137.7	115.31	145.94	P
30	64	119.2	92.88	102.5	P
31	72	137.1	106.25	135.61	P
32	73	92.83	144.4	126.37	P
33	69	131.4	105.47	81.14	P
34	72	100.9	127.65	86.16	P
35	54	100.9	90.91	91.06	P
36	49	153.7	115.9	137.63	P
37	42	122.5	118.57	112.49	P
38	65	104.9	95.01	140.39	P
39	68	135.5	119.8	133.23	P
40	71	136.5	123.94	120.28	P
41	76	85.83	142.09	144.16	P
42	58	154.5	121.48	108.96	P
43	44	131	115.51	93.61	P
44	53	134.2	123.82	100.88	P
45	71	121.6	97.97	73.5	P
46	75	118.3	90.74	117.58	P
47	77	132.6	125.48	100.53	P
48	62	127.8	137.05	106.08	C
49	79	135.5	118.96	126.52	C
50	71	145.9	104.66	135.09	C

Table 3 Classification according to anatomical location and pterygium morphology

A/A	age	anatomical location	morphology	pathological
1	84	K	A	P
2	72	K	A	P
3	69	S	E	P
3	75	S	A	P
5	89	S	A	P
6	73	K	A	P
7	45	K	E	P
8	57	K	A	P
9	72	S	E	P
10	62	S	E	P
11	82	K	A	P
12	51	K	E	P
13	49	S	A	P
14	58	K	E	P
15	66	S	E	P
16	53	S	E	P
17	60	K	A	P
18	55	S	A	P
19	59	S	A	P

20	68	K	A	P
21	64	S	A	P
22	72	K	A	P
23	73	S	E	P
24	69	K	A	P
25	72	K	E	P
26	54	S	A	P
27	49	K	E	P
28	42	K	E	P
29	65	K	A	P
30	68	S	A	P
31	71	K	A	P
32	76	S	E	P
33	58	S	A	P
34	44	K	A	P
35	53	S	E	P
36	71	S	E	P
37	75	K	A	P
38	77	S	E	P

S: Sclerocorneal limbus

K: Corneal involvement

E: fleshy pterygium

A: flat, atrophic pterygium

Statistical data processing was performed using the IBM SPSS v25 statistical software (SPSS Inc, Chicago, IL, USA) 0. Pearson Chi-Square (x2) test and Fisher's exact tests were used to compare between immunocytochemical molecular analysis and clinicopathological features, respectively. The level of statistical significance between the above correlations was set at $p<0.05$.

***Table 4** p53 vs PTEN*

Double input matrix

		PTEN			
		Expression		Low Expression	
		Count	Row N %	Count	Row N %
p53	Expression	19	82.6%	4	17.4%
	Low Expression	14	51.9%	13	48.1%
	Total	33	66.0%	17	34.0%

χ^2 control result

Pearson Chi-Square Tests

		PTEN
P53	Chi-square	5.236
	df	1
	Sig.	.022*

Results are based on nonempty rows and columns in each innermost sub table.

The Chi-square statistic is significant at the ,05 level.

Correlation coefficient value Phi (Φ)

Symmetric Measures

		Value	Approx. Sig.
Nominal by Nominal	Phi	.324	.022
	Cramer's V	.324	.022
N of Valid Cases		50	

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

The control of the two categorical variables P53 and PTEN confirms the existence of a relationship, as evidenced by the statistical chi-square test and determined by the value of X 21 = 5.236, with p = 0.05. According to the value of the correlation coefficient Phi (Φ) which is equal to 0.324 we can say that there is a weak to moderate positive correlation between them.

Table 5 p53 vs Control/Lesion

Double input matrix

		Control / Lesion			
		control		lesion	
		Count	Row N %	Count	Row N %
p53	Expression	1	4.3%	22	95.7%
	Low Expression	11	40.7%	16	59.3%
	Total	12	24.0%	38	76.0%

χ^2 control result

Pearson Chi-Square Tests

		Control / Lesion
p53	Chi-square	9.018
	df	1
	Sig.	.003*

Results are based on nonempty rows and columns in each innermost sub table

The Chi-square statistic is significant at the ,05 level.

Correlation coefficient value Phi (Φ)

Symmetric Measures

		Value	Approx. Sig.
Nominal by Nominal	Phi	.425	.003
	Cramer'sV	.425	.003
N of Valid Cases		50	

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

Table 6 p53 vs Survivin

Double input matrix

		Survivin			
		Expression		Low Expression	
		Count	Row N %	Count	Row N %
p53	Expression	16	69,6%	7	30,4%
	Low Expression	18	66,7%	9	33,3%

$\chi 2$ control result

Pearson Chi-Square Tests

		Survivin
p53	Chi-square	,048
	df	1
	Sig.	,827

Results are based on nonempty rows and columns in each innermost sub table.

The control of the two categorical variables p53 and survivin, does not confirm a relationship between them.

Table 7 PTEN vs Survivin

Double input matrix

		Survivin			
		Expression		Low Expression	
		Count	Row N %	Count	Row N %
PTEN	Expression	22	66,7%	11	33,3%
	Low Expression	12	70,6%	5	29,4%

$\chi 2$ control result

Pearson Chi-Square Tests

		Survivin
PTEN	Chi-square	,079
	df	1
	Sig.	,778

Results are based on nonempty rows and columns in each innermost sub table

The Chi-square statistic is significant at the ,05 level.

Table 8 Survivin vs Peripheral-Central

Double Input Matrix

		Peripheral / Central location			
		Count	Row N %	Count	Row N %
Survivin	Expression	17	50,0%	17	50,0%
	Low Expression	8	50,0%	8	50,0%

χ2 control result

PearsonChi-SquareTests

		Peripheral / Central
Survivin	Chi-square	,000
	df	1
	Sig.	1,000

Results are based on nonempty rows and columns in each innermost subtable.

The control of the two categorical variables Survivin and Peripheral / Central location, does not confirm a relationship between them

Table 9 PTEN vs Fleshy / Flat

Double Input Matrix

		Fleshy / Flat			
		Count	Row N %	Count	Row N %
PTEN	Expression	19	57,6%	14	42,4%
	Low Expression	9	52,9%	8	47,1%

χ2 control result
PearsonChi-SquareTests

		Fleshy / Flat
PTEN	Chi-square	,098
	df	1
	Sig.	,754

Results are based on nonempty rows and columns in each innermost subtable.

The control of the two categorical variables PTEN and Fleshy / Flat does not confirm a relationship between them

Table 10 Total results of the examined markers and also p values

n=50	p53			PTEN			Survivin		
	L	OE	p-value	L	OE	p-value	L	OE	p-value
pterygia (n=38)	16	22	0.03	13	25	0.456	8	30	0.019
type			NS			NS			NS
flat (n=22)	10	12		9	13		5	17	
fleshy (n=16)	6	10		4	12		3	13	
localization			NS			NS			NS
central (n=18)	10	8		11	7		4	14	
peripheral (n=20)	8	12		2	18		7	13	
control* (n=12)	1	11		4	8		9	3	

L: low expression OE: over expression NS: no statistical significance

* Control: normal conjunctival epithelia

p53 mean values** (pterygia: 126.4, control: 147.9)

PTEN mean values** (pterygia: 121.6, control: 139.5)

Survivin mean values** (pterygia: 113.7, control: 141.7)

p53/PTEN co-expression (p=0.022)

**Mean values: staining intensity levels (range 0-255)

12. Discussion

Pterygium is one of the most commonly observed, diseases in ophthalmology. It is a proliferative, invasive and highly vascularized tissue, that progressively stretches from the conjunctiva onto the nasal, temporal, or both aspects of the cornea, in a wing-like shape [24]. It can impair vision through altered tear film, photophobia, epiphora, induced irregular astigmatism or even binocular diplopia due to contraction of Tenon's capsule and in severe cases, central corneal stromal scarring. Exposure to high levels of ultraviolet (UV) radiation is believed to be one of the main causative factors, alongside reflected scattered light and other environmental elements, like dust and chronic inflammation [509]. This exposure causes increased damage through oxidative stress to the respective epithelial and limbal stem cells and acts as the main mechanism in the transformation of their cellular microenvironment [510]. Although this pathological condition of the conjunctival epithelium was traditionally considered as a benign degenerative process, it exhibits traits similar to tumors and neoplasia, such as epithelial proliferation, goblet cell hyperplasia, angiogenesis, inflammation, elastosis and most importantly, recurrence after resection. Progressive dysplasia is observed combined with the development of an infiltrative pattern [511]. For these reasons, the classification of the pterygium as a pathological entity is relatively difficult due to its nature and evolution. As manifestations of the same pathology, both the degeneration and reconstruction of the epithelium, as well as the neoplastic diversion of the pterygium, have been proposed [512].

A significant number of studies were conducted that support the notion of tumor behavior, providing some evidence to the pathogenetic mechanisms [513, 514]. Substrates investigated include

-Growth factors that are molecules that stimulate cell growth. They have the ability to promote mitosis and proliferation, in cell cycle. Numbers of growth factors, such as transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), nerve growth factor (NGF) insulin-like growth factor (IGF), and connective tissue growth factor (CTGF) have all been discovered, in pterygium.

The VEGF family control pathological angiogenesis and increase vascular permeability in ocular diseases [515]. Compared to normal conjunctiva, pterygium presents higher levels of VEGF and vascular endothelial growth factor receptor (VEGFR)-2, -3 [516]. Upregulation of VEGF-C mRNA level probably leads to lymphangiogenesis in pterygium, especially in recurrent pterygium [517]. Lately, it was clarified that TNF-α mediated the expression of VEGF-C [518].

TGF-β is responsible for regulating many of the processes that are common to both tissue repair and disease, including fibroblast proliferation, angiogenesis, fibroblast proliferation, degradation of matrix proteins like collagen and fibronectin and controlled synthesis. TGF-β1 and TGF-β2 were found to be upregulated while transforming growth factor-beta receptor 1, 2 (TGFR-β1, -β2) were downregulated in pterygium, compared to normal bulbar conjunctival tissues [519].

Insulin-like Growth Factor (IGF), capable of promoting cell mitosis, stimulating cell proliferation and inhibiting apoptosis, contributes to the growth of pterygium. Insulin-like growth factor binding protein-2 (IGFBP2) was overexpressed in pterygium's body fibroblasts [520]. However, insulin-like growth factor binding protein-3 (IGFBP3) was markedly decreased in pterygium compared to normal conjunctiva. Downregulation of IGFBP3 is known to be closely associated with the occurrence of cancer. This could mean that low level of IGFBP3 may be relevant to the out-of-control cell proliferation, signifying that downregulation of IGFBP3 is possibly, linked to the continuing growth of pterygium [521, 522].

Nerve Growth Factor (NGF), is a peptide that regulates growth, proliferation and survival of certain neuronal cells. Epithelial cells, fibroblasts and vascular endothelial cells of pterygium overexpress NGF [69]. Connective Tissue Growth Factor (CTGF), involved in cell adhesion, migration and chemotaxis is also present in the epithelium of pterygium but absent in normal conjunctiva [523].

-Tumor suppressor genes like p63, p16 and p27 appear to participate in pterygium pathology, as well [41, 524]. P63 is increasingly expressed, in the basal and parabasal layers of primary pterygium, similarly in the full thickness of the epithelium in recurrent pterygium. Increased expression of p16 protein was

observed in pterygium. Yet, p63 and p16 seemed to express rarely in normal conjunctiva [55].

-Proliferation related proteins, such as Ki-67, proliferating cell nuclear antigen (PCNA) and cyclin D1, play important roles in the process of cell growth. The Ki-67 protein is known as a cellular marker for proliferation. It is suggested that Ki-67 might be a sensitive marker for ocular malignant tumors [525]. Expression of Ki-67 was found increased in both primary and recurrent pterygium, which supports the proliferative nature of pterygium [51, 526].

PCNA is a DNA clamp and achieves its role by surrounding the DNA, where it functions as a scaffold to engage proteins involved in DNA replication, DNA repair, chromatin remodeling and epigenetics [527]. It was reported that the expression of PCNA and Ki-67 was significantly higher in pterygium than normal conjunctiva [528].

Cyclin D1 is a protein that is essential for progression through the G1 phase of the cell cycle [529]. Recent studies found that PCNA and cyclin D1 were overexpressed in the epithelial cells of the limbal part of pterygium, compared with normal conjunctivas. This event might lead to limbal micro-environmental anomaly such as hyper-proliferation of resident epithelial cells and it may explain how these two proteins promote the initiation of the disease [52].

Significant increase of Glutathione S-transferase expression in pterygium observed in a study, may reflect the activation of GST in response to excessive free radical formation from ultraviolet exposure to maintain antioxidant capacity in pterygium [530]. Although, best known for their ability to detoxify cellular environments, GSTs are also capable of binding nonsubstrate ligands, with important cell signaling implications. Several GST isozymes from various classes have been shown to inhibit the function of a kinase involved in the MAPK pathway that regulates cell proliferation and death[531]. β-Catenin overexpressed in nuclei/cytoplasm could increase cyclin D1 protein expression, which could contribute to proliferation of pterygium cells [53].

-Apoptosis related proteins, Bcl-2, Bax and Bcl-w, are known to be involved in the regulation of cell apoptosis. Bcl-2, a protein encoded in humans, by the Bcl-2 gene,

is the founding member of the Bcl-2 family that controls apoptosis by either inducing or inhibiting apoptosis. Bax, known as an apoptotic activator protein, comes from the Bcl-2 gene family as well [532]. Notable expression of Bcl-2 was observed in the basal epithelial layer of all pterygium epithelial cells, while normal conjunctiva showed no evidence of Bcl-2. Bax expression though, seemed to be similar in both pterygium and normal conjunctiva [55]. Bcl-w, another member of Bcl-2 family, protects cell from apoptosis and was reported to be overexpressed, in pterygium [46].

It is evident, that so far various molecular analyzes of the tissue substrate from pterygia have identified specific genes involved in cell proliferation, apoptotic death and the transmission of signals from the periphery to the nucleus - whose deregulation appears to lead to the gradual transformation from normal epithelium to the pterygium shaping.

Among them, survivin, PTEN and p53 genes are important regulators of cellular and overall tissue homeostasis, most likely, through their effect on apoptotic pathways [533]. Survivin, (gene locus: 17q25), is a member of the family of proteins that inhibit apoptosis (IAP) through negative interaction and inhibition of the activity of the initiating and effector caspases [534]. At the subcellular organelle level, Survivin interacts with the microtubules at the level of the mitotic sphincter and competes with mitochondrial-dependent apoptosis [535]. Overexpression of Survivin results in inhibition of both intrinsic and extrinsic pathways of apoptosis. Decrease of survivin in the human cells produces defects in apoptosis and multiple defects in cell division [536, 537]. Although the exact mechanism of inhibition of apoptosis by Survivin is still largely undiscovered, both direct and indirect binding of survivin to initiator or effector caspases is believed to contribute to inhibition of apoptosis by Survivin [538]. Binding of survivin directly to effector caspase-3 has been hypothesized, however, unlike other IAPs, survivin does not seem to possess the structural moiety responsible for docking of caspase-3 to the BIR domain [539]. Some studies suggest that survivin can bind directly to caspase-9 and inhibits its activity [540]. There is considerable evidence to support the dual function of survivin i.e., as a regulator of cell division and inhibitor of apoptosis. The C-terminus of survivin is necessary for cell division and the N-

terminus is dispensable for apoptosis [541]. Though, appreciated as two separate functions, it may be possible that survivin expression acts as a vital checkpoint for initiation of programmed cell death, in those cells that are undergoing aberrant cell division. More studies are required to demonstrate, whether survivin specifically acts as a propagator of cytokinesis, or cell death in normal cells and whether either of these functions becomes prevalent, during tumor progression [115].

Survivin occupies a central position among IAPs as being both the regulator of cell division and apoptosis. Nevertheless, normal differentiated cells present very low or no expression of survivin. But, does low or no expression of survivin on normal cells has any correlation with the cellular functions or does it signify less cell division/apoptosis in normal cells? Survivin knock-out mice exhibit embryonic lethality, loss of self-renewing bone marrow progenitor cells and bone marrow ablation [542, 543]. Conditional deletion of survivin in thymus gland leads to a developmental arrest in thymocytes at double negative stage and the appearance of immature T cells in periphery [544]. Survivin also presents as a key regulator of clonal expansion of T effector cells, proliferation of activated mature B cells and B cell progenitors, erythroid and megakaryocyte differentiation [122, 178, 545]. Survivin emerges as an intriguing protein that regulates functions of various immune cells [546].

The coordination of cytokinesis and mitosis, is a fundamental conserved role of survivin in all eukaryotes, from yeast to human. In particular, it exhibits an overexpression in the G2 / M phases and undergoes the G1 phase of the cell cycle [547].

When survivin (BIRC5) was first identified, its discovery generated a considerable interest from cell biologists and oncologists, an interest that continues today. For oncologists looking for new anti-cancer targets, proteins that are required for cell proliferation rate very high on their 'most wanted' list [541].

Proteins that are involved in programmed cell death (i.e., apoptosis) are similarly appealing. This is the intended response of tumor cells to traditional chemo- or radio-therapies. Therefore, as a protein that is, at the same time, able to inhibit apoptosis and is essential for mitosis, survivin seemed, at first glance, a promising

new target. Indeed, the most attractive targets are those that are differentially expressed in cancer versus normal cells. Moreover, survivin is highly abundant in cancer, but absent from most normal somatic cells [548]. Therefore, from this point of view, it seemed an ideal candidate, as well. In spite of these advantageous characteristics, rather disappointingly, an accurate and specific anti-survivin agent has not yet reached the clinic. It may be that survivin on its own, has no enzymatic activity; instead, it fulfils most of its tasks in conjunction with other proteins and is most likely best characterized as an adaptor protein that interplays with, or shuttles its partners to their destinations [549]. This is truly the case during mitosis, when survivin targets the chromosomal passenger complex (CPC) at the centromeres, and thus enables aurora-B kinase to phosphorylate a number of proteins that are responsible for ensuring that finally, the chromosomes are aligned properly, before they are segregated, at anaphase [550].

PTEN (phosphatase and tensin homolog deleted in chromosome 10), which is encoded by the corresponding gene (gene locus: 10q23.3) acts as a tumor suppressant. Over the past years, it has been suggested, that the changes regarding tumor stroma metabolism are powerful inducers of tumor growth [205]. In the stages of cancer induction and progression, the normal fibroblasts that are neighboring the tumor cells undergo metabolic reprogramming and alter their phenotype. Cancer Associated Fibroblasts (CAFs) support the growth of adjacent epithelial cancer cells, causing in turn oxidative stress and senescence in adjacent CAFs [551]. In senescent CAFs, initiation of autophagy and mitophagy potentiate a change in aerobic glycolysis producing biochemical molecules that drive oxidative phosphorylation based, anabolic growth in the tumor cells [552].

Overexpression of PTEN has been of significant interest, both in biologic and clinical significance terms. Studies that are conducted in non-small cell lung carcinoma cell lines have shown that overexpression of PTEN was connected to the inhibition of tumor growth, an increase in levels of cleaved caspase-3 and cell arrest in G0/G1 phase, thus presenting a role for PTEN, as a potential target [553]. In these patients, improvement based on the efficacy of pemetrexed, a first-line chemotherapy drug, has been observed [554]. Several studies processing different types of cancer cell lines, have revealed, that PTEN overexpression can augment

apoptosis in glioblastoma, inhibit proliferation and promote apoptosis of hepatocytes and reduce cisplatin resistance of ovarian cancer, by upregulating a downstream molecule termed as keratin, type I cytoskeletal 10 (KRT10) [555-557]. It can also, have a cooperative role with lithium for reducing colorectal cancer, suggesting their potential combination as a novel treatment [558].

It becomes evident that in this respect, numerous mechanisms are potentially able of transcriptional and post-transcriptional regulation of PTEN expression, including transcriptional repression, epigenetic silencing, regulation by miRNAs and disruption of competitive endogenous RNA (ceRNA) networks. All of them have been shown to play a role in regulating PTEN levels [181]. Furthermore, PTEN is subject to a wide range of post-translational modifications that ultimately regulate its protein levels, activity and function, including overexpression of PTEN interacting proteins, dimerization, and secretion [181]. Deregulation of the gene through deletion, mutation, or epigenetic changes (methylation of the gene promoter) into a wide variety of malignancies leads to a synchronization of the internal cytoplasm of molecular signaling pathways to the nucleus [559]. Normally the produced protein exerts a suppressive effect on cell proliferation by promoting the cell in the Go dormant phase.

It appears that, through autophagy PTEN determines the fate of the tumor [246]. Furthermore, the biology of the immune system permits tumor initiation and progression through the balance between effector and tolerance response, modulated by autophagy [560, 561]. Autophagy affects various biological functions of different cell types of the immune system such as dendritic cells, natural killer cells, T and B lymphocytes and macrophages. It can adjust the production of cytokines and antibodies which also have effects on the autophagic process. In addition, transforming growth factor -β, interferon-γ, and several interleukins (IL) act as stimulators, while IL-4, IL-10, and IL-13 counteract autophagy [562]. Autophagy can be prompted by the activity of the innate immune receptors, such as Toll-like receptors, and in adaptive immunity, it is an important determinant for lymphocyte differentiation, antigen presentation and cytokine secretion, with onco-suppressor activity [563, 564].

Recent studies explore the onco-suppressor role of PTEN in the tumor microenvironment regulation, affecting autophagy and metabolic reprogramming. In fact, specific genetic alterations including those of PTEN in cancer cells may affect the immune composition of the tumor microenvironment and such infiltrating immune cells may in turn act to inhibit or sustain cancer cells proliferation [552].

The aforementioned p53 protein is biochemically a 53 kDa phosphoprotein and plays a key role as a genome "gate keeper" [565, 566]. The encoding gene is located on chromosome 17 (gene locus: 17p13). It acts as a transcription factor for critical genes, inhibiting abnormal DNA duplication and together with the base repair system (DNA MMR) prevents the replication of abnormal nucleotides, as well as regulates apoptosis [306]. The pathological protein resulting mainly from point mutations changes the role of the molecule from tumor suppressor to oncogene [426].

By definition, tumor suppressor genes control metabolic functions, that limit inappropriate cell expansion and their inactivation promote tumor initiation or progression. Taking into account, the many processes that p53 controls, which of its effector functions are crucial for tumor suppression, has been proven the topic of much debate between scientists [306]. Senescence and apoptosis are clearly observed in tumors and when these processes are initiated, they are definitely tumor suppressive. Nevertheless, a recent body of studies suggests that apoptosis and senescence can be nonessential for tumor suppression and that, in some specific contexts, other non-canonical p53 functions may be more critical [567].

There is no clear consensus on which p53-dependent process is the most important. The only relevant measure of "tumor suppression" is whether a gene impedes the onset or progression of tumors emerging, in vivo [568]. As exemplified by the p53 knockout mouse, which is a powerful model that develops thymic lymphoma (and sometimes sarcoma) at complete penetrance [569].

To address which p53 function is critical for tumor suppression, mutant strains have been developed, in an attempt to separate the specific p53 functions. Furthermore, the resulting animal cohorts were continuously monitored for tumors, over time. If the removal of a p53-driven function permits tumorigenesis,

it is self-evident. that the underlying process is crucial for the tumor suppressive activity of p53. If it does not, it is considered dispensable [306]. However, it should be taken into consideration, that thymic lymphoma rarely occurs in people, so that the prerequisites for suppressing this rare form of cancer, may not necessarily apply to other systems [570].

One line of investigation has analyzed tumor onset and pathology between mice bearing knockouts of p53 target genes versus p53 itself. For example, mice that are deficient for p21, Noxa and Puma do not develop thymic lymphoma, implying that p53-mediated cell cycle arrest and apoptosis might be inessential for tumor suppression [571]. Still, it is possible that p53 target genes may be, already expressed at basal levels so p53-null cells, for example, are by no means p21-null [572]. Accordingly, this approach could overestimate the importance of a particular p53 effector to the null phenotype. Additionally, since numerous effectors mediate most of p53 outputs, mouse strains that are deficient for selected p53 effector genes do not fully incapacitate the relevant p53 effector program (e.g., p21 loss does not completely negate p53-mediated cell cycle arrest) [573, 574]. However, this view could underestimate the input of the targeted processes to tumor suppression. Changes in feedback loops and compensatory mechanisms presenting as a result of manipulating the pathway could complicate even more, the interpretation of such studies [575].

Another way to approach this subject, was to attempt to isolate p53 effects, through separation-of-function mutants that selectively retain or lose their ability to modulate specific subsets of p53 target genes and activities. For example, the tumor-derived p53R175P and p53E180R alleles exhibit defects in apoptosis while retaining the capability to evoke cell cycle arrest, thus mice harboring the relevant mutations show extended tumor-free survival as compared to p53- null animals [576].

Furthermore, the tumors that do appear in these mice appear to demonstrate far less chromosomal instability (CIN) than p53-null tumors. This observation indicates that different p53 mutants may impinge selectively on downstream effector pathways [576]. On the other hand, engineered structure and function mutants that disarray p53 transcriptional domains or are deficient in their

acetylation, can separate critical p53 functions, at least in vitro [577]. Although, these studies support the importance of p53-mediated transcription for tumor suppression, they do not determine a single key process. While, such lines of investigative research regarding structure and function, are appealing, they also present limitations. Mutant p53 proteins appear to be more stable or less stable than the wild-type protein and thus, the variability in phenotypes may reflect quantitative, as well as qualitative modifications [578].

The majority of structure-function mutants have only been isolated in a limited number of cell types, and with the context limitations, it cannot be presumed, that these results can effectuate tumorigenesis, in all cell tissues. Maybe, this is why, studies that are technically sound, have been unable to reach a common consensus, on the mechanisms involved [306].

Quite a few studies have evaded the issues by focusing on specific functions, peripheral to p53. So, rather than measuring the onset of tumor itself, upon p53 loss, they instead took advantage of mouse models harboring "switchable" p53 alleles. In these studies, attempts were made to stimulate endogenous p53 in already established tumors. In all of these situations, p53 remodeling resulted in an accentuated anti-tumor response, the nature of which was depended on the model employed [579, 580]. In liver carcinomas and sarcomas, the response is senescence. In Myc-expressing B cell lymphomas, the response is massive apoptosis. Under other conditions, p53 reactivation can trigger cellular differentiation and a loss of self-renewal [581]. It becomes evident that, the result of p53 reactivation in an existing cancer may not mirror the same processes that were lost during tumorigenesis and these studies support the belief, that the p53 response varies, depending on the context [582].

Previous studies established the critical relevance of p53 in the autophagy network, in order to promote cell survival. At the same time, p53 can also trigger autophagic cell death in various severe stress conditions [583]. p53 can regulate mitophagy as well as macro-autophagy, a process that leads to the synthesis of double-membrane vesicles and their integration into lysosomes, that recycle macromolecules and preserve intracellular pools of metabolites [584]. p53 can inhibit or stimulate autophagy, depending on its subcellular localization, its

mutational status, and stress type, through multiple mechanisms that include the transcriptional control of multiple autophagy-related genes, the modification of the mTORC1 kinase [585]. Kinase mTORCH1 firmly controls the autophagic process conforming to the intracellular nutrient and energy levels, by the regulation of BCL-2 family members which also control autophagy, or by direct interaction with the key autophagic regulator BECLIN 1 [586].

Several studies propose that the link between the p53 pathway and autophagy extends further, to other vital components of this cascade, including E4F1 and MDM2 [587]. Therefore, genetic inactivation of E4f1 has been shown to induce autophagy in leukemic cells and MDM2 was also proven to be regulated upon accumulation of the autophagy substrate p62/SQSTM1 in KRASG12D- driven pancreatic cancer cells [588, 589].

In addition, MDM2 supervises the balance between apoptosis and autophagy, in Nutlin-treated cells. In fact, Nutlin arrests autophagy and promotes apoptosis in MDM2-amplified cancer cells, but it promotes autophagy in MDM2 non-amplified cells. This diverse effect is linked with αKG levels and the transcriptional regulation of ATG genes, through an epigenetic mechanism implying the regulation of the αKG-dependent demethylase JMJD2b [590].

So eventually, what are the most critical p53 activities necessary for tumor suppression. Obviously, the above-mentioned notions exclude generalities without considering the necessity for context-specificity. For example, the importance of context is readily documented, in mouse studies demonstrating that Puma suppression compares to p53 loss in driving Myc-induced lymphomagenesis but not in promoting thymic lymphoma [591]. By embracing this notion, it should enable the demonstration of tumor-specific processes of tumor suppression and pave the way for restoring the most applicable p53 functions, in individual tumors.

It becomes evident, that evolution has opted for a delicate balance of p53 activity, since too little of p53 leads to early onset cancer and too much of p53 accelerates aging. The danger of excess p53 is evident in other pathologies, beyond cancer, including injury, aging and degeneration [592] .

As all animals age, the cost of eliminating potentially dangerous cells, is the gradual weakening of the stem cells responsible for tissue homeostasis. In a rapid process, patients presenting the heritable DNA repair deficiency syndrome Fanconi anemia, overstimulate p53 in an effort to resolve accumulating DNA damage and finally, experience bone marrow failure owing to progressive HSC loss [593]. Excessive p53-dependent apoptosis can also lead to developmental disorders of the brain and aging-associated neurodegenerative diseases, namely Alzheimer's and Parkinson's diseases [594].

As a regulator of cell death, it has been documented that p53 is involved in the pathological response to cardiac and cerebral ischemia; p53 manipulation and inhibition has been proposed as a protective strategy in the acute phase following injury [592]. Collectively, the characteristics of p53 function in normal physiology and non-cancer pathologies disclosed additional regulatory processes, downstream functions and possible therapeutic targets.

Studies on the presence of p53 in pterygia reveal increased expression [35, 595, 596]. In normal cells, p53 protein is short lived and maintained at low, often undetectable levels [36]. But mutations in p53 gene result in increased stability of its protein derivative in the cell, that can be readily detected, by antibodies to several epitopes of p53.

Many researchers have found abnormal levels of p53 protein in the epithelium in both primary and recurrent pterygia [43]. The reported prevalence of p53 positive staining by immunohistochemistry ranges from 7,9% to 100% [35]. Interestingly, p53 overexpression didn't block cell proliferation or cause apoptosis, implying that normal p53 functions were inactivated. UV radiation can cause mutations in the p53 gene, with loss of heterozygosity, resulting in cell proliferation and genomic instability, instead [29].

In our experimental study, as part of this doctoral dissertation, we analyzed the expression of the three aforementioned molecules p53, survivin and PTEN in pterygium epithelium. Our aim is to explore the possible role, they play in the evolution and transformation of the normal conjunctival epithelium to pathological pterygium tissue. In the study of this doctoral thesis, a combination of exfoliating cytology was applied and the analysis of proteins was performed on a liquid phase

substrate with an objective assessment of the expression of the molecules through digital image analysis. A similar application of immunocytochemistry has been used in another study at the cytological level for the analysis of squamous cell carcinomas of the eye [597].

Extensive molecular studies have shown that tissue structural stability and functional corresponding homeostasis are achieved through a balance between cell proliferation and apoptotic death [598]. Decoding the transition and transformation of the normal conjunctival epithelium into a pterygium, as a clinically specific entity, it reinforces the notion, that this balance is deregulated.

Some studies have suggested based on their experimental data that the progressive development of the lesion is probably due to the synchronization of the molecules that promote and respectively inhibit this planned process. In particular, a combined overexpression of the p53 protein and the corresponding mitochondrial bcl2 and bax, an inhibitor and an apoptosis promoter, respectively, has been observed [599]. This mechanism of combined protein expression is not observed in normal epithelium [512]. In fact, the purpose of p53 is to prevent the abnormal expression of these genes. In combination with this mechanism, survivin promotes the suppression of apoptosis. It has been observed that in the pterygium epithelium, the increased transcription of these genes is consistent with their protein overexpression [55].

A study, analyzing the survivin molecule at the m-RNA and protein levels combined concluded that elevated levels of the phosphorylated molecule were found mainly in the cytoplasm relative to the nuclear space [600]. However, it was only weakly expressed in the cytosol of normal conjunctival epithelium. Furthermore, knockdown of survivin by siRNA, suppressed propagation of pterygium epithelial cells, along with downregulation of p63 and upregulation of p57 and p21 expressions [600]. It was demonstrated that oxidative stress could cause activation of survivin expression, inducing a hyperproliferative condition, which might be a crucial event in the growth of pterygium [42]. Survivin had an essential connection with COX-2 in primary pterygium, suggesting that pterygium might originate via an anti-apoptotic mechanism [44].

An interesting observation was that the protein produced, could be the target for targeted treatment strategies through the development and application of specialized siRNAs such as in vitro tested in mixed cultures of normal keratinocytes and pterygium epithelial cells [600]. At the same time, another similarly important study reported as a result that the combined overexpression of the proteins p53 and survivin is associated not only with the initial development of pterygium but also with its regeneration and recurrence after surgical excision [43].

According to the results of the protein analysis of our study, the increased expression of surviving, PTEN, mainly and of p53 to a lesser extent, were associated with the pathological epithelium of the conjunctiva, on pterygium substrate. This co-expression strongly supports, the role of these molecules in the deregulation of the apoptotic mechanism in pathological epithelia. In addition, these proteins appear to interact with others including cyclooxygenase-2 (COX-2) and cyclic adeno-monophosphatase binding protein (AMP-response CREB) which also contribute to pterygium formation [44, 601].

Survivin also, shows an indirect synergy in oxidative stress which leads to DNA pathology, forming an activation circuit of the molecule which facilitates the formation of the pterygium [42].

At the same time, the exact role of the p53 protein in the process of ''neoplastic'' adaptation and change of the normal epithelium to the pterygium type is under investigation. There are various published, controversial experimental data, on this view. Some of these studies support the idea that despite the fact of its overexpression in the primary pterygia, the same does not happen with their recurrence after surgical treatment [36, 528]. Based on the results of our experimental study, the total co-expression (overexpression) of p53 / PTEN molecules is correlated with the pterygium phenotype. Especially for the PTEN protein, for which there is very limited data regarding its role in the formation and growth of the pterygium, this correlation is very important.

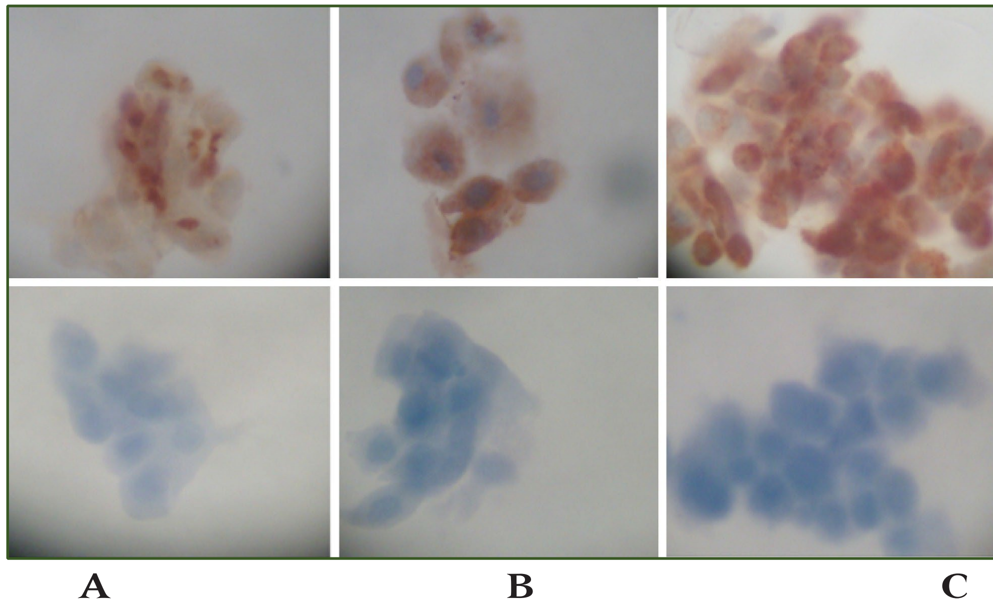


Figure 27 Immunocytochemistry analysis for the markers **A:** p53 expression (nuclear staining pattern). **B:** PTEN expression (cytoplasmic mainly and nuclear staining pattern). **C:** survivin (nuclear predominantly and cytoplasmic staining pattern). Second row: negative expression, original magnification 400×.

Embryo-histogenetic studies have shown that the protein critically regulates the architecture and homeostasis of ocular tissues and also in cases of traumatic corneal damage and conjunctivitis increases the migration of new cells and tissue healing through the promotion of PI3K/AKT signal [602, 603]. In addition, experimental analyzes in early organisms such as Drosophila and mice have shown that the effect of the molecule is catalytic during growth by regulating the balance between proliferation and apoptosis [604]. Regarding pterygium, PTEN overexpression leads to suppression of signaling, i.e., the signal transduction from the cell membrane to the nucleus and thus acts as an adjunct to epithelial apoptosis. In fact, other similar protein analysis studies of the molecules tested, have shown that PTEN indirectly suppresses survivin [23, 605, 606].

13. Conclusions

Survivin, PTEN and p53 proteins are important biomarker molecules, involved in the emergence and propagation of cancer cells. The specific analysis of their co-expression in our study, supports the growing belief, that pterygium should no longer be considered as a conjunctival inflammatory degenerative disease. Instead, pterygium should warrant the attention of premalignant invasive pathologies with the potential to rarely transform in a true cancerous lesion [596].

Our study demonstrated, in keeping with other publications, that survivin, through its overexpression leads to the inhibition of the apoptotic potential of the epithelium in the area of pterygium [37]. In addition, overexpression of p53 is observed in these epithelia but the impact of the molecule on the formation and evolution of tissue conversion from normal to their pathological phenotype seems to be through its mutated self, leading to increased stability and inactivity. Mutant p53 appears to shed its protective role as a genome gate keeper and it is very strongly suggested that it may even possess tumorigenic properties, in its mutated form [607]. Additionally, the role of PTEN as a key nodal point is confirmed, based on the findings of protein analysis. Although not explicitly clarified, it seems to indirectly regulate the balance between the other two molecules examined, by favoring apoptosis. So far, no studies exist to investigate the importance of PTEN expression in pterygium and our study, is the first to our knowledge, to demonstrate the significant correlation of p53 and PTEN expression, to the pathological tissue of pterygia.

Additional molecular analysis relating to apoptotic death pathways, signal transduction to the nucleus, and cell cycle regulation will demonstrate the true molecular basis of pterygium.

Regarding the treatment of pterygium, new lines of investigation have already been undertaken, in an effort to identify the therapeutic agent or agents that would arrest growth and/or prevent recurrences, thereby relieving both patients and national health systems, of a significant burden. To this end, immunosuppressing agents, like mycophenolate mofetil have been employed, showing promising results with suppression of pterygium fibroblast proliferation

after short exposure to the acid, in vitro [608]. More published data, support the use of antioxidants, like curcumin, a yellow-colored polyphenol, also known as diferuloylmethane, from the Curcuma Longa plant. Curcumin is contemplated as a potent anticancer drug. In a study conducted, exposing cultured pterygium fibroblasts to curcumin for 48h, a remarkable inhibitory effect on fibroblasts and their VEGF expression, was noted [609].

p53, survivin, and PTEN have already been targeted for cancer treatment, either separately or in conjunction with other forms of therapy, i.e. chemotherapy or irradiation and they show promising results, with studies entering phase I and II [115, 610, 611]. Still, given the diversity of their context-specific actions and the tremendous heterogeneity of human cancer, a lot of research remains to be undertaken, in order to achieve the necessary level of efficacy and productiveness, to fend off cancer.

We propose that these molecules may be of considerable value, in discovering new effective therapeutic modalities, for pterygium. Since conventional treatment strategies have failed, so far, to prevent recurrences [612], it becomes self-evident that future investigations should be undertaken to identify the usefulness and practicality of survivin, PTEN, and p53, either as prognostic biomarkers or therapeutic targets, in future pterygium treatment options [613].

14. Abstract

Purpose: Concerning pterygium pathogenesis, high levels of ultraviolet (UV) exposure are considered as the major risk factor. However, the role of specific gene deregulations, including apoptotic/anti-apoptotic factors and significant suppressor genes, in signaling transduction pathways remains unclear. In the current study, we co-analyzed p53, Survivin, and PTEN proteins in pterygia and normal conjunctiva.

Methods: Using a liquid-based cytology assay, fifty (n=50) cell specimens were obtained by a smooth scraping on conjunctiva epithelia and fixed accordingly. Among them, thirty-eight (n=38) were referred to pterygia and the others (n=12) to normal epithelia (control group). Immunocytochemistry assays were implemented on the corresponding slides by applying anti-p53, -Survivin, and -PTEN antibodies. Digital image analysis was performed for objectively evaluating the corresponding immunostaining intensity levels.

Results: The majority of the examined pterygia cases overexpressed the markers (p53: 22/38-57.9%. Survivin: 30/38-78.9%. and PTEN: 25/38-65.7%. respectively). Interestingly, overall p53/PTEN co-expression was found to be statistically significant (p=0.022). Overexpression of Survivin, PTEN mainly and of p53 to a lesser extent, was associated to abnormal conjunctiva epithelia (p=0.019, p=0.03, respectively). No statistical significance was observed regarding the anatomical localization of the pterygia (central/peripheral) or their type (fleshy/flat).

Conclusions: Survivin overexpression leads to an increased anti-apoptotic activity playing a central molecular role in the pathogenesis and progression of pterygia. Furthermore, p53 expression is also observed in these lesions, but its impact seems to be low compared to Survivin's influence on them. Although unexplored, the role of PTEN in the current process is potentially significant, providing a suppressor balance to the p53/survivin complex.

Keywords: pterygia, genes, cytology, apoptosis, image analysis

15. Περίληψη

Σκοπός: Στη παθογένεια του πτερυγίου, τα υψηλά επίπεδα έκθεσης στην υπεριώδη ακτινοβολία, φαίνεται να έχουν τον πρωτεύοντα ρόλο, για την εμφάνιση του. Η σημασία όμως, της απορρύθμισης συγκεκριμένων γονίδιων, όπως είναι οι αποπτωτικοί και αντιαποπτωτικοί παράγοντες και κατασταλτικά της ογκογένεσης γονίδια, στη διαταραχή των μεταγωγικών σημάτων στο εσωτερικό του κυττάρου, δεν έχει διασαφηνιστεί.

Στη παρούσα μελέτη, συν αναλύουμε τα πρωτεϊνικά προϊόντα των γονίδιων Survivin, p53 και PTEN, σε οφθαλμικά πτερύγια συγκριτικά με το επιθήλιο του υγιή επιπεφυκότα.

Μέθοδος: Με κυτταρολογία υγρής φάσης, αναλυθήκαν πενήντα (50) κυτταρικά δείγματα, που λήφθηκαν με ήπια απόξεση του επιθήλιου του επιπεφυκότα. Από αυτά τα δείγματα, τριάντα οκτώ (38) προέρχονταν από παθολογικό ιστό, από την περιοχή του πτερυγίου και τα υπόλοιπα δώδεκα (12) δείγματα, ήταν από φυσιολογικό επιπεφυκότα.

Έγινε ανοσοκυτταροχημική μελέτη των αντίστοιχων πλακιδίων με εφαρμογή αντι- p53, -Survivin και -PTEN αντισωμάτων και ψηφιακή ανάλυση της εικόνας για την ακριβή εκτίμηση της έντασης της ανοσοχημικής χρώσης.

Αποτελέσματα: Η πλειοψηφία των εξετασθέντων ιστών, από πτερύγια, εμφάνισαν υπερέκφραση των πρωτεϊνών (p53: 22/38 – 57.9%, survivin: 30/38 – 78.9%, PTEN: 25/38 – 65.7%, αντίστοιχα). Ενδιαφέρον έχει, η στατιστική σημασία που εντοπίσαμε στη συνδυασμένη υπερέκφραση των p53 και PTEN, με τιμή σημαντικότητας (p = 0.022). Η υπερέκφραση της Survivin και η υπερέκφραση της p53, σχετιζόταν με τα παθολογικά επιθήλια, με τιμή σημαντικότητας (p = 0.019 και p = 0.03, αντίστοιχα). Δεν διαπιστώθηκε, στατιστικά σημαντική σχέση ανάμεσα στην έκφραση των συγκεκριμένων πρωτεϊνών και την ανατομική θέση (επεκτεινόμενο κεντρικά/περιοριζόμενο στο ΣΚΟ), η μορφολογία (συμπαγές/ατροφικό) των πτερυγίων.

Συμπεράσματα: Η υπερεκφραση της survivin, οδηγεί το κύτταρο σε αυξημένη αντιαποπτωτική δραστηριότητα και αποδεικνύει το σημαντικό ρόλο της στην εμφάνιση και εξέλιξη του πτερυγίου. Επιπρόσθετα, η σε μικρότερο βαθμό έκφραση του

p53 , ενισχύει την άποψη ότι η επίδραση του είναι αμφιλεγόμενη, σε σύγκριση με την survivin. Η σημασία του PTEN, από τη μελέτη μας, αναδεικνύεται σημαντική, καθώς φαίνεται να επιτελεί ένα κατασταλτικό - ρυθμιστικό ρόλο στην ισορροπία του συμπλέγματος survivin / p53, πάρα την έλλειψη άλλων μελετών.

Λέξεις- Κλειδιά: πτερύγιο, γονίδια, απόπτωση, ανάλυση εικόνας.

16. References - Bibliography

1. John V. Forrester, A.D.D., Paul G. McMenamin, William R. Lee, *The Eye* Second ed. Vol. 1. 2002, London, UK: Harcourt Publishers Limited. 447.
2. Richard S. Snell, M.A.L., *Clinical Anatomy of the Eye*. Second ed. Vol. 1. 1998, U.S.A.: Blackwell Science. 423.
3. Caleb L. Shumway, M.M., Matthew Wade, *Anatomy, Head and Neck, Eye Conjunctiva*. 2020: StatPearls Publishing. 5.
4. Michael A. Lemp, J.S.P., *Adler's Physiology of the Eye*. Ninth ed, ed. L. Craven. Vol. 1. 1992, U.S.A.: George S. Stamathis. 46.
5. Ramos, T., D. Scott, and S. Ahmad, *An Update on Ocular Surface Epithelial Stem Cells: Cornea and Conjunctiva.* Stem Cells Int, 2015. **2015**: p. 601731.
6. Herbert E. Kaufman, B.A.B., Marguerite B. McDonald, Stephen C. Kaufman, *Companion Handbook to the Cornea*, ed. H.E. Kaufman. Vol. 1. 2000, U.S.A.: Butterworth-Heinemann. 14.
7. Myron Yanoff, J.S.D., *Ophthalmology*. Second ed. Vol. 1. 2004, U.S.A.: Mosby. 1651.
8. Yam, J.C. and A.K. Kwok, *Ultraviolet light and ocular diseases.* Int Ophthalmol, 2014. **34**(2): p. 383-400.
9. Bikbov, M.M., R.M. Zainullin, G.M. Kazakbaeva, T.R. Gilmanshin, V.F. Salavatova, Arslangareeva, II, et al., *Pterygium Prevalence and Its Associations in a Russian Population: The Ural Eye and Medical Study.* Am J Ophthalmol, 2019. **205**: p. 27-34.
10. Todani, A. and S.A. Melki, *Pterygium: current concepts in pathogenesis and treatment.* Int Ophthalmol Clin, 2009. **49**(1): p. 21-30.
11. Ha, J., S.L. Cremers, M. Korchak, J. Koppinger, and J.A. Martinez, *A New Automated Method to Grade Pterygium Severity Using Scheimpflug Imaging.* Ophthalmology, 2016. **123**(11): p. 2435-2436.
12. Wanzeler, A.C.V., I.A.F. Barbosa, B. Duarte, D. Borges, E.B. Barbosa, D. Kamiji, et al., *Mechanisms and biomarker candidates in pterygium development.* Arq Bras Oftalmol, 2019. **82**(6): p. 528-536.
13. Julio, G., S. Lluch, P. Pujol, and D. Merindano, *Ocular discomfort in pterygium patients.* Optom Vis Sci, 2013. **90**(3): p. 269-74.
14. Vanathi, M., S. Goel, A. Ganger, T. Agarwal, T. Dada, and S. Khokhar, *Corneal tomography and biomechanics in primary pterygium.* Int Ophthalmol, 2018. **38**(2): p. 663-671.
15. Gumus, K., A. Guven, M. Altinkaynak, and M. Keskin, *Comparison of Different Measurement Tools and Dimensional Parameters of Pterygium to Investigate its Impact on Refractive Indices and Ocular Aberrations.* Eye Contact Lens, 2018. **44**(2): p. 118-124.
16. Chan, C.M., Y.P. Liu, and D.T. Tan, *Ocular surface changes in pterygium.* Cornea, 2002. **21**(1): p. 38-42.
17. Wu, C.W., Y.W. Cheng, N.Y. Hsu, K.T. Yeh, Y.Y. Tsai, C.C. Chiang, et al., *MiRNA-221 negatively regulated downstream p27Kip1 gene expression involvement in pterygium pathogenesis.* Mol Vis, 2014. **20**: p. 1048-56.

18. Dzunic, B., P. Jovanovic, G. Zlatanovic, D. Veselinovic, A. Petrovic, and I. Stefanovic, *[Comparative analysis of histopathological and clinical characteristics of pterygium].* Vojnosanit Pregl, 2010. **67**(2): p. 159-65.
19. Boban Džunić, P.J., Dragan Veselinović, and I.S. Aleksandar Petrović, Igor Kovačević, *Analysis of pathohistological characteristics of pterygium.* BOSNIAN JOURNAL OF BASIC MEDICAL SCIENCES 2010. **10**(4): p. 307-313.
20. Lu, C.W., J.L. Hao, L. Yao, H.J. Li, and D.D. Zhou, *Efficacy of curcumin in inducing apoptosis and inhibiting the expression of VEGF in human pterygium fibroblasts.* Int J Mol Med, 2017. **39**(5): p. 1149-1154.
21. Marcovich, A.L., Y. Morad, J. Sandbank, M. Huszar, M. Rosner, A. Pollack, et al., *Angiogenesis in pterygium: morphometric and immunohistochemical study.* Curr Eye Res, 2002. **25**(1): p. 17-22.
22. Ip, M.H., J.J. Chui, L. Tat, and M.T. Coroneo, *Significance of Fuchs Flecks in Patients With Pterygium/Pinguecula: Earliest Indicator of Ultraviolet Light Damage.* Cornea, 2015. **34**(12): p. 1560-3.
23. Cardenas-Cantu, E., J. Zavala, J. Valenzuela, and J.E. Valdez-Garcia, *Molecular Basis of Pterygium Development.* Semin Ophthalmol, 2016. **31**(6): p. 567-83.
24. Chui, J., N. Di Girolamo, D. Wakefield, and M.T. Coroneo, *The pathogenesis of pterygium: current concepts and their therapeutic implications.* Ocul Surf, 2008. **6**(1): p. 24-43.
25. Dupont, E., J. Gomez, and D. Bilodeau, *Beyond UV radiation: a skin under challenge.* Int J Cosmet Sci, 2013. **35**(3): p. 224-32.
26. Bernerd, F., C. Marionnet, and C. Duval, *Solar ultraviolet radiation induces biological alterations in human skin in vitro: relevance of a well-balanced UVA/UVB protection.* Indian J Dermatol Venereol Leprol, 2012. **78 Suppl 1**: p. S15-23.
27. Chui, J., M.T. Coroneo, L.T. Tat, R. Crouch, D. Wakefield, and N. Di Girolamo, *Ophthalmic pterygium: a stem cell disorder with premalignant features.* Am J Pathol, 2011. **178**(2): p. 817-27.
28. Nolan, T.M., N. DiGirolamo, N.H. Sachdev, T. Hampartzoumian, M.T. Coroneo, and D. Wakefield, *The role of ultraviolet irradiation and heparin-binding epidermal growth factor-like growth factor in the pathogenesis of pterygium.* Am J Pathol, 2003. **162**(2): p. 567-74.
29. Cimpean, A.M., M.P. Sava, and M. Raica, *DNA damage in human pterygium: one-shot multiple targets.* Mol Vis, 2013. **19**: p. 348-56.
30. Tsai, Y.Y., C.C. Chang, C.C. Chiang, K.T. Yeh, P.L. Chen, C.H. Chang, et al., *HPV infection and p53 inactivation in pterygium.* Mol Vis, 2009. **15**: p. 1092-7.
31. Rodrigues, F.W., J.T. Arruda, R.E. Silva, and K.K. Moura, *TP53 gene expression, codon 72 polymorphism and human papillomavirus DNA associated with pterygium.* Genet Mol Res, 2008. **7**(4): p. 1251-8.
32. Chalkia, A.K., D.A. Spandidos, and E.T. Detorakis, *Viral involvement in the pathogenesis and clinical features of ophthalmic pterygium (Review).* Int J Mol Med, 2013. **32**(3): p. 539-43.

33. Kilic-Toprak, E., I. Toprak, S. Caliskan, Y. Ozdemir, O. Demirtas, F. Altintas, et al., *Oxidative Stress and Genotoxicity in Pterygium: A Systemic Investigation.* Eye Contact Lens, 2019. **45**(6): p. 399-404.
34. Kim, K.W., S.H. Park, and J.C. Kim, *Fibroblast biology in pterygia.* Exp Eye Res, 2016. **142**: p. 32-9.
35. Tsai, Y.Y., K.C. Chang, C.L. Lin, H. Lee, F.J. Tsai, Y.W. Cheng, et al., *p53 Expression in pterygium by immunohistochemical analysis: a series report of 127 cases and review of the literature.* Cornea, 2005. **24**(5): p. 583-6.
36. Weinstein, O., G. Rosenthal, H. Zirkin, T. Monos, T. Lifshitz, and S. Argov, *Overexpression of p53 tumor suppressor gene in pterygia.* Eye (Lond), 2002. **16**(5): p. 619-21.
37. Konstantopoulou, K., E. Tsiambas, E. Baliou, A.C. Lazaris, N. Kavantzas, A. Karameris, et al., *Deregulation of p53/survivin apoptotic markers correlated to PTEN expression in pterygium neoplastic cells.* J BUON, 2018. **23**(3): p. 826-831.
38. Arora, R., *Commentary: Expression of p53 and Ki-67 proteins in patients with increasing severity and duration of pterygium.* Indian J Ophthalmol, 2021. **69**(4): p. 851.
39. Detorakis, E.T. and D.A. Spandidos, *Pathogenetic mechanisms and treatment options for ophthalmic pterygium: trends and perspectives (Review).* Int J Mol Med, 2009. **23**(4): p. 439-47.
40. Kase, S., S. Takahashi, I. Sato, K. Nakanishi, K. Yoshida, and S. Ohno, *Expression of p27(KIP1) and cyclin D1, and cell proliferation in human pterygium.* Br J Ophthalmol, 2007. **91**(7): p. 958-61.
41. Ramalho, F.S., C. Maestri, L.N. Ramalho, A. Ribeiro-Silva, and E. Romao, *Expression of p63 and p16 in primary and recurrent pterygia.* Graefes Arch Clin Exp Ophthalmol, 2006. **244**(10): p. 1310-4.
42. Maxia, C., M.T. Perra, P. Demurtas, L. Minerba, D. Murtas, F. Piras, et al., *Expression of survivin protein in pterygium and relationship with oxidative DNA damage.* J Cell Mol Med, 2008. **12**(6A): p. 2372-80.
43. Zhang, L.W., B.H. Chen, X.H. Xi, Q.Q. Han, and L.S. Tang, *Survivin and p53 expression in primary and recurrent pterygium in Chinese patients.* Int J Ophthalmol, 2011. **4**(4): p. 388-92.
44. Maxia, C., M.T. Perra, P. Demurtas, L. Minerba, D. Murtas, F. Piras, et al., *Relationship between the expression of cyclooxygenase-2 and survivin in primary pterygium.* Mol Vis, 2009. **15**: p. 458-63.
45. Kun Liang, Z.J., Bi-qing Ding, Ping Cheng, Da-ke Huang, Li-ming Tao, *Expression of cell proliferation and apoptosis biomarkers in pterygia and normal conjunctiva.* Molecular Vision 2011. **17**: p. 1687-1693.
46. Cui, Y.H., H.Y. Li, Z.X. Gao, N. Liang, S.S. Ma, F.J. Meng, et al., *Regulation of Apoptosis by miR-122 in Pterygium via Targeting Bcl-w.* Invest Ophthalmol Vis Sci, 2016. **57**(8): p. 3723-30.
47. Liu, Y., H. Xu, and M. An, *mTORC1 regulates apoptosis and cell proliferation in pterygium via targeting autophagy and FGFR3.* Sci Rep, 2017. **7**(1): p. 7339.

48. Zhao, X.R., M.C. Zhang, H.T. Xie, N. Ji, and X.Q. Ma, *Expression of mTOR in Primary Pterygium and its Correlation with alpha-Smooth Muscle Actin.* Eur J Ophthalmol, 2017. **27**(6): p. 664-669.
49. Kase, S., M. Osaki, I. Sato, S. Takahashi, K. Nakanishi, K. Yoshida, et al., *Immunolocalisation of E-cadherin and beta-catenin in human pterygium.* Br J Ophthalmol, 2007. **91**(9): p. 1209-12.
50. Beden, U., M. Irkec, D. Orhan, and M. Orhan, *The roles of T-lymphocyte subpopulations (CD4 and CD8), intercellular adhesion molecule-1 (ICAM-1), HLA-DR receptor, and mast cells in etiopathogenesis of pterygium.* Ocul Immunol Inflamm, 2003. **11**(2): p. 115-22.
51. Mahesh, M., S.K. Mittal, S. Kishore, A. Singh, N. Gupta, and R. Rana, *Expression of p53 and Ki-67 proteins in patients with increasing severity and duration of pterygium.* Indian J Ophthalmol, 2021. **69**(4): p. 847-850.
52. Das, P., A. Gokani, K. Bagchi, G. Bhaduri, S. Chaudhuri, and S. Law, *Limbal epithelial stem-microenvironmental alteration leads to pterygium development.* Mol Cell Biochem, 2015. **402**(1-2): p. 123-39.
53. Tung, J.N., C.C. Chiang, Y.Y. Tsai, Y.Y. Chou, K.T. Yeh, H. Lee, et al., *CyclinD1 protein expressed in pterygia is associated with beta-catenin protein localization.* Mol Vis, 2010. **16**: p. 2733-8.
54. Mei-Ling Peng, Y.-Y.T., Chun-Chi Chiang, Ying-Che Huang, Ming-Chih Chou, Kun-Tu Yeh, Huei Lee, Ya-Wen Cheng, *CYP1A1 protein activity is associated with allelic variation in pterygium tissues and cells.* Molecular Vision 2012, 2012. **18**: p. 1937-1943.
55. Feng, Q.Y., Z.X. Hu, X.L. Song, and H.W. Pan, *Aberrant expression of genes and proteins in pterygium and their implications in the pathogenesis.* Int J Ophthalmol, 2017. **10**(6): p. 973-981.
56. Lanneau, D., A. de Thonel, S. Maurel, C. Didelot, and C. Garrido, *Apoptosis versus cell differentiation: role of heat shock proteins HSP90, HSP70 and HSP27.* Prion, 2007. **1**(1): p. 53-60.
57. Yoshida, Y., Y. Ban, and S. Kinoshita, *Tight junction transmembrane protein claudin subtype expression and distribution in human corneal and conjunctival epithelium.* Invest Ophthalmol Vis Sci, 2009. **50**(5): p. 2103-8.
58. Dogan, A.S., E. Onder, A.T. Arikok, T. Bicer, and C. Gurdal, *Claudin-1 expressions decrease in pterygium with respect to normal conjunctiva.* Cutan Ocul Toxicol, 2016. **35**(4): p. 315-8.
59. Perez-Rico, C., G. Pascual, S. Sotomayor, M.A. Montes-Mollon, C. Trejo, T. Sasaki, et al., *Tropoelastin and fibulin overexpression in the subepithelial connective tissue of human pterygium.* Am J Ophthalmol, 2011. **151**(1): p. 44-52.
60. Xu, N., Y. Cui, J. Dong, and L. Huang, *Exploring the Molecular Mechanisms of Pterygium by Constructing lncRNA-miRNA-mRNA Regulatory Network.* Invest Ophthalmol Vis Sci, 2020. **61**(8): p. 12.
61. Xu, K., T. Tao, J. Jie, X. Lu, X. Li, M.A. Mehmood, et al., *Increased importin 13 activity is associated with the pathogenesis of pterygium.* Mol Vis, 2013. **19**: p. 604-13.

62. Seet, L.F., L. Tong, R. Su, and T.T. Wong, *Involvement of SPARC and MMP-3 in the pathogenesis of human pterygium.* Invest Ophthalmol Vis Sci, 2012. **53**(2): p. 587-95.
63. Schellini, S.A., E. Hoyama, D.E. Oliveira, C.E. Bacchi, and C.R. Padovani, *Matrix metalloproteinase-9 expression in pterygium.* Arq Bras Oftalmol, 2006. **69**(2): p. 161-4.
64. Hoyama, S.A.S.E. and D.E.O.C.E.B.C.R. Padovani, *Matrix metalloproteinase-9 expression in pterygium.* Arq Bras Oftalmol., 2006. **69**: p. 161-164.
65. Sarkar, P. and K. Tripathy, *Pterygium*, in *StatPearls*. 2021: Treasure Island (FL).
66. Di Girolamo, N., R.K. Kumar, M.T. Coroneo, and D. Wakefield, *UVB-mediated induction of interleukin-6 and -8 in pterygia and cultured human pterygium epithelial cells.* Invest Ophthalmol Vis Sci, 2002. **43**(11): p. 3430-7.
67. Baser, G., O.N. Sivrikoz, E. Karahan, E.S. Un, and H. Yildirim, *The Influence of Chemokine CXCR4 and Cyclooxygenase-2 in the Recurrence of Pterygium.* Ocul Immunol Inflamm, 2017. **25**(3): p. 328-332.
68. Kim, K.W., H.S. Ha, and J.C. Kim, *Ischemic tissue injury and progenitor cell tropism: significant contributors to the pathogenesis of pterygium.* Histol Histopathol, 2015. **30**(3): p. 311-20.
69. Ribatti, D., B. Nico, M.T. Perra, C. Maxia, F. Piras, D. Murtas, et al., *Correlation between NGF/TrkA and microvascular density in human pterygium.* Int J Exp Pathol, 2009. **90**(6): p. 615-20.
70. Timothy M. Nolan, N.D. and T.H. Nitin H. Sachdev, Minas T. Coroneo, and Denis Wakefield, *The Role of Ultraviolet Irradiation and Heparin-Binding Epidermal Growth Factor-Like Growth Factor in the Pathogenesis of Pterygium.* American Journal of Pathology 2003. **162**(2): p. 567-574.
71. S., S.A., *Pterygium.* Br J Ophthalmol, 2006. **90**(6): p. 664-5.
72. Gum, S.I., Y.H. Kim, J.C. Jung, I.G. Kim, J.S. Lee, K.W. Lee, et al., *Cyclosporine A inhibits TGF-beta2-induced myofibroblasts of primary cultured human pterygium fibroblasts.* Biochem Biophys Res Commun, 2017. **482**(4): p. 1148-1153.
73. Kilic, R., A. Kurt, M. Tad, and S. Tasdemir, *Endocan Overexpression in Pterygium.* Cornea, 2017. **36**(6): p. 696-699.
74. Peng, M.L., Y.Y. Tsai, J.N. Tung, C.C. Chiang, Y.C. Huang, H. Lee, et al., *Vascular endothelial growth factor gene polymorphism and protein expression in the pathogenesis of pterygium.* Br J Ophthalmol, 2014. **98**(4): p. 556-61.
75. Bianchi, E., F. Scarinci, C. Grande, R. Plateroti, P. Plateroti, A.M. Plateroti, et al., *Immunohistochemical profile of VEGF, TGF-beta and PGE(2) in human pterygium and normal conjunctiva: experimental study and review of the literature.* Int J Immunopathol Pharmacol, 2012. **25**(3): p. 607-15.
76. Stainer, G.A., F.S. Brightbill, P. Holm, and D. Laux, *The development of pseudopterygia in hard contact lens wearers.* Contact Intraocul Lens Med J, 1981. **7**(1): p. 1-4.

77. Hirst, L.W., R.A. Axelsen, and I. Schwab, *Pterygium and associated ocular surface squamous neoplasia.* Arch Ophthalmol, 2009. **127**(1): p. 31-2.
78. Graue-Hernandez, E.O., A. Cordoba, A. Jimenez-Corona, A. Ramirez-Miranda, A. Navas, J.C. Serna-Ojeda, et al., *Practice Patterns in the Management of Primary Pterygium: A Survey Study.* Cornea, 2019. **38**(11): p. 1339-1344.
79. Wanzeler, A.C.V., I.A.F. Barbosa, B. Duarte, E.B. Barbosa, D.A. Borges, and M. Alves, *Impact of pterygium on the ocular surface and meibomian glands.* PLoS One, 2019. **14**(9): p. e0213956.
80. Singh, S.K., *Pterygium: epidemiology prevention and treatment.* Community Eye Health, 2017. **30**(99): p. S5-S6.
81. Maheshwari, S., *Pterygium-induced corneal refractive changes.* Indian J Ophthalmol, 2007. **55**(5): p. 383-6.
82. Nuzzi, R. and F. Tridico, *How to minimize pterygium recurrence rates: clinical perspectives.* Clin Ophthalmol, 2018. **12**: p. 2347-2362.
83. Hirst, L.W. and D. Battistuta, *Eight-year trends in the Australian surgical approach to pterygium removal.* Clin Exp Ophthalmol, 2019. **47**(1): p. 15-19.
84. Ghoz, N., M. Elalfy, D. Said, and H. Dua, *Healing of autologous conjunctival grafts in pterygium surgery.* Acta Ophthalmol, 2018. **96**(8): p. e979-e988.
85. Rock, T., M. Bramkamp, K.U. Bartz-Schmidt, and D. Rock, *A Retrospective Study to Compare the Recurrence Rate After Treatment of Pterygium by Conjunctival Autograft, Primary Closure, and Amniotic Membrane Transplantation.* Med Sci Monit, 2019. **25**: p. 7976-7981.
86. Forbes, J., R. Collin, and J. Dart, *Split thickness buccal mucous membrane grafts and beta irradiation in the treatment of recurrent pterygium.* Br J Ophthalmol, 1998. **82**(12): p. 1420-3.
87. Rosen, R., *Amniotic Membrane Grafts to Reduce Pterygium Recurrence.* Cornea, 2018. **37**(2): p. 189-193.
88. Clearfield, E., B.S. Hawkins, and I.C. Kuo, *Conjunctival Autograft Versus Amniotic Membrane Transplantation for Treatment of Pterygium: Findings From a Cochrane Systematic Review.* Am J Ophthalmol, 2017. **182**: p. 8-17.
89. Mahar, P.S. and N. Manzar, *Pterygium recurrence related to its size and corneal involvement.* J Coll Physicians Surg Pak, 2013. **23**(2): p. 120-3.
90. Luanratanakorn, P., T. Ratanapakorn, O. Suwan-Apichon, and R.S. Chuck, *Randomised controlled study of conjunctival autograft versus amniotic membrane graft in pterygium excision.* Br J Ophthalmol, 2006. **90**(12): p. 1476-80.
91. Tan, J., U. Vollmer-Conna, L. Tat, and M. Coroneo, *Dry-Eye Disease in Recurrent Pterygium.* Ophthalmic Res, 2019. **61**(4): p. 199-203.
92. Hirst, L.W., *Recurrent pterygium surgery using pterygium extended removal followed by extended conjunctival transplant: recurrence rate and cosmesis.* Ophthalmology, 2009. **116**(7): p. 1278-86.
93. Raiskup, F., A. Solomon, D. Landau, M. Ilsar, and J. Frucht-Pery, *Mitomycin C for pterygium: long term evaluation.* Br J Ophthalmol, 2004. **88**(11): p. 1425-8.

94. Said, D.G., L.A. Faraj, M.S. Elalfy, A. Yeung, A. Miri, U. Fares, et al., *Intra-lesional 5 fluorouracil for the management of recurrent pterygium.* Eye (Lond), 2013. **27**(10): p. 1123-9.
95. Bekibele, C.O., A.M. Baiyeroju, B.A. Olusanya, A.O. Ashaye, and T.S. Oluleye, *Pterygium treatment using 5-FU as adjuvant treatment compared to conjunctiva autograft.* Eye (Lond), 2008. **22**(1): p. 31-4.
96. Pikkel, J., Y. Porges, and A. Ophir, *Halting pterygium recurrence by postoperative 5-fluorouracil.* Cornea, 2001. **20**(2): p. 168-71.
97. Ozulken, K., M. Koc, O. Ayar, and H. Hasiripi, *Topical cyclosporine A administration after pterygium surgery.* Eur J Ophthalmol, 2012. **22 Suppl 7**: p. S5-10.
98. Meneghim, R., L.H. Satto, K.L. Natsuaki, A.C. Oliveira, C.R. Padovani, M.M.H. Viveiros, et al., *Topical cyclosporine A 0.05% before and after surgery to prevent pterygium recurrence.* Arq Bras Oftalmol, 2019. **82**(5): p. 372-376.
99. Rose, L., J.M. Byrd, and Y. Qaseem, *Subtenon Injections of Ranibizumab Arrest Growth in Early Recurrent Pterygium.* Eye Contact Lens, 2017. **43**(6): p. 399-405.
100. Sarac, O., S. Demirel, and R. Oltulu, *Efficacy of intralesional bevacizumab administration in primary pterygium: a quantitative analysis.* Eye Contact Lens, 2014. **40**(1): p. 46-50.
101. Stival, L.R., A.M. Lago, M.N. Figueiredo, R.H. Bittar, M.L. Machado, and J.J. Nassaralla Junior, *Efficacy and safety of subconjunctival bevacizumab for recurrent pterygium.* Arq Bras Oftalmol, 2014. **77**(1): p. 4-7.
102. Murube, J., *Pterygium: its treatment with beta therapy.* Ocul Surf, 2009. **7**(1): p. 3-9.
103. Krag, S. and N. Ehlers, *Excimer laser treatment of pterygium.* Acta Ophthalmol (Copenh), 1992. **70**(4): p. 530-3.
104. Murube, J., *Pterygium: evolution of medical and surgical treatments.* Ocul Surf, 2008. **6**(4): p. 155-61.
105. Goossens, N., S. Nakagawa, X. Sun, and Y. Hoshida, *Cancer biomarker discovery and validation.* Transl Cancer Res, 2015. **4**(3): p. 256-269.
106. Mishra, A. and M. Verma, *Cancer biomarkers: are we ready for the prime time?* Cancers (Basel), 2010. **2**(1): p. 190-208.
107. Strimbu, K. and J.A. Tavel, *What are biomarkers?* Curr Opin HIV AIDS, 2010. **5**(6): p. 463-6.
108. Henry, N.L. and D.F. Hayes, *Cancer biomarkers.* Mol Oncol, 2012. **6**(2): p. 140-6.
109. Vacante, M., A.M. Borzi, F. Basile, and A. Biondi, *Biomarkers in colorectal cancer: Current clinical utility and future perspectives.* World J Clin Cases, 2018. **6**(15): p. 869-881.
110. Anderson, D.C. and K. Kodukula, *Biomarkers in pharmacology and drug discovery.* Biochem Pharmacol, 2014. **87**(1): p. 172-88.
111. Elmore, S., *Apoptosis: a review of programmed cell death.* Toxicol Pathol, 2007. **35**(4): p. 495-516.

112. Altieri, S.P.W.a.D.C., *Survivin at a glance.* Journal of Cell Science, 2019. **132**(7): p. 1-8.
113. Lalaoui, N. and D.L. Vaux, *Recent advances in understanding inhibitor of apoptosis proteins.* F1000Res, 2018. **7**.
114. Rothe, M., M.G. Pan, W.J. Henzel, T.M. Ayres, and D.V. Goeddel, *The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins.* Cell, 1995. **83**(7): p. 1243-52.
115. Garg, H., P. Suri, J.C. Gupta, G.P. Talwar, and S. Dubey, *Survivin: a unique target for tumor therapy.* Cancer Cell Int, 2016. **16**: p. 49.
116. Fukuda, S. and L.M. Pelus, *Survivin, a cancer target with an emerging role in normal adult tissues.* Mol Cancer Ther, 2006. **5**(5): p. 1087-98.
117. Schimmer, A.D., *Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice.* Cancer Res, 2004. **64**(20): p. 7183-90.
118. Deveraux, Q.L. and J.C. Reed, *IAP family proteins--suppressors of apoptosis.* Genes Dev, 1999. **13**(3): p. 239-52.
119. Clem, R.J. and L.K. Miller, *Control of programmed cell death by the baculovirus genes p35 and iap.* Mol Cell Biol, 1994. **14**(8): p. 5212-22.
120. Gyrd-Hansen, M., M. Darding, M. Miasari, M.M. Santoro, L. Zender, W. Xue, et al., *IAPs contain an evolutionarily conserved ubiquitin-binding domain that regulates NF-kappaB as well as cell survival and oncogenesis.* Nat Cell Biol, 2008. **10**(11): p. 1309-17.
121. Song, Z., S. Liu, H. He, N. Hoti, Y. Wang, S. Feng, et al., *A single amino acid change (Asp 53 --> Ala53) converts Survivin from anti-apoptotic to pro-apoptotic.* Mol Biol Cell, 2004. **15**(3): p. 1287-96.
122. Song, J., T. So, M. Cheng, X. Tang, and M. Croft, *Sustained survivin expression from OX40 costimulatory signals drives T cell clonal expansion.* Immunity, 2005. **22**(5): p. 621-31.
123. Ambrosini, G., C. Adida, G. Sirugo, and D.C. Altieri, *Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting.* J Biol Chem, 1998. **273**(18): p. 11177-82.
124. Li, F., I. Aljahdali, and X. Ling, *Cancer therapeutics using survivin BIRC5 as a target: what can we do after over two decades of study?* J Exp Clin Cancer Res, 2019. **38**(1): p. 368.
125. Sampath, J. and L.M. Pelus, *Alternative splice variants of survivin as potential targets in cancer.* Curr Drug Discov Technol, 2007. **4**(3): p. 174-91.
126. Mahotka, C., J. Liebmann, M. Wenzel, C.V. Suschek, M. Schmitt, H.E. Gabbert, et al., *Differential subcellular localization of functionally divergent survivin splice variants.* Cell Death Differ, 2002. **9**(12): p. 1334-42.
127. Necochea-Campion, R., C.S. Chen, S. Mirshahidi, F.D. Howard, and N.R. Wall, *Clinico-pathologic relevance of Survivin splice variant expression in cancer.* Cancer Lett, 2013. **339**(2): p. 167-74.
128. Suga, K., T. Yamamoto, Y. Yamada, S. Miyatake, T. Nakagawa, and N. Tanigawa, *Correlation between transcriptional expression of survivin isoforms and clinicopathological findings in human colorectal carcinomas.* Oncol Rep, 2005. **13**(5): p. 891-7.

129. Li, F., *Role of survivin and its splice variants in tumorigenesis.* Br J Cancer, 2005. **92**(2): p. 212-6.
130. Doucette, T., K. Latha, Y. Yang, G.N. Fuller, A. Rao, and G. Rao, *Survivin transcript variant 2 drives angiogenesis and malignant progression in proneural gliomas.* Neuro Oncol, 2014. **16**(9): p. 1220-8.
131. Chiou, S.K., M.K. Jones, and A.S. Tarnawski, *Survivin - an anti-apoptosis protein: its biological roles and implications for cancer and beyond.* Med Sci Monit, 2003. **9**(4): p. PI25-9.
132. Warrier, N.M., P. Agarwal, and P. Kumar, *Emerging Importance of Survivin in Stem Cells and Cancer: the Development of New Cancer Therapeutics.* Stem Cell Rev Rep, 2020. **16**(5): p. 828-852.
133. Li, F., J. Yang, N. Ramnath, M.M. Javle, and D. Tan, *Nuclear or cytoplasmic expression of survivin: what is the significance?* Int J Cancer, 2005. **114**(4): p. 509-12.
134. Dohi, T., E. Beltrami, N.R. Wall, J. Plescia, and D.C. Altieri, *Mitochondrial survivin inhibits apoptosis and promotes tumorigenesis.* J Clin Invest, 2004. **114**(8): p. 1117-27.
135. Khan, S., J.M. Jutzy, J.R. Aspe, D.W. McGregor, J.W. Neidigh, and N.R. Wall, *Survivin is released from cancer cells via exosomes.* Apoptosis, 2011. **16**(1): p. 1-12.
136. Khan, S., J.M. Jutzy, M.M. Valenzuela, D. Turay, J.R. Aspe, A. Ashok, et al., *Plasma-derived exosomal survivin, a plausible biomarker for early detection of prostate cancer.* PLoS One, 2012. **7**(10): p. e46737.
137. Li, F., E.J. Ackermann, C.F. Bennett, A.L. Rothermel, J. Plescia, S. Tognin, et al., *Pleiotropic cell-division defects and apoptosis induced by interference with survivin function.* Nat Cell Biol, 1999. **1**(8): p. 461-6.
138. Kitagawa, M. and S.H. Lee, *The chromosomal passenger complex (CPC) as a key orchestrator of orderly mitotic exit and cytokinesis.* Front Cell Dev Biol, 2015. **3**: p. 14.
139. Stauber, R.H., W. Mann, and S.K. Knauer, *Nuclear and cytoplasmic survivin: molecular mechanism, prognostic, and therapeutic potential.* Cancer Res, 2007. **67**(13): p. 5999-6002.
140. Lens, S.M., G. Vader, and R.H. Medema, *The case for Survivin as mitotic regulator.* Curr Opin Cell Biol, 2006. **18**(6): p. 616-22.
141. Rosa, J., P. Canovas, A. Islam, D.C. Altieri, and S.J. Doxsey, *Survivin modulates microtubule dynamics and nucleation throughout the cell cycle.* Mol Biol Cell, 2006. **17**(3): p. 1483-93.
142. Fernandez, J.G., D.A. Rodriguez, M. Valenzuela, C. Calderon, U. Urzua, D. Munroe, et al., *Survivin expression promotes VEGF-induced tumor angiogenesis via PI3K/Akt enhanced beta-catenin/Tcf-Lef dependent transcription.* Mol Cancer, 2014. **13**: p. 209.
143. Li, Q.X., J. Zhao, J.Y. Liu, L.T. Jia, H.Y. Huang, Y.M. Xu, et al., *Survivin stable knockdown by siRNA inhibits tumor cell growth and angiogenesis in breast and cervical cancers.* Cancer Biol Ther, 2006. **5**(7): p. 860-6.
144. Tran, J., Z. Master, J.L. Yu, J. Rak, D.J. Dumont, and R.S. Kerbel, *A role for survivin in chemoresistance of endothelial cells mediated by VEGF.* Proc Natl Acad Sci U S A, 2002. **99**(7): p. 4349-54.

145. Virrey, J.J., S. Guan, W. Li, A.H. Schonthal, T.C. Chen, and F.M. Hofman, *Increased survivin expression confers chemoresistance to tumor-associated endothelial cells.* Am J Pathol, 2008. **173**(2): p. 575-85.
146. McKenzie, J.A. and D. Grossman, *Role of the apoptotic and mitotic regulator survivin in melanoma.* Anticancer Res, 2012. **32**(2): p. 397-404.
147. Mehrotra, S., L.R. Languino, C.M. Raskett, A.M. Mercurio, T. Dohi, and D.C. Altieri, *IAP regulation of metastasis.* Cancer Cell, 2010. **17**(1): p. 53-64.
148. Cai, X., S. Ma, M. Gu, C. Zu, W. Qu, and X. Zheng, *Survivin regulates the expression of VEGF-C in lymphatic metastasis of breast cancer.* Diagn Pathol, 2012. **7**: p. 52.
149. Chu, X.Y., L.B. Chen, J.H. Wang, Q.S. Su, J.R. Yang, Y. Lin, et al., *Overexpression of survivin is correlated with increased invasion and metastasis of colorectal cancer.* J Surg Oncol, 2012. **105**(6): p. 520-8.
150. Adida, C., P.L. Crotty, J. McGrath, D. Berrebi, J. Diebold, and D.C. Altieri, *Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation.* Am J Pathol, 1998. **152**(1): p. 43-9.
151. Fukuda, S. and L.M. Pelus, *Regulation of the inhibitor-of-apoptosis family member survivin in normal cord blood and bone marrow CD34(+) cells by hematopoietic growth factors: implication of survivin expression in normal hematopoiesis.* Blood, 2001. **98**(7): p. 2091-100.
152. Fukuda, S., R.G. Foster, S.B. Porter, and L.M. Pelus, *The antiapoptosis protein survivin is associated with cell cycle entry of normal cord blood CD34(+) cells and modulates cell cycle and proliferation of mouse hematopoietic progenitor cells.* Blood, 2002. **100**(7): p. 2463-71.
153. Filion, T.M., M. Qiao, P.N. Ghule, M. Mandeville, A.J. van Wijnen, J.L. Stein, et al., *Survival responses of human embryonic stem cells to DNA damage.* J Cell Physiol, 2009. **220**(3): p. 586-92.
154. Gil-Kulik, P., A. Krzyzanowski, E. Dudzinska, J. Karwat, P. Chomik, M. Swistowska, et al., *Potential Involvement of BIRC5 in Maintaining Pluripotency and Cell Differentiation of Human Stem Cells.* Oxid Med Cell Longev, 2019. **2019**: p. 8727925.
155. Mull, A.N., A. Klar, and C.S. Navara, *Differential localization and high expression of SURVIVIN splice variants in human embryonic stem cells but not in differentiated cells implicate a role for SURVIVIN in pluripotency.* Stem Cell Res, 2014. **12**(2): p. 539-49.
156. Singh, P., S. Fukuda, L. Liu, B.R. Chitteti, and L.M. Pelus, *Survivin Is Required for Mouse and Human Bone Marrow Mesenchymal Stromal Cell Function.* Stem Cells, 2018. **36**(1): p. 123-129.
157. Blum, B. and N. Benvenisty, *The tumorigenicity of diploid and aneuploid human pluripotent stem cells.* Cell Cycle, 2009. **8**(23): p. 3822-30.
158. Labarrade, F., J.M. Botto, and N. Domloge, *CRM1 and chromosomal passenger complex component survivin are essential to normal mitosis progress and to preserve keratinocytes from mitotic abnormalities.* Int J Cosmet Sci, 2016. **38**(5): p. 452-61.
159. Dallaglio, K., A. Marconi, and C. Pincelli, *Survivin: a dual player in healthy and diseased skin.* J Invest Dermatol, 2012. **132**(1): p. 18-27.

160. Nakaya, T., S. Ogawa, I. Manabe, M. Tanaka, M. Sanada, T. Sato, et al., *KLF5 regulates the integrity and oncogenicity of intestinal stem cells.* Cancer Res, 2014. **74**(10): p. 2882-91.
161. Marconi, A., K. Dallaglio, R. Lotti, C. Vaschieri, F. Truzzi, F. Fantini, et al., *Survivin identifies keratinocyte stem cells and is downregulated by anti-betal integrin during anoikis.* Stem Cells, 2007. **25**(1): p. 149-55.
162. Feng, R., S. Zhou, Y. Liu, D. Song, Z. Luan, X. Dai, et al., *Sox2 protects neural stem cells from apoptosis via up-regulating survivin expression.* Biochem J, 2013. **450**(3): p. 459-68.
163. Li, C., Y. Yan, W. Ji, L. Bao, H. Qian, L. Chen, et al., *OCT4 positively regulates Survivin expression to promote cancer cell proliferation and leads to poor prognosis in esophageal squamous cell carcinoma.* PLoS One, 2012. **7**(11): p. e49693.
164. Fukuda, S., M. Abe, C. Onishi, T. Taketani, J. Purevsuren, S. Yamaguchi, et al., *Survivin selectively modulates genes deregulated in human leukemia stem cells.* J Oncol, 2011. **2011**: p. 946936.
165. Altieri, D.C., *Survivin, versatile modulation of cell division and apoptosis in cancer.* Oncogene, 2003. **22**(53): p. 8581-9.
166. Altieri, D.C., *Survivin - The inconvenient IAP.* Semin Cell Dev Biol, 2015. **39**: p. 91-6.
167. Ferrandina, G., F. Legge, E. Martinelli, F.O. Ranelletti, G.F. Zannoni, L. Lauriola, et al., *Survivin expression in ovarian cancer and its correlation with clinico-pathological, surgical and apoptosis-related parameters.* Br J Cancer, 2005. **92**(2): p. 271-7.
168. Liu, J.L., W. Gao, Q.M. Kang, X.J. Zhang, and S.G. Yang, *Prognostic value of survivin in patients with gastric cancer: a systematic review with meta-analysis.* PLoS One, 2013. **8**(8): p. e71930.
169. Salz, W., D. Eisenberg, J. Plescia, D.S. Garlick, R.M. Weiss, X.R. Wu, et al., *A survivin gene signature predicts aggressive tumor behavior.* Cancer Res, 2005. **65**(9): p. 3531-4.
170. Shariat, S.F., Y. Lotan, H. Saboorian, S.M. Khoddami, C.G. Roehrborn, K.M. Slawin, et al., *Survivin expression is associated with features of biologically aggressive prostate carcinoma.* Cancer, 2004. **100**(4): p. 751-7.
171. Rodel, F., J. Hoffmann, L. Distel, M. Herrmann, T. Noisternig, T. Papadopoulos, et al., *Survivin as a radioresistance factor, and prognostic and therapeutic target for radiotherapy in rectal cancer.* Cancer Res, 2005. **65**(11): p. 4881-7.
172. Asanuma, K., R. Moriai, T. Yajima, A. Yagihashi, M. Yamada, D. Kobayashi, et al., *Survivin as a radioresistance factor in pancreatic cancer.* Jpn J Cancer Res, 2000. **91**(11): p. 1204-9.
173. Duffy, M.J., N. O'Donovan, D.J. Brennan, W.M. Gallagher, and B.M. Ryan, *Survivin: a promising tumor biomarker.* Cancer Lett, 2007. **249**(1): p. 49-60.
174. Smith, S.D., M.A. Wheeler, J. Plescia, J.W. Colberg, R.M. Weiss, and D.C. Altieri, *Urine detection of survivin and diagnosis of bladder cancer.* JAMA, 2001. **285**(3): p. 324-8.
175. Yie, S.M., B. Luo, N.Y. Ye, K. Xie, and S.R. Ye, *Detection of Survivin-expressing circulating cancer cells in the peripheral blood of breast cancer*

patients by a RT-PCR ELISA. Clin Exp Metastasis, 2006. **23**(5-6): p. 279-89.

176. Sugahara, K., A. Uemura, H. Harasawa, H. Nagai, Y. Hirakata, M. Tomonaga, et al., *Clinical relevance of survivin as a biomarker in neoplasms, especially in adult T-cell leukemias and acute leukemias.* Int J Hematol, 2004. **80**(1): p. 52-8.
177. Mahotka, C., M. Wenzel, E. Springer, H.E. Gabbert, and C.D. Gerharz, *Survivin-deltaEx3 and survivin-2B: two novel splice variants of the apoptosis inhibitor survivin with different antiapoptotic properties.* Cancer Res, 1999. **59**(24): p. 6097-102.
178. Gurbuxani, S., Y. Xu, G. Keerthivasan, A. Wickrema, and J.D. Crispino, *Differential requirements for survivin in hematopoietic cell development.* Proc Natl Acad Sci U S A, 2005. **102**(32): p. 11480-5.
179. Steck, P.A., M.A. Pershouse, S.A. Jasser, W.K. Yung, H. Lin, A.H. Ligon, et al., *Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers.* Nat Genet, 1997. **15**(4): p. 356-62.
180. Vidwans, S.J., M.L. Turski, F. Janku, I. Garrido-Laguna, J. Munoz, R. Schwab, et al., *A framework for genomic biomarker actionability and its use in clinical decision making.* Oncoscience, 2014. **1**(10): p. 614-623.
181. Lee, Y.R., M. Chen, and P.P. Pandolfi, *The functions and regulation of the PTEN tumour suppressor: new modes and prospects.* Nat Rev Mol Cell Biol, 2018. **19**(9): p. 547-562.
182. Pagni, F., E. Guerini-Rocco, A.M. Schultheis, G. Grazia, E. Rijavec, M. Ghidini, et al., *Targeting Immune-Related Biological Processes in Solid Tumors: We do Need Biomarkers.* Int J Mol Sci, 2019. **20**(21).
183. Fusco, N., E. Sajjadi, K. Venetis, G. Gaudioso, G. Lopez, C. Corti, et al., *PTEN Alterations and Their Role in Cancer Management: Are We Making Headway on Precision Medicine?* Genes (Basel), 2020. **11**(7).
184. Masson, G.R. and R.L. Williams, *Structural Mechanisms of PTEN Regulation.* Cold Spring Harb Perspect Med, 2020. **10**(3).
185. Haynie, D.T. and B. Xue, *Superdomains in the protein structure hierarchy: The case of PTP-C2.* Protein Sci, 2015. **24**(5): p. 874-82.
186. Sun, Z., C. Huang, J. He, K.L. Lamb, X. Kang, T. Gu, et al., *PTEN C-terminal deletion causes genomic instability and tumor development.* Cell Rep, 2014. **6**(5): p. 844-54.
187. Chen, C.Y., J. Chen, L. He, and B.L. Stiles, *PTEN: Tumor Suppressor and Metabolic Regulator.* Front Endocrinol (Lausanne), 2018. **9**: p. 338.
188. Xu, W., Z. Yang, S.F. Zhou, and N. Lu, *Posttranslational regulation of phosphatase and tensin homolog (PTEN) and its functional impact on cancer behaviors.* Drug Des Devel Ther, 2014. **8**: p. 1745-51.
189. Lawrence, M.S., P. Stojanov, C.H. Mermel, J.T. Robinson, L.A. Garraway, T.R. Golub, et al., *Discovery and saturation analysis of cancer genes across 21 tumour types.* Nature, 2014. **505**(7484): p. 495-501.
190. Hoxhaj, G. and B.D. Manning, *The PI3K-AKT network at the interface of oncogenic signalling and cancer metabolism.* Nat Rev Cancer, 2020. **20**(2): p. 74-88.

191. Porta, C., C. Paglino, and A. Mosca, *Targeting PI3K/Akt/mTOR Signaling in Cancer.* Front Oncol, 2014. **4**: p. 64.
192. Bilanges, B., Y. Posor, and B. Vanhaesebroeck, *PI3K isoforms in cell signalling and vesicle trafficking.* Nat Rev Mol Cell Biol, 2019. **20**(9): p. 515-534.
193. Thorpe, L.M., H. Yuzugullu, and J.J. Zhao, *PI3K in cancer: divergent roles of isoforms, modes of activation and therapeutic targeting.* Nat Rev Cancer, 2015. **15**(1): p. 7-24.
194. Zbuk, K.M. and C. Eng, *Cancer phenomics: RET and PTEN as illustrative models.* Nat Rev Cancer, 2007. **7**(1): p. 35-45.
195. Papa, A. and P.P. Pandolfi, *The PTEN(-)PI3K Axis in Cancer.* Biomolecules, 2019. **9**(4).
196. Song, M.S., L. Salmena, and P.P. Pandolfi, *The functions and regulation of the PTEN tumour suppressor.* Nat Rev Mol Cell Biol, 2012. **13**(5): p. 283-96.
197. Spinelli, L. and N.R. Leslie, *Assays to Measure PTEN Lipid Phosphatase Activity In Vitro from Purified Enzyme or Immunoprecipitates.* Methods Mol Biol, 2016. **1447**: p. 95-105.
198. Revathidevi, S. and A.K. Munirajan, *Akt in cancer: Mediator and more.* Semin Cancer Biol, 2019. **59**: p. 80-91.
199. Lee, J.H., R. Liu, J. Li, C. Zhang, Y. Wang, Q. Cai, et al., *Stabilization of phosphofructokinase 1 platelet isoform by AKT promotes tumorigenesis.* Nat Commun, 2017. **8**(1): p. 949.
200. Beg, M., N. Abdullah, F.S. Thowfeik, N.K. Altorki, and T.E. McGraw, *Distinct Akt phosphorylation states are required for insulin regulated Glut4 and Glut1-mediated glucose uptake.* Elife, 2017. **6**.
201. Mossmann, D., S. Park, and M.N. Hall, *mTOR signalling and cellular metabolism are mutual determinants in cancer.* Nat Rev Cancer, 2018. **18**(12): p. 744-757.
202. Kelsey, I. and B.D. Manning, *mTORC1 status dictates tumor response to targeted therapeutics.* Sci Signal, 2013. **6**(294): p. pe31.
203. Ben-Sahra, I., J.J. Howell, J.M. Asara, and B.D. Manning, *Stimulation of de novo pyrimidine synthesis by growth signaling through mTOR and S6K1.* Science, 2013. **339**(6125): p. 1323-8.
204. Chalhoub, N. and S.J. Baker, *PTEN and the PI3-kinase pathway in cancer.* Annu Rev Pathol, 2009. **4**: p. 127-50.
205. Aquila, S., M. Santoro, A. Caputo, M.L. Panno, V. Pezzi, and F. De Amicis, *The Tumor Suppressor PTEN as Molecular Switch Node Regulating Cell Metabolism and Autophagy: Implications in Immune System and Tumor Microenvironment.* Cells, 2020. **9**(7).
206. Unterlass, J.E. and N.J. Curtin, *Warburg and Krebs and related effects in cancer.* Expert Rev Mol Med, 2019. **21**: p. e4.
207. Lyssiotis, C.A. and D. Nagrath, *Metabolic Reprogramming and Vulnerabilities in Cancer.* Cancers (Basel), 2019. **12**(1).
208. Phadngam, S., A. Castiglioni, A. Ferraresi, F. Morani, C. Follo, and C. Isidoro, *PTEN dephosphorylates AKT to prevent the expression of GLUT1*

on plasmamembrane and to limit glucose consumption in cancer cells. Oncotarget, 2016. **7**(51): p. 84999-85020.
209. Marin-Hernandez, A., J.C. Gallardo-Perez, S. Rodriguez-Enriquez, R. Encalada, R. Moreno-Sanchez, and E. Saavedra, *Modeling cancer glycolysis.* Biochim Biophys Acta, 2011. **1807**(6): p. 755-67.
210. Granchi, C. and F. Minutolo, *Anticancer agents that counteract tumor glycolysis.* ChemMedChem, 2012. **7**(8): p. 1318-50.
211. Tramontano, D. and F. De Amicis, *Is the secret for a successful aging to keep track of cancer pathways?* J Cell Physiol, 2018. **233**(11): p. 8467-8476.
212. Qiao, X., D.I. Kim, H. Jun, Y. Ma, A.J. Knights, M.J. Park, et al., *Protein Arginine Methyltransferase 1 Interacts With PGC1alpha and Modulates Thermogenic Fat Activation.* Endocrinology, 2019. **160**(12): p. 2773-2786.
213. Ortega-Molina, A. and M. Serrano, *PTEN in cancer, metabolism, and aging.* Trends Endocrinol Metab, 2013. **24**(4): p. 184-9.
214. Wang, L., H. Xiong, F. Wu, Y. Zhang, J. Wang, L. Zhao, et al., *Hexokinase 2-mediated Warburg effect is required for PTEN- and p53-deficiency-driven prostate cancer growth.* Cell Rep, 2014. **8**(5): p. 1461-74.
215. He, R. and H. Liu, *TRIM59 knockdown blocks cisplatin resistance in A549/DDP cells through regulating PTEN/AKT/HK2.* Gene, 2020. **747**: p. 144553.
216. Wong, N., J. De Melo, and D. Tang, *PKM2, a Central Point of Regulation in Cancer Metabolism.* Int J Cell Biol, 2013. **2013**: p. 242513.
217. Talesa, V.N., I. Ferri, G. Bellezza, H.D. Love, A. Sidoni, and C. Antognelli, *Glyoxalase 2 Is Involved in Human Prostate Cancer Progression as Part of a Mechanism Driven By PTEN/PI3K/AKT/mTOR Signaling With Involvement of PKM2 and ERalpha.* Prostate, 2017. **77**(2): p. 196-210.
218. Herranz, D., A. Ambesi-Impiombato, J. Sudderth, M. Sanchez-Martin, L. Belver, V. Tosello, et al., *Metabolic reprogramming induces resistance to anti-NOTCH1 therapies in T cell acute lymphoblastic leukemia.* Nat Med, 2015. **21**(10): p. 1182-9.
219. Qu, X., J. Sun, Y. Zhang, J. Li, J. Hu, K. Li, et al., *c-Myc-driven glycolysis via TXNIP suppression is dependent on glutaminase-MondoA axis in prostate cancer.* Biochem Biophys Res Commun, 2018. **504**(2): p. 415-421.
220. Hu, W., C. Zhang, R. Wu, Y. Sun, A. Levine, and Z. Feng, *Glutaminase 2, a novel p53 target gene regulating energy metabolism and antioxidant function.* Proc Natl Acad Sci U S A, 2010. **107**(16): p. 7455-60.
221. Suzuki, S., T. Tanaka, M.V. Poyurovsky, H. Nagano, T. Mayama, S. Ohkubo, et al., *Phosphate-activated glutaminase (GLS2), a p53-inducible regulator of glutamine metabolism and reactive oxygen species.* Proc Natl Acad Sci U S A, 2010. **107**(16): p. 7461-6.
222. Wei, Z., L. Cui, Z. Mei, M. Liu, and D. Zhang, *miR-181a mediates metabolic shift in colon cancer cells via the PTEN/AKT pathway.* FEBS Lett, 2014. **588**(9): p. 1773-9.
223. Antico Arciuch, V.G., M.A. Russo, K.S. Kang, and A. Di Cristofano, *Inhibition of AMPK and Krebs cycle gene expression drives metabolic remodeling of Pten-deficient preneoplastic thyroid cells.* Cancer Res, 2013. **73**(17): p. 5459-72.

224. Garcia-Cao, I., M.S. Song, R.M. Hobbs, G. Laurent, C. Giorgi, V.C. de Boer, et al., *Systemic elevation of PTEN induces a tumor-suppressive metabolic state.* Cell, 2012. **149**(1): p. 49-62.
225. Bankoglu, E.E., O. Tschopp, J. Schmitt, P. Burkard, D. Jahn, A. Geier, et al., *Role of PTEN in Oxidative Stress and DNA Damage in the Liver of Whole-Body Pten Haplodeficient Mice.* PLoS One, 2016. **11**(11): p. e0166956.
226. Peyrou, M., L. Bourgoin, and M. Foti, *PTEN in liver diseases and cancer.* World J Gastroenterol, 2010. **16**(37): p. 4627-33.
227. Wang, K.C., Y.C. Liu, M. El-Shazly, S.P. Shih, Y.C. Du, Y.M. Hsu, et al., *The Antioxidant from Ethanolic Extract of Rosa cymosa Fruits Activates Phosphatase and Tensin Homolog In Vitro and In Vivo: A New Insight on Its Antileukemic Effect.* Int J Mol Sci, 2019. **20**(8).
228. Ho, J., E.S. Cruise, R.J.O. Dowling, and V. Stambolic, *PTEN Nuclear Functions.* Cold Spring Harb Perspect Med, 2020. **10**(5).
229. Milella, M., I. Falcone, F. Conciatori, U. Cesta Incani, A. Del Curatolo, N. Inzerilli, et al., *PTEN: Multiple Functions in Human Malignant Tumors.* Front Oncol, 2015. **5**: p. 24.
230. Malaney, P., E. Palumbo, J. Semidey-Hurtado, J. Hardee, K. Stanford, J.J. Kathiriya, et al., *PTEN Physically Interacts with and Regulates E2F1-mediated Transcription in Lung Cancer.* Cell Cycle, 2018. **17**(8): p. 947-962.
231. Ming, M. and Y.Y. He, *PTEN in DNA damage repair.* Cancer Lett, 2012. **319**(2): p. 125-129.
232. Puc, J., M. Keniry, H.S. Li, T.K. Pandita, A.D. Choudhury, L. Memeo, et al., *Lack of PTEN sequesters CHK1 and initiates genetic instability.* Cancer Cell, 2005. **7**(2): p. 193-204.
233. Iyer, D.R. and N. Rhind, *The Intra-S Checkpoint Responses to DNA Damage.* Genes (Basel), 2017. **8**(2).
234. Brandmaier, A., S.Q. Hou, and W.H. Shen, *Cell Cycle Control by PTEN.* J Mol Biol, 2017. **429**(15): p. 2265-2277.
235. Liu, T., Y. Wang, Y. Wang, and A.M. Chan, *Multifaceted Regulation of PTEN Subcellular Distributions and Biological Functions.* Cancers (Basel), 2019. **11**(9).
236. Chen, Z.H., M. Zhu, J. Yang, H. Liang, J. He, S. He, et al., *PTEN interacts with histone H1 and controls chromatin condensation.* Cell Rep, 2014. **8**(6): p. 2003-2014.
237. Fan, X., J. Kraynak, J.P.S. Knisely, S.C. Formenti, and W.H. Shen, *PTEN as a Guardian of the Genome: Pathways and Targets.* Cold Spring Harb Perspect Med, 2020. **10**(9).
238. Alvarez-Garcia, V., Y. Tawil, H.M. Wise, and N.R. Leslie, *Mechanisms of PTEN loss in cancer: It's all about diversity.* Semin Cancer Biol, 2019. **59**: p. 66-79.
239. Cancer Genome Atlas Research, N., C. Kandoth, N. Schultz, A.D. Cherniack, R. Akbani, Y. Liu, et al., *Integrated genomic characterization of endometrial carcinoma.* Nature, 2013. **497**(7447): p. 67-73.

240. Lopez, G., M. Noale, C. Corti, G. Gaudioso, E. Sajjadi, K. Venetis, et al., *PTEN Expression as a Complementary Biomarker for Mismatch Repair Testing in Breast Cancer.* Int J Mol Sci, 2020. **21**(4).
241. Chang, H., Z. Cai, and T.M. Roberts, *The Mechanisms Underlying PTEN Loss in Human Tumors Suggest Potential Therapeutic Opportunities.* Biomolecules, 2019. **9**(11).
242. Djordjevic, B., B.A. Barkoh, R. Luthra, and R.R. Broaddus, *Relationship between PTEN, DNA mismatch repair, and tumor histotype in endometrial carcinoma: retained positive expression of PTEN preferentially identifies sporadic non-endometrioid carcinomas.* Mod Pathol, 2013. **26**(10): p. 1401-12.
243. Lemery, S., P. Keegan, and R. Pazdur, *First FDA Approval Agnostic of Cancer Site - When a Biomarker Defines the Indication.* N Engl J Med, 2017. **377**(15): p. 1409-1412.
244. Davies, H., S. Morganella, C.A. Purdie, S.J. Jang, E. Borgen, H. Russnes, et al., *Whole-Genome Sequencing Reveals Breast Cancers with Mismatch Repair Deficiency.* Cancer Res, 2017. **77**(18): p. 4755-4762.
245. Fusco, N., G. Lopez, C. Corti, C. Pesenti, P. Colapietro, G. Ercoli, et al., *Mismatch Repair Protein Loss as a Prognostic and Predictive Biomarker in Breast Cancers Regardless of Microsatellite Instability.* JNCI Cancer Spectr, 2018. **2**(4): p. pky056.
246. Wen, X. and D.J. Klionsky, *At a glance: A history of autophagy and cancer.* Semin Cancer Biol, 2020. **66**: p. 3-11.
247. Qu, X., J. Yu, G. Bhagat, N. Furuya, H. Hibshoosh, A. Troxel, et al., *Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene.* J Clin Invest, 2003. **112**(12): p. 1809-20.
248. Shaw, R.J., *LKB1 and AMP-activated protein kinase control of mTOR signalling and growth.* Acta Physiol (Oxf), 2009. **196**(1): p. 65-80.
249. Errafiy, R., C. Aguado, G. Ghislat, J.M. Esteve, A. Gil, M. Loutfi, et al., *PTEN increases autophagy and inhibits the ubiquitin-proteasome pathway in glioma cells independently of its lipid phosphatase activity.* PLoS One, 2013. **8**(12): p. e83318.
250. Yang, A., N.V. Rajeshkumar, X. Wang, S. Yabuuchi, B.M. Alexander, G.C. Chu, et al., *Autophagy is critical for pancreatic tumor growth and progression in tumors with p53 alterations.* Cancer Discov, 2014. **4**(8): p. 905-13.
251. Rosenfeldt, M.T., J. O'Prey, J.P. Morton, C. Nixon, G. MacKay, A. Mrowinska, et al., *p53 status determines the role of autophagy in pancreatic tumour development.* Nature, 2013. **504**(7479): p. 296-300.
252. Zhu, L., L. Li, Q. Zhang, X. Yang, Z. Zou, B. Hao, et al., *NOS1 S-nitrosylates PTEN and inhibits autophagy in nasopharyngeal carcinoma cells.* Cell Death Discov, 2017. **3**: p. 17011.
253. Cai, J., R. Li, X. Xu, L. Zhang, R. Lian, L. Fang, et al., *CK1alpha suppresses lung tumour growth by stabilizing PTEN and inducing autophagy.* Nat Cell Biol, 2018. **20**(4): p. 465-478.

254. Ueno, T., W. Sato, Y. Horie, M. Komatsu, I. Tanida, M. Yoshida, et al., *Loss of Pten, a tumor suppressor, causes the strong inhibition of autophagy without affecting LC3 lipidation.* Autophagy, 2008. **4**(5): p. 692-700.
255. Wesselborg, S. and B. Stork, *Autophagy signal transduction by ATG proteins: from hierarchies to networks.* Cell Mol Life Sci, 2015. **72**(24): p. 4721-57.
256. Peng, W., J.Q. Chen, C. Liu, S. Malu, C. Creasy, M.T. Tetzlaff, et al., *Loss of PTEN Promotes Resistance to T Cell-Mediated Immunotherapy.* Cancer Discov, 2016. **6**(2): p. 202-16.
257. Coussens, L.M. and Z. Werb, *Inflammation and cancer.* Nature, 2002. **420**(6917): p. 860-7.
258. Wichmann, G., C. Gaede, S. Melzer, J. Bocsi, S. Henger, C. Engel, et al., *Discrimination of Head and Neck Squamous Cell Carcinoma Patients and Healthy Adults by 10-Color Flow Cytometry: Development of a Score Based on Leukocyte Subsets.* Cancers (Basel), 2019. **11**(6).
259. Dumitru, C.A., K. Moses, S. Trellakis, S. Lang, and S. Brandau, *Neutrophils and granulocytic myeloid-derived suppressor cells: immunophenotyping, cell biology and clinical relevance in human oncology.* Cancer Immunol Immunother, 2012. **61**(8): p. 1155-67.
260. Xu, C., C.M. Fillmore, S. Koyama, H. Wu, Y. Zhao, Z. Chen, et al., *Loss of Lkb1 and Pten leads to lung squamous cell carcinoma with elevated PD-L1 expression.* Cancer Cell, 2014. **25**(5): p. 590-604.
261. Chen, H., W. Chong, C. Teng, Y. Yao, X. Wang, and X. Li, *The immune response-related mutational signatures and driver genes in non-small-cell lung cancer.* Cancer Sci, 2019. **110**(8): p. 2348-2356.
262. Wartewig, T., Z. Kurgyis, S. Keppler, K. Pechloff, E. Hameister, R. Ollinger, et al., *PD-1 is a haploinsufficient suppressor of T cell lymphomagenesis.* Nature, 2017. **552**(7683): p. 121-125.
263. Zhao, J., A.X. Chen, R.D. Gartrell, A.M. Silverman, L. Aparicio, T. Chu, et al., *Immune and genomic correlates of response to anti-PD-1 immunotherapy in glioblastoma.* Nat Med, 2019. **25**(3): p. 462-469.
264. Cretella, D., G. Digiacomo, E. Giovannetti, and A. Cavazzoni, *PTEN Alterations as a Potential Mechanism for Tumor Cell Escape from PD-1/PD-L1 Inhibition.* Cancers (Basel), 2019. **11**(9).
265. Atri, C., F.Z. Guerfali, and D. Laouini, *Role of Human Macrophage Polarization in Inflammation during Infectious Diseases.* Int J Mol Sci, 2018. **19**(6).
266. Quatromoni, J.G. and E. Eruslanov, *Tumor-associated macrophages: function, phenotype, and link to prognosis in human lung cancer.* Am J Transl Res, 2012. **4**(4): p. 376-89.
267. Vergadi, E., E. Ieronymaki, K. Lyroni, K. Vaporidi, and C. Tsatsanis, *Akt Signaling Pathway in Macrophage Activation and M1/M2 Polarization.* J Immunol, 2017. **198**(3): p. 1006-1014.
268. Sahin, E., S. Haubenwallner, M. Kuttke, I. Kollmann, A. Halfmann, A.M. Dohnal, et al., *Macrophage PTEN regulates expression and secretion of arginase I modulating innate and adaptive immune responses.* J Immunol, 2014. **193**(4): p. 1717-27.

269. Johnson, T.A. and D.K. Singla, *PTEN inhibitor VO-OHpic attenuates inflammatory M1 macrophages and cardiac remodeling in doxorubicin-induced cardiomyopathy.* Am J Physiol Heart Circ Physiol, 2018. **315**(5): p. H1236-H1249.
270. Junttila, M.R. and F.J. de Sauvage, *Influence of tumour micro-environment heterogeneity on therapeutic response.* Nature, 2013. **501**(7467): p. 346-54.
271. Zamarron, B.F. and W. Chen, *Dual roles of immune cells and their factors in cancer development and progression.* Int J Biol Sci, 2011. **7**(5): p. 651-8.
272. Sahai, E., I. Astsaturov, E. Cukierman, D.G. DeNardo, M. Egeblad, R.M. Evans, et al., *A framework for advancing our understanding of cancer-associated fibroblasts.* Nat Rev Cancer, 2020. **20**(3): p. 174-186.
273. Lane, D.P. and L.V. Crawford, *T antigen is bound to a host protein in SV40-transformed cells.* Nature, 1979. **278**(5701): p. 261-3.
274. Olivier, M., M. Hollstein, and P. Hainaut, *TP53 mutations in human cancers: origins, consequences, and clinical use.* Cold Spring Harb Perspect Biol, 2010. **2**(1): p. a001008.
275. Laptenko, O. and C. Prives, *Transcriptional regulation by p53: one protein, many possibilities.* Cell Death Differ, 2006. **13**(6): p. 951-61.
276. Rivlin, N., R. Brosh, M. Oren, and V. Rotter, *Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis.* Genes Cancer, 2011. **2**(4): p. 466-74.
277. Haupt, Y., R. Maya, A. Kazaz, and M. Oren, *Mdm2 promotes the rapid degradation of p53.* Nature, 1997. **387**(6630): p. 296-9.
278. Shieh, S.Y., M. Ikeda, Y. Taya, and C. Prives, *DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2.* Cell, 1997. **91**(3): p. 325-34.
279. Zhang, Y., Y. Xiong, and W.G. Yarbrough, *ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways.* Cell, 1998. **92**(6): p. 725-34.
280. el-Deiry, W.S., T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, et al., *WAF1, a potential mediator of p53 tumor suppression.* Cell, 1993. **75**(4): p. 817-25.
281. Serrano, M., A.W. Lin, M.E. McCurrach, D. Beach, and S.W. Lowe, *Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a.* Cell, 1997. **88**(5): p. 593-602.
282. Miyashita, T., S. Krajewski, M. Krajewska, H.G. Wang, H.K. Lin, D.A. Liebermann, et al., *Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo.* Oncogene, 1994. **9**(6): p. 1799-805.
283. Livingstone, L.R., A. White, J. Sprouse, E. Livanos, T. Jacks, and T.D. Tlsty, *Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53.* Cell, 1992. **70**(6): p. 923-35.
284. Lowe, S.W., T. Jacks, D.E. Housman, and H.E. Ruley, *Abrogation of oncogene-associated apoptosis allows transformation of p53-deficient cells.* Proc Natl Acad Sci U S A, 1994. **91**(6): p. 2026-30.
285. Lane, D.P., *Cancer. p53, guardian of the genome.* Nature, 1992. **358**(6381): p. 15-6.

286. Williams, A.B. and B. Schumacher, *p53 in the DNA-Damage-Repair Process.* Cold Spring Harb Perspect Med, 2016. **6**(5).
287. Ciriello, G., M.L. Miller, B.A. Aksoy, Y. Senbabaoglu, N. Schultz, and C. Sander, *Emerging landscape of oncogenic signatures across human cancers.* Nat Genet, 2013. **45**(10): p. 1127-33.
288. Vitre, B.D. and D.W. Cleveland, *Centrosomes, chromosome instability (CIN) and aneuploidy.* Curr Opin Cell Biol, 2012. **24**(6): p. 809-15.
289. Schvartzman, J.M., P.H. Duijf, R. Sotillo, C. Coker, and R. Benezra, *Mad2 is a critical mediator of the chromosome instability observed upon Rb and p53 pathway inhibition.* Cancer Cell, 2011. **19**(6): p. 701-14.
290. Vitale, I., L. Senovilla, M. Jemaa, M. Michaud, L. Galluzzi, O. Kepp, et al., *Multipolar mitosis of tetraploid cells: inhibition by p53 and dependency on Mos.* EMBO J, 2010. **29**(7): p. 1272-84.
291. Eischen, C.M., *Genome Stability Requires p53.* Cold Spring Harb Perspect Med, 2016. **6**(6).
292. Ganem, N.J., H. Cornils, S.Y. Chiu, K.P. O'Rourke, J. Arnaud, D. Yimlamai, et al., *Cytokinesis failure triggers hippo tumor suppressor pathway activation.* Cell, 2014. **158**(4): p. 833-848.
293. Cancer Genome Atlas Research, N., *Comprehensive molecular characterization of clear cell renal cell carcinoma.* Nature, 2013. **499**(7456): p. 43-9.
294. Soto, M., J.A. Raaijmakers, B. Bakker, D.C.J. Spierings, P.M. Lansdorp, F. Foijer, et al., *p53 Prohibits Propagation of Chromosome Segregation Errors that Produce Structural Aneuploidies.* Cell Rep, 2017. **19**(12): p. 2423-2431.
295. Tang, Y.C., B.R. Williams, J.J. Siegel, and A. Amon, *Identification of aneuploidy-selective antiproliferation compounds.* Cell, 2011. **144**(4): p. 499-512.
296. Hayashi, M.T., A.J. Cesare, J.A. Fitzpatrick, E. Lazzerini-Denchi, and J. Karlseder, *A telomere-dependent DNA damage checkpoint induced by prolonged mitotic arrest.* Nat Struct Mol Biol, 2012. **19**(4): p. 387-94.
297. Maciejowski, J., Y. Li, N. Bosco, P.J. Campbell, and T. de Lange, *Chromothripsis and Kataegis Induced by Telomere Crisis.* Cell, 2015. **163**(7): p. 1641-54.
298. Rausch, T., D.T. Jones, M. Zapatka, A.M. Stutz, T. Zichner, J. Weischenfeldt, et al., *Genome sequencing of pediatric medulloblastoma links catastrophic DNA rearrangements with TP53 mutations.* Cell, 2012. **148**(1-2): p. 59-71.
299. Levine, A.J., D.T. Ting, and B.D. Greenbaum, *P53 and the defenses against genome instability caused by transposons and repetitive elements.* Bioessays, 2016. **38**(6): p. 508-13.
300. Wylie, A., W.J. Lu, A. D'Brot, M. Buszczak, and J.M. Abrams, *p53 activity is selectively licensed in the Drosophila stem cell compartment.* Elife, 2014. **3**: p. e01530.
301. Chang, N.T., W.K. Yang, H.C. Huang, K.W. Yeh, and C.W. Wu, *The transcriptional activity of HERV-I LTR is negatively regulated by its cis-elements and wild type p53 tumor suppressor protein.* J Biomed Sci, 2007. **14**(2): p. 211-22.

302. Tubio, J.M.C., Y. Li, Y.S. Ju, I. Martincorena, S.L. Cooke, M. Tojo, et al., *Mobile DNA in cancer. Extensive transduction of nonrepetitive DNA mediated by L1 retrotransposition in cancer genomes.* Science, 2014. **345**(6196): p. 1251343.
303. Ting, D.T., D. Lipson, S. Paul, B.W. Brannigan, S. Akhavanfard, E.J. Coffman, et al., *Aberrant overexpression of satellite repeats in pancreatic and other epithelial cancers.* Science, 2011. **331**(6017): p. 593-6.
304. Wylie, A., A.E. Jones, A. D'Brot, W.J. Lu, P. Kurtz, J.V. Moran, et al., *p53 genes function to restrain mobile elements.* Genes Dev, 2016. **30**(1): p. 64-77.
305. McGranahan, N. and C. Swanton, *Clonal Heterogeneity and Tumor Evolution: Past, Present, and the Future.* Cell, 2017. **168**(4): p. 613-628.
306. Kastenhuber, E.R. and S.W. Lowe, *Putting p53 in Context.* Cell, 2017. **170**(6): p. 1062-1078.
307. Hager, K.M. and W. Gu, *Understanding the non-canonical pathways involved in p53-mediated tumor suppression.* Carcinogenesis, 2014. **35**(4): p. 740-6.
308. Aylon, Y. and M. Oren, *The Paradox of p53: What, How, and Why?* Cold Spring Harb Perspect Med, 2016. **6**(10).
309. Pappas, K., J. Xu, S. Zairis, L. Resnick-Silverman, F. Abate, N. Steinbach, et al., *p53 Maintains Baseline Expression of Multiple Tumor Suppressor Genes.* Mol Cancer Res, 2017. **15**(8): p. 1051-1062.
310. Sullivan, K.D., M.D. Galbraith, Z. Andrysik, and J.M. Espinosa, *Mechanisms of transcriptional regulation by p53.* Cell Death Differ, 2018. **25**(1): p. 133-143.
311. Mirza, A., Q. Wu, L. Wang, T. McClanahan, W.R. Bishop, F. Gheyas, et al., *Global transcriptional program of p53 target genes during the process of apoptosis and cell cycle progression.* Oncogene, 2003. **22**(23): p. 3645-54.
312. Allen, M.A., Z. Andrysik, V.L. Dengler, H.S. Mellert, A. Guarnieri, J.A. Freeman, et al., *Global analysis of p53-regulated transcription identifies its direct targets and unexpected regulatory mechanisms.* Elife, 2014. **3**: p. e02200.
313. Olivos, D.J. and L.D. Mayo, *Emerging Non-Canonical Functions and Regulation by p53: p53 and Stemness.* Int J Mol Sci, 2016. **17**(12).
314. Fischer, M., *Census and evaluation of p53 target genes.* Oncogene, 2017. **36**(28): p. 3943-3956.
315. Waters, A.M. and C.J. Der, *KRAS: The Critical Driver and Therapeutic Target for Pancreatic Cancer.* Cold Spring Harb Perspect Med, 2018. **8**(9).
316. Beckerman, R. and C. Prives, *Transcriptional regulation by p53.* Cold Spring Harb Perspect Biol, 2010. **2**(8): p. a000935.
317. Morachis, J.M., C.M. Murawsky, and B.M. Emerson, *Regulation of the p53 transcriptional response by structurally diverse core promoters.* Genes Dev, 2010. **24**(2): p. 135-47.
318. Kruiswijk, F., C.F. Labuschagne, and K.H. Vousden, *p53 in survival, death and metabolic health: a lifeguard with a licence to kill.* Nat Rev Mol Cell Biol, 2015. **16**(7): p. 393-405.

319. Zhang, C., J. Liu, Y. Liang, R. Wu, Y. Zhao, X. Hong, et al., *Tumour-associated mutant p53 drives the Warburg effect.* Nat Commun, 2013. **4**: p. 2935.
320. Kim, H.R., J.S. Roe, J.E. Lee, E.J. Cho, and H.D. Youn, *p53 regulates glucose metabolism by miR-34a.* Biochem Biophys Res Commun, 2013. **437**(2): p. 225-31.
321. Stambolsky, P., L. Weisz, I. Shats, Y. Klein, N. Goldfinger, M. Oren, et al., *Regulation of AIF expression by p53.* Cell Death Differ, 2006. **13**(12): p. 2140-9.
322. Jiang, P., W. Du, A. Mancuso, K.E. Wellen, and X. Yang, *Reciprocal regulation of p53 and malic enzymes modulates metabolism and senescence.* Nature, 2013. **493**(7434): p. 689-93.
323. Wang, P.Y., W. Ma, J.Y. Park, F.S. Celi, R. Arena, J.W. Choi, et al., *Increased oxidative metabolism in the Li-Fraumeni syndrome.* N Engl J Med, 2013. **368**(11): p. 1027-32.
324. Young, A.R., M. Narita, M. Ferreira, K. Kirschner, M. Sadaie, J.F. Darot, et al., *Autophagy mediates the mitotic senescence transition.* Genes Dev, 2009. **23**(7): p. 798-803.
325. Gao, M., P. Monian, Q. Pan, W. Zhang, J. Xiang, and X. Jiang, *Ferroptosis is an autophagic cell death process.* Cell Res, 2016. **26**(9): p. 1021-32.
326. Thorburn, J., Z. Andrysik, L. Staskiewicz, J. Gump, P. Maycotte, A. Oberst, et al., *Autophagy controls the kinetics and extent of mitochondrial apoptosis by regulating PUMA levels.* Cell Rep, 2014. **7**(1): p. 45-52.
327. Duan, L., R.E. Perez, B. Davaadelger, E.N. Dedkova, L.A. Blatter, and C.G. Maki, *p53-regulated autophagy is controlled by glycolysis and determines cell fate.* Oncotarget, 2015. **6**(27): p. 23135-56.
328. Kumari, R., S. Kohli, and S. Das, *p53 regulation upon genotoxic stress: intricacies and complexities.* Mol Cell Oncol, 2014. **1**(3): p. e969653.
329. Stewart-Ornstein, J. and G. Lahav, *p53 dynamics in response to DNA damage vary across cell lines and are shaped by efficiency of DNA repair and activity of the kinase ATM.* Sci Signal, 2017. **10**(476).
330. Gomes, N.P. and J.M. Espinosa, *Disparate chromatin landscapes and kinetics of inactivation impact differential regulation of p53 target genes.* Cell Cycle, 2010. **9**(17): p. 3428-37.
331. Su, D., X. Wang, M.R. Campbell, L. Song, A. Safi, G.E. Crawford, et al., *Interactions of chromatin context, binding site sequence content, and sequence evolution in stress-induced p53 occupancy and transactivation.* PLoS Genet, 2015. **11**(1): p. e1004885.
332. Gomes, N.P. and J.M. Espinosa, *Gene-specific repression of the p53 target gene PUMA via intragenic CTCF-Cohesin binding.* Genes Dev, 2010. **24**(10): p. 1022-34.
333. Itahana, Y., J. Zhang, J. Goke, L.A. Vardy, R. Han, K. Iwamoto, et al., *Histone modifications and p53 binding poise the p21 promoter for activation in human embryonic stem cells.* Sci Rep, 2016. **6**: p. 28112.
334. Cooks, T., C.C. Harris, and M. Oren, *Caught in the cross fire: p53 in inflammation.* Carcinogenesis, 2014. **35**(8): p. 1680-90.

335. Sullivan, K.D., V.V. Palaniappan, and J.M. Espinosa, *ATM regulates cell fate choice upon p53 activation by modulating mitochondrial turnover and ROS levels.* Cell Cycle, 2015. **14**(1): p. 56-63.
336. Lahalle, A., M. Lacroix, C. De Blasio, M.Y. Cisse, L.K. Linares, and L. Le Cam, *The p53 Pathway and Metabolism: The Tree That Hides the Forest.* Cancers (Basel), 2021. **13**(1).
337. Holley, A.K. and D.K. St Clair, *Watching the watcher: regulation of p53 by mitochondria.* Future Oncol, 2009. **5**(1): p. 117-30.
338. Lacroix, M., R. Riscal, G. Arena, L.K. Linares, and L. Le Cam, *Metabolic functions of the tumor suppressor p53: Implications in normal physiology, metabolic disorders, and cancer.* Mol Metab, 2020. **33**: p. 2-22.
339. Mancini, F. and F. Moretti, *Mitochondrial MDM4 (MDMX): an unpredicted role in the p53-mediated intrinsic apoptotic pathway.* Cell Cycle, 2009. **8**(23): p. 3854-9.
340. Arena, G., M.Y. Cisse, S. Pyrdziak, L. Chatre, R. Riscal, M. Fuentes, et al., *Mitochondrial MDM2 Regulates Respiratory Complex I Activity Independently of p53.* Mol Cell, 2018. **69**(4): p. 594-609 e8.
341. Itahana, K., H.V. Clegg, and Y. Zhang, *ARF in the mitochondria: the last frontier?* Cell Cycle, 2008. **7**(23): p. 3641-6.
342. Banerjee Mustafi, S., N. Aznar, S.K. Dwivedi, P.K. Chakraborty, R. Basak, P. Mukherjee, et al., *Mitochondrial BMI1 maintains bioenergetic homeostasis in cells.* FASEB J, 2016. **30**(12): p. 4042-4055.
343. Kook, S., X. Zhan, K. Thibeault, M.R. Ahmed, V.V. Gurevich, and E.V. Gurevich, *Mdm2 enhances ligase activity of parkin and facilitates mitophagy.* Sci Rep, 2020. **10**(1): p. 5028.
344. Hoshino, A., M. Ariyoshi, Y. Okawa, S. Kaimoto, M. Uchihashi, K. Fukai, et al., *Inhibition of p53 preserves Parkin-mediated mitophagy and pancreatic beta-cell function in diabetes.* Proc Natl Acad Sci U S A, 2014. **111**(8): p. 3116-21.
345. Song, Y.M., W.K. Lee, Y.H. Lee, E.S. Kang, B.S. Cha, and B.W. Lee, *Metformin Restores Parkin-Mediated Mitophagy, Suppressed by Cytosolic p53.* Int J Mol Sci, 2016. **17**(1).
346. Kitamura, N., Y. Nakamura, Y. Miyamoto, T. Miyamoto, K. Kabu, M. Yoshida, et al., *Mieap, a p53-inducible protein, controls mitochondrial quality by repairing or eliminating unhealthy mitochondria.* PLoS One, 2011. **6**(1): p. e16060.
347. Liu, J., L. Cao, J. Chen, S. Song, I.H. Lee, C. Quijano, et al., *Bmi1 regulates mitochondrial function and the DNA damage response pathway.* Nature, 2009. **459**(7245): p. 387-392.
348. Rodier, G., O. Kirsh, M. Baraibar, T. Houles, M. Lacroix, H. Delpech, et al., *The transcription factor E4F1 coordinates CHK1-dependent checkpoint and mitochondrial functions.* Cell Rep, 2015. **11**(2): p. 220-33.
349. Reef, S., E. Zalckvar, O. Shifman, S. Bialik, H. Sabanay, M. Oren, et al., *A short mitochondrial form of p19ARF induces autophagy and caspase-independent cell death.* Mol Cell, 2006. **22**(4): p. 463-75.
350. Gray, L.R., S.C. Tompkins, and E.B. Taylor, *Regulation of pyruvate metabolism and human disease.* Cell Mol Life Sci, 2014. **71**(14): p. 2577-604.

351. McCommis, K.S. and B.N. Finck, *Mitochondrial pyruvate transport: a historical perspective and future research directions.* Biochem J, 2015. **466**(3): p. 443-54.
352. Berkers, C.R., O.D. Maddocks, E.C. Cheung, I. Mor, and K.H. Vousden, *Metabolic regulation by p53 family members.* Cell Metab, 2013. **18**(5): p. 617-33.
353. Contractor, T. and C.R. Harris, *p53 negatively regulates transcription of the pyruvate dehydrogenase kinase Pdk2.* Cancer Res, 2012. **72**(2): p. 560-7.
354. Way, L., J. Faktor, P. Dvorakova, J. Nicholson, B. Vojtesek, D. Graham, et al., *Rearrangement of mitochondrial pyruvate dehydrogenase subunit dihydrolipoamide dehydrogenase protein-protein interactions by the MDM2 ligand nutlin-3.* Proteomics, 2016. **16**(17): p. 2327-44.
355. Li, X., K.K.Y. Cheng, Z. Liu, J.K. Yang, B. Wang, X. Jiang, et al., *The MDM2-p53-pyruvate carboxylase signalling axis couples mitochondrial metabolism to glucose-stimulated insulin secretion in pancreatic beta-cells.* Nat Commun, 2016. **7**: p. 11740.
356. Polyak, K., Y. Xia, J.L. Zweier, K.W. Kinzler, and B. Vogelstein, *A model for p53-induced apoptosis.* Nature, 1997. **389**(6648): p. 300-5.
357. Riscal, R., E. Schrepfer, G. Arena, M.Y. Cisse, F. Bellvert, M. Heuillet, et al., *Chromatin-Bound MDM2 Regulates Serine Metabolism and Redox Homeostasis Independently of p53.* Mol Cell, 2016. **62**(6): p. 890-902.
358. Riscal, R., L. Le Cam, and L.K. Linares, *Chromatin-bound MDM2, a new player in metabolism.* Mol Cell Oncol, 2016. **3**(5): p. e1210560.
359. Wei, C.L., Q. Wu, V.B. Vega, K.P. Chiu, P. Ng, T. Zhang, et al., *A global map of p53 transcription-factor binding sites in the human genome.* Cell, 2006. **124**(1): p. 207-19.
360. Chaneton, B., P. Hillmann, L. Zheng, A.C.L. Martin, O.D.K. Maddocks, A. Chokkathukalam, et al., *Serine is a natural ligand and allosteric activator of pyruvate kinase M2.* Nature, 2012. **491**(7424): p. 458-462.
361. Ou, Y., S.J. Wang, L. Jiang, B. Zheng, and W. Gu, *p53 Protein-mediated regulation of phosphoglycerate dehydrogenase (PHGDH) is crucial for the apoptotic response upon serine starvation.* J Biol Chem, 2015. **290**(1): p. 457-66.
362. Yang, M. and K.H. Vousden, *Serine and one-carbon metabolism in cancer.* Nat Rev Cancer, 2016. **16**(10): p. 650-62.
363. Cisse, M.Y., S. Pyrdziak, N. Firmin, L. Gayte, M. Heuillet, F. Bellvert, et al., *Targeting MDM2-dependent serine metabolism as a therapeutic strategy for liposarcoma.* Sci Transl Med, 2020. **12**(547).
364. Parrales, A. and T. Iwakuma, *p53 as a Regulator of Lipid Metabolism in Cancer.* Int J Mol Sci, 2016. **17**(12).
365. Goldstein, I. and V. Rotter, *Regulation of lipid metabolism by p53 - fighting two villains with one sword.* Trends Endocrinol Metab, 2012. **23**(11): p. 567-75.
366. Liu, Y., Y. He, A. Jin, A.P. Tikunov, L. Zhou, L.A. Tollini, et al., *Ribosomal protein-Mdm2-p53 pathway coordinates nutrient stress with lipid*

metabolism by regulating MCD and promoting fatty acid oxidation. Proc Natl Acad Sci U S A, 2014. **111**(23): p. E2414-22.

367. Goldstein, I., O. Ezra, N. Rivlin, A. Molchadsky, S. Madar, N. Goldfinger, et al., *p53, a novel regulator of lipid metabolism pathways.* J Hepatol, 2012. **56**(3): p. 656-62.
368. Li, T., N. Kon, L. Jiang, M. Tan, T. Ludwig, Y. Zhao, et al., *Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence.* Cell, 2012. **149**(6): p. 1269-83.
369. Kon, N., D. Wang, T. Li, L. Jiang, L. Qiang, and W. Gu, *Inhibition of Mdmx (Mdm4) in vivo induces anti-obesity effects.* Oncotarget, 2018. **9**(7): p. 7282-7297.
370. Hallenborg, P., S. Feddersen, S. Francoz, I. Murano, U. Sundekilde, R.K. Petersen, et al., *Mdm2 controls CREB-dependent transactivation and initiation of adipocyte differentiation.* Cell Death Differ, 2012. **19**(8): p. 1381-9.
371. Hu, T., A. Kitano, V. Luu, B. Dawson, K.A. Hoegenauer, B.H. Lee, et al., *Bmi1 Suppresses Adipogenesis in the Hematopoietic Stem Cell Niche.* Stem Cell Reports, 2019. **13**(3): p. 545-558.
372. Zhang, J. and X. Chen, *p53 tumor suppressor and iron homeostasis.* FEBS J, 2019. **286**(4): p. 620-629.
373. Weizer-Stern, O., K. Adamsky, O. Margalit, O. Ashur-Fabian, D. Givol, N. Amariglio, et al., *Hepcidin, a key regulator of iron metabolism, is transcriptionally activated by p53.* Br J Haematol, 2007. **138**(2): p. 253-62.
374. Funauchi, Y., C. Tanikawa, P.H. Yi Lo, J. Mori, Y. Daigo, A. Takano, et al., *Regulation of iron homeostasis by the p53-ISCU pathway.* Sci Rep, 2015. **5**: p. 16497.
375. Hwang, P.M., F. Bunz, J. Yu, C. Rago, T.A. Chan, M.P. Murphy, et al., *Ferredoxin reductase affects p53-dependent, 5-fluorouracil-induced apoptosis in colorectal cancer cells.* Nat Med, 2001. **7**(10): p. 1111-7.
376. Shimizu, R., N.N. Lan, T.T. Tai, Y. Adachi, A. Kawazoe, A. Mu, et al., *p53 directly regulates the transcription of the human frataxin gene and its lack of regulation in tumor cells decreases the utilization of mitochondrial iron.* Gene, 2014. **551**(1): p. 79-85.
377. Liu, G. and X. Chen, *The ferredoxin reductase gene is regulated by the p53 family and sensitizes cells to oxidative stress-induced apoptosis.* Oncogene, 2002. **21**(47): p. 7195-204.
378. Shen, J., X. Sheng, Z. Chang, Q. Wu, S. Wang, Z. Xuan, et al., *Iron metabolism regulates p53 signaling through direct heme-p53 interaction and modulation of p53 localization, stability, and function.* Cell Rep, 2014. **7**(1): p. 180-93.
379. Lee, J.H., H. Jang, E.J. Cho, and H.D. Youn, *Ferritin binds and activates p53 under oxidative stress.* Biochem Biophys Res Commun, 2009. **389**(3): p. 399-404.
380. Dongiovanni, P., A.L. Fracanzani, G. Cairo, C.P. Megazzini, S. Gatti, R. Rametta, et al., *Iron-dependent regulation of MDM2 influences p53 activity and hepatic carcinogenesis.* Am J Pathol, 2010. **176**(2): p. 1006-17.

381. Gnanapradeepan, K., S. Basu, T. Barnoud, A. Budina-Kolomets, C.P. Kung, and M.E. Murphy, *The p53 Tumor Suppressor in the Control of Metabolism and Ferroptosis.* Front Endocrinol (Lausanne), 2018. **9**: p. 124.
382. Kang, R., G. Kroemer, and D. Tang, *The tumor suppressor protein p53 and the ferroptosis network.* Free Radic Biol Med, 2019. **133**: p. 162-168.
383. Venkatesh, D., N.A. O'Brien, F. Zandkarimi, D.R. Tong, M.E. Stokes, D.E. Dunn, et al., *MDM2 and MDMX promote ferroptosis by PPARalpha-mediated lipid remodeling.* Genes Dev, 2020. **34**(7-8): p. 526-543.
384. Kaymak, I., C.R. Maier, W. Schmitz, A.D. Campbell, B. Dankworth, C.P. Ade, et al., *Mevalonate Pathway Provides Ubiquinone to Maintain Pyrimidine Synthesis and Survival in p53-Deficient Cancer Cells Exposed to Metabolic Stress.* Cancer Res, 2020. **80**(2): p. 189-203.
385. Sermeus, A. and C. Michiels, *Reciprocal influence of the p53 and the hypoxic pathways.* Cell Death Dis, 2011. **2**: p. e164.
386. An, W.G., M. Kanekal, M.C. Simon, E. Maltepe, M.V. Blagosklonny, and L.M. Neckers, *Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha.* Nature, 1998. **392**(6674): p. 405-8.
387. Chen, D., M. Li, J. Luo, and W. Gu, *Direct interactions between HIF-1 alpha and Mdm2 modulate p53 function.* J Biol Chem, 2003. **278**(16): p. 13595-8.
388. Zhu, Y., X.O. Mao, Y. Sun, Z. Xia, and D.A. Greenberg, *p38 Mitogen-activated protein kinase mediates hypoxic regulation of Mdm2 and p53 in neurons.* J Biol Chem, 2002. **277**(25): p. 22909-14.
389. Lai, Y., M. Song, K. Hakala, S.T. Weintraub, and Y. Shiio, *Proteomic dissection of the von Hippel-Lindau (VHL) interactome.* J Proteome Res, 2011. **10**(11): p. 5175-82.
390. Zhao, R.Z., S. Jiang, L. Zhang, and Z.B. Yu, *Mitochondrial electron transport chain, ROS generation and uncoupling (Review).* Int J Mol Med, 2019. **44**(1): p. 3-15.
391. Deisenroth, C. and Y. Zhang, *The Ribosomal Protein-Mdm2-p53 Pathway and Energy Metabolism: Bridging the Gap between Feast and Famine.* Genes Cancer, 2011. **2**(4): p. 392-403.
392. Paliwal, S., R.C. Kovi, B. Nath, Y.W. Chen, B.C. Lewis, and S.R. Grossman, *The alternative reading frame tumor suppressor antagonizes hypoxia-induced cancer cell migration via interaction with the COOH-terminal binding protein corepressor.* Cancer Res, 2007. **67**(19): p. 9322-9.
393. Lee, J.H., Y. Jin, G. He, S.X. Zeng, Y.V. Wang, G.M. Wahl, et al., *Hypoxia activates tumor suppressor p53 by inducing ATR-Chk1 kinase cascade-mediated phosphorylation and consequent 14-3-3gamma inactivation of MDMX protein.* J Biol Chem, 2012. **287**(25): p. 20898-903.
394. White, M.C., D.M. Holman, J.E. Boehm, L.A. Peipins, M. Grossman, and S.J. Henley, *Age and cancer risk: a potentially modifiable relationship.* Am J Prev Med, 2014. **46**(3 Suppl 1): p. S7-15.
395. Pearson, B.J. and A. Sanchez Alvarado, *A planarian p53 homolog regulates proliferation and self-renewal in adult stem cell lineages.* Development, 2010. **137**(2): p. 213-21.

396. Dejosez, M., H. Ura, V.L. Brandt, and T.P. Zwaka, *Safeguards for cell cooperation in mouse embryogenesis shown by genome-wide cheater screen.* Science, 2013. **341**(6153): p. 1511-4.
397. Merkle, F.T., S. Ghosh, N. Kamitaki, J. Mitchell, Y. Avior, C. Mello, et al., *Human pluripotent stem cells recurrently acquire and expand dominant negative P53 mutations.* Nature, 2017. **545**(7653): p. 229-233.
398. Jackson-Grusby, L., C. Beard, R. Possemato, M. Tudor, D. Fambrough, G. Csankovszki, et al., *Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation.* Nat Genet, 2001. **27**(1): p. 31-9.
399. Feng, Z., C. Zhang, H.J. Kang, Y. Sun, H. Wang, A. Naqvi, et al., *Regulation of female reproduction by p53 and its family members.* FASEB J, 2011. **25**(7): p. 2245-55.
400. Danilova, N., K.M. Sakamoto, and S. Lin, *p53 family in development.* Mech Dev, 2008. **125**(11-12): p. 919-31.
401. Li, H., S. Wang, Y. Chen, K. Lu, B. Yin, S. Li, et al., *Identification of two p53 isoforms from Litopenaeus vannamei and their interaction with NF-kappaB to induce distinct immune response.* Sci Rep, 2017. **7**: p. 45821.
402. Lane, D.P., A. Madhumalar, A.P. Lee, B.H. Tay, C. Verma, S. Brenner, et al., *Conservation of all three p53 family members and Mdm2 and Mdm4 in the cartilaginous fish.* Cell Cycle, 2011. **10**(24): p. 4272-9.
403. Horvath, M.M., X. Wang, M.A. Resnick, and D.A. Bell, *Divergent evolution of human p53 binding sites: cell cycle versus apoptosis.* PLoS Genet, 2007. **3**(7): p. e127.
404. Abegglen, L.M., A.F. Caulin, A. Chan, K. Lee, R. Robinson, M.S. Campbell, et al., *Potential Mechanisms for Cancer Resistance in Elephants and Comparative Cellular Response to DNA Damage in Humans.* JAMA, 2015. **314**(17): p. 1850-60.
405. Tosoni, D., S. Zecchini, M. Coazzoli, I. Colaluca, G. Mazzarol, A. Rubio, et al., *The Numb/p53 circuitry couples replicative self-renewal and tumor suppression in mammary epithelial cells.* J Cell Biol, 2015. **211**(4): p. 845-62.
406. Friedmann-Morvinski, D., E.A. Bushong, E. Ke, Y. Soda, T. Marumoto, O. Singer, et al., *Dedifferentiation of neurons and astrocytes by oncogenes can induce gliomas in mice.* Science, 2012. **338**(6110): p. 1080-4.
407. Tschaharganeh, D.F., W. Xue, D.F. Calvisi, M. Evert, T.V. Michurina, L.E. Dow, et al., *p53-dependent Nestin regulation links tumor suppression to cellular plasticity in liver cancer.* Cell, 2014. **158**(3): p. 579-92.
408. Menendez, S., S. Camus, and J.C. Izpisua Belmonte, *p53: guardian of reprogramming.* Cell Cycle, 2010. **9**(19): p. 3887-91.
409. Charni, M., R. Aloni-Grinstein, A. Molchadsky, and V. Rotter, *p53 on the crossroad between regeneration and cancer.* Cell Death Differ, 2017. **24**(1): p. 8-14.
410. Ritschka, B., M. Storer, A. Mas, F. Heinzmann, M.C. Ortells, J.P. Morton, et al., *The senescence-associated secretory phenotype induces cellular plasticity and tissue regeneration.* Genes Dev, 2017. **31**(2): p. 172-183.

411. Yun, M.H., P.B. Gates, and J.P. Brockes, *Regulation of p53 is critical for vertebrate limb regeneration.* Proc Natl Acad Sci U S A, 2013. **110**(43): p. 17392-7.
412. Spike, B.T. and G.M. Wahl, *p53, Stem Cells, and Reprogramming: Tumor Suppression beyond Guarding the Genome.* Genes Cancer, 2011. **2**(4): p. 404-19.
413. Mizuno, H., B.T. Spike, G.M. Wahl, and A.J. Levine, *Inactivation of p53 in breast cancers correlates with stem cell transcriptional signatures.* Proc Natl Acad Sci U S A, 2010. **107**(52): p. 22745-50.
414. Steensma, D.P., R. Bejar, S. Jaiswal, R.C. Lindsley, M.A. Sekeres, R.P. Hasserjian, et al., *Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes.* Blood, 2015. **126**(1): p. 9-16.
415. Lujambio, A., L. Akkari, J. Simon, D. Grace, D.F. Tschaharganeh, J.E. Bolden, et al., *Non-cell-autonomous tumor suppression by p53.* Cell, 2013. **153**(2): p. 449-60.
416. Laberge, R.M., P. Awad, J. Campisi, and P.Y. Desprez, *Epithelial-mesenchymal transition induced by senescent fibroblasts.* Cancer Microenviron, 2012. **5**(1): p. 39-44.
417. Baugh, E.H., H. Ke, A.J. Levine, R.A. Bonneau, and C.S. Chan, *Why are there hotspot mutations in the TP53 gene in human cancers?* Cell Death Differ, 2018. **25**(1): p. 154-160.
418. Levine, A.J., *The many faces of p53: something for everyone.* J Mol Cell Biol, 2019. **11**(7): p. 524-530.
419. Shirole, N.H., D. Pal, E.R. Kastenhuber, S. Senturk, J. Boroda, P. Pisterzi, et al., *TP53 exon-6 truncating mutations produce separation of function isoforms with pro-tumorigenic functions.* Elife, 2016. **5**.
420. Liu, Y., C. Chen, Z. Xu, C. Scuoppo, C.D. Rillahan, J. Gao, et al., *Deletions linked to TP53 loss drive cancer through p53-independent mechanisms.* Nature, 2016. **531**(7595): p. 471-475.
421. Watanabe, H., K. Shimokado, T. Asahara, K. Dohi, and O. Niwa, *Analysis of the c-myc, K-ras and p53 genes in methylcholanthrene-induced mouse sarcomas.* Jpn J Cancer Res, 1999. **90**(1): p. 40-7.
422. Grzes, M., M. Oron, Z. Staszczak, A. Jaiswar, M. Nowak-Niezgoda, and D. Walerych, *A Driver Never Works Alone-Interplay Networks of Mutant p53, MYC, RAS, and Other Universal Oncogenic Drivers in Human Cancer.* Cancers (Basel), 2020. **12**(6).
423. Oren, M. and V. Rotter, *Mutant p53 gain-of-function in cancer.* Cold Spring Harb Perspect Biol, 2010. **2**(2): p. a001107.
424. Aschauer, L. and P.A. Muller, *Novel targets and interaction partners of mutant p53 Gain-Of-Function.* Biochem Soc Trans, 2016. **44**(2): p. 460-6.
425. Olive, K.P., D.A. Tuveson, Z.C. Ruhe, B. Yin, N.A. Willis, R.T. Bronson, et al., *Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome.* Cell, 2004. **119**(6): p. 847-60.
426. Freed-Pastor, W.A. and C. Prives, *Mutant p53: one name, many proteins.* Genes Dev, 2012. **26**(12): p. 1268-86.
427. Muller, P.A. and K.H. Vousden, *Mutant p53 in cancer: new functions and therapeutic opportunities.* Cancer Cell, 2014. **25**(3): p. 304-17.

428. Mantovani, F., L. Collavin, and G. Del Sal, *Mutant p53 as a guardian of the cancer cell.* Cell Death Differ, 2019. **26**(2): p. 199-212.
429. Zhu, J., M.A. Sammons, G. Donahue, Z. Dou, M. Vedadi, M. Getlik, et al., *Gain-of-function p53 mutants co-opt chromatin pathways to drive cancer growth.* Nature, 2015. **525**(7568): p. 206-11.
430. Weissmueller, S., E. Manchado, M. Saborowski, J.P.t. Morris, E. Wagenblast, C.A. Davis, et al., *Mutant p53 drives pancreatic cancer metastasis through cell-autonomous PDGF receptor beta signaling.* Cell, 2014. **157**(2): p. 382-394.
431. Pfister, N.T., V. Fomin, K. Regunath, J.Y. Zhou, W. Zhou, L. Silwal-Pandit, et al., *Mutant p53 cooperates with the SWI/SNF chromatin remodeling complex to regulate VEGFR2 in breast cancer cells.* Genes Dev, 2015. **29**(12): p. 1298-315.
432. Senturk, S., Z. Yao, M. Camiolo, B. Stiles, T. Rathod, A.M. Walsh, et al., *p53Psi is a transcriptionally inactive p53 isoform able to reprogram cells toward a metastatic-like state.* Proc Natl Acad Sci U S A, 2014. **111**(32): p. E3287-96.
433. Candeias, M.M., M. Hagiwara, and M. Matsuda, *Cancer-specific mutations in p53 induce the translation of Delta160p53 promoting tumorigenesis.* EMBO Rep, 2016. **17**(11): p. 1542-1551.
434. Tschaharganeh, D.F., B. Bosbach, and S.W. Lowe, *Coordinated Tumor Suppression by Chromosome 8p.* Cancer Cell, 2016. **29**(5): p. 617-619.
435. Brandtzaeg, P., *The increasing power of immunohistochemistry and immunocytochemistry.* J Immunol Methods, 1998. **216**(1-2): p. 49-67.
436. Duraiyan, J., R. Govindarajan, K. Kaliyappan, and M. Palanisamy, *Applications of immunohistochemistry.* J Pharm Bioallied Sci, 2012. **4**(Suppl 2): p. S307-9.
437. Matos, L.L., D.C. Trufelli, M.G. de Matos, and M.A. da Silva Pinhal, *Immunohistochemistry as an important tool in biomarkers detection and clinical practice.* Biomark Insights, 2010. **5**: p. 9-20.
438. Hofman, F., *Immunohistochemistry.* Curr Protoc Immunol, 2002. **Chapter 21**: p. Unit 21 4.
439. Marrack, J.R., *Derived Antigens as a Means of Studying the relation of Specific Combination to Chemical Structure: (Section of Therapeutics and Pharmacology).* Proc R Soc Med, 1934. **27**(8): p. 1063-5.
440. Towbin, H., T. Staehelin, and J. Gordon, *Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.* Proc Natl Acad Sci U S A, 1979. **76**(9): p. 4350-4.
441. Haines, D.M. and K.H. West, *Immunohistochemistry: forging the links between immunology and pathology.* Vet Immunol Immunopathol, 2005. **108**(1-2): p. 151-6.
442. Sternberger, L.A., P.H. Hardy, Jr., J.J. Cuculis, and H.G. Meyer, *The unlabeled antibody enzyme method of immunohistochemistry: preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes.* J Histochem Cytochem, 1970. **18**(5): p. 315-33.

443. Mason, D.Y. and R. Sammons, *Alkaline phosphatase and peroxidase for double immunoenzymatic labelling of cellular constituents.* J Clin Pathol, 1978. **31**(5): p. 454-60.
444. Singer, S.J., *Preparation of an electron-dense antibody conjugate.* Nature, 1959. **183**(4674): p. 1523-4.
445. Sternberger, L.A., *Electron microscopic immunocytochemistry: a review.* J Histochem Cytochem, 1967. **15**(3): p. 139-59.
446. Faulk, W.P. and G.M. Taylor, *An immunocolloid method for the electron microscope.* Immunochemistry, 1971. **8**(11): p. 1081-3.
447. Heitz, P.U., *Report on workshop 1 "immunocytochemistry--techniques, problems and possible applications".* Acta Histochem Suppl, 1982. **25**: p. 45-6.
448. Huang, S.N., H. Minassian, and J.D. More, *Application of immunofluorescent staining on paraffin sections improved by trypsin digestion.* Lab Invest, 1976. **35**(4): p. 383-90.
449. Hsu, S.M. and L. Raine, *Protein A, avidin, and biotin in immunohistochemistry.* J Histochem Cytochem, 1981. **29**(11): p. 1349-53.
450. Hsu, S.M., L. Raine, and H. Fanger, *The use of antiavidin antibody and avidin-biotin-peroxidase complex in immunoperoxidase technics.* Am J Clin Pathol, 1981. **75**(6): p. 816-21.
451. Bodey, B., *The significance of immunohistochemistry in the diagnosis and therapy of neoplasms.* Expert Opin Biol Ther, 2002. **2**(4): p. 371-93.
452. Schrohl, A.S., M. Holten-Andersen, F. Sweep, M. Schmitt, N. Harbeck, J. Foekens, et al., *Tumor markers: from laboratory to clinical utility.* Mol Cell Proteomics, 2003. **2**(6): p. 378-87.
453. Im, K., S. Mareninov, M.F.P. Diaz, and W.H. Yong, *An Introduction to Performing Immunofluorescence Staining.* Methods Mol Biol, 2019. **1897**: p. 299-311.
454. Morimoto, J., M. Sarkar, S. Kenrick, and T. Kodadek, *Dextran as a generally applicable multivalent scaffold for improving immunoglobulin-binding affinities of peptide and peptidomimetic ligands.* Bioconjug Chem, 2014. **25**(8): p. 1479-91.
455. Ivell, R., K. Teerds, and G.E. Hoffman, *Proper application of antibodies for immunohistochemical detection: antibody crimes and how to prevent them.* Endocrinology, 2014. **155**(3): p. 676-87.
456. Lipman, N.S., L.R. Jackson, L.J. Trudel, and F. Weis-Garcia, *Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources.* ILAR J, 2005. **46**(3): p. 258-68.
457. Schroeder, H.W., Jr. and L. Cavacini, *Structure and function of immunoglobulins.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S41-52.
458. Chiu, M.L., D.R. Goulet, A. Teplyakov, and G.L. Gilliland, *Antibody Structure and Function: The Basis for Engineering Therapeutics.* Antibodies (Basel), 2019. **8**(4).
459. Pradidarcheep, W., W.T. Labruyere, N.F. Dabhoiwala, and W.H. Lamers, *Lack of specificity of commercially available antisera: better specifications needed.* J Histochem Cytochem, 2008. **56**(12): p. 1099-111.

460. Barwick, B.G., V.A. Gupta, P.M. Vertino, and L.H. Boise, *Cell of Origin and Genetic Alterations in the Pathogenesis of Multiple Myeloma.* Front Immunol, 2019. **10**: p. 1121.
461. Milstein, C. and A.C. Cuello, *Hybrid hybridomas and their use in immunohistochemistry.* Nature, 1983. **305**(5934): p. 537-40.
462. Parray, H.A., S. Shukla, S. Samal, T. Shrivastava, S. Ahmed, C. Sharma, et al., *Hybridoma technology a versatile method for isolation of monoclonal antibodies, its applicability across species, limitations, advancement and future perspectives.* Int Immunopharmacol, 2020. **85**: p. 106639.
463. Tate, J. and G. Ward, *Interferences in immunoassay.* Clin Biochem Rev, 2004. **25**(2): p. 105-20.
464. Laboratory, Q.s., *Principles of Immunohistochemistry Queen's Laboratory For Molecular Pathology*, L. Boudreau, Editor. 2011. p. 12.
465. Heyderman, E., *Immunoperoxidase technique in histopathology: applications, methods, and controls.* J Clin Pathol, 1979. **32**(10): p. 971-8.
466. Ramos-Vara, J.A., *Technical aspects of immunohistochemistry.* Vet Pathol, 2005. **42**(4): p. 405-26.
467. Janardhan, K.S., H. Jensen, N.P. Clayton, and R.A. Herbert, *Immunohistochemistry in Investigative and Toxicologic Pathology.* Toxicol Pathol, 2018. **46**(5): p. 488-510.
468. Chen, X., D.B. Cho, and P.C. Yang, *Double staining immunohistochemistry.* N Am J Med Sci, 2010. **2**(5): p. 241-5.
469. Bratthauer, G.L., *The peroxidase-antiperoxidase (PAP) method and other all-immunologic detection methods.* Methods Mol Biol, 2010. **588**: p. 243-55.
470. Jain, A. and K. Cheng, *The principles and applications of avidin-based nanoparticles in drug delivery and diagnosis.* J Control Release, 2017. **245**: p. 27-40.
471. Helppolainen, S.H., K.P. Nurminen, J.A. Maatta, K.K. Halling, J.P. Slotte, T. Huhtala, et al., *Rhizavidin from Rhizobium etli: the first natural dimer in the avidin protein family.* Biochem J, 2007. **405**(3): p. 397-405.
472. Wilchek, M. and E.A. Bayer, *Introduction to avidin-biotin technology.* Methods Enzymol, 1990. **184**: p. 5-13.
473. Korpela, J., *Avidin, a high affinity biotin-binding protein, as a tool and subject of biological research.* Med Biol, 1984. **62**(1): p. 5-26.
474. Bayer, E.A., E. Skutelsky, and M. Wilchek, *The avidin-biotin complex in affinity cytochemistry.* Methods Enzymol, 1979. **62**: p. 308-15.
475. Waldrop, G.L., H.M. Holden, and M. St Maurice, *The enzymes of biotin dependent CO(2) metabolism: what structures reveal about their reaction mechanisms.* Protein Sci, 2012. **21**(11): p. 1597-619.
476. Millar, T.J., R. Knighton, and J.A. Chuck, *The use of biotin to demonstrate immunohistochemistry, Western blotting, and dot blots in university practical classes.* Biochem Mol Biol Educ, 2012. **40**(4): p. 246-53.
477. Gonzalez, M., C.E. Argarana, and G.D. Fidelio, *Extremely high thermal stability of streptavidin and avidin upon biotin binding.* Biomol Eng, 1999. **16**(1-4): p. 67-72.

478. Rybak, J.N., S.B. Scheurer, D. Neri, and G. Elia, *Purification of biotinylated proteins on streptavidin resin: a protocol for quantitative elution.* Proteomics, 2004. **4**(8): p. 2296-9.
479. Elia, G., *Biotinylation reagents for the study of cell surface proteins.* Proteomics, 2008. **8**(19): p. 4012-24.
480. Bratthauer, G.L., *The avidin-biotin complex (ABC) method and other avidin-biotin binding methods.* Methods Mol Biol, 2010. **588**: p. 257-70.
481. Haugland, R.P. and W.W. You, *Coupling of antibodies with biotin.* Methods Mol Biol, 2008. **418**: p. 13-24.
482. Kurzban, G.P., E.A. Bayer, M. Wilchek, and P.M. Horowitz, *The quaternary structure of streptavidin in urea.* J Biol Chem, 1991. **266**(22): p. 14470-7.
483. Krenacs, T., L. Krenacs, and M. Raffeld, *Multiple antigen immunostaining procedures.* Methods Mol Biol, 2010. **588**: p. 281-300.
484. Kanehira, K., J. Hu, T. Pier, L. Sebree, and W. Huang, *High endogenous avidin binding activity: an inexpensive and readily available marker for the differential diagnosis of kidney neoplasms.* Int J Clin Exp Pathol, 2008. **1**(5): p. 435-9.
485. Tsutsumi, Y., A. Serizawa, and K. Kawai, *Enhanced polymer one-step staining (EPOS) for proliferating cell nuclear antigen (PCNA) and Ki-67 antigen: application to intra-operative frozen diagnosis.* Pathol Int, 1995. **45**(2): p. 108-15.
486. Kammerer, U., M. Kapp, A.M. Gassel, T. Richter, C. Tank, J. Dietl, et al., *A new rapid immunohistochemical staining technique using the EnVision antibody complex.* J Histochem Cytochem, 2001. **49**(5): p. 623-30.
487. Wolff, A.C., M.E. Hammond, J.N. Schwartz, K.L. Hagerty, D.C. Allred, R.J. Cote, et al., *American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer.* Arch Pathol Lab Med, 2007. **131**(1): p. 18-43.
488. Skoog, L. and E. Tani, *Immunocytochemistry: an indispensable technique in routine cytology.* Cytopathology, 2011. **22**(4): p. 215-29.
489. Pinheiro, C., R. Roque, A. Adriano, P. Mendes, M. Praca, I. Reis, et al., *Optimization of immunocytochemistry in cytology: comparison of two protocols for fixation and preservation on cytospin and smear preparations.* Cytopathology, 2015. **26**(1): p. 38-43.
490. Sauter, J.L., A.B. Ambaye, and S.L. Mount, *Increased utilization, verification, and clinical implications of immunocytochemistry: Experience in a northern New England hospital.* Diagn Cytopathol, 2015. **43**(9): p. 688-95.
491. Srebotnik Kirbis, I., R. Rodrigues Roque, M. Bongiovanni, M. Strojan Flezar, and B. Cochand-Priollet, *Immunocytochemistry practices in European cytopathology laboratories-Review of European Federation of Cytology Societies (EFCS) online survey results with best practice recommendations.* Cancer Cytopathol, 2020. **128**(10): p. 757-766.
492. Mize, R.R., *Quantitative image analysis for immunocytochemistry and in situ hybridization.* J Neurosci Methods, 1994. **54**(2): p. 219-37.

493. Hurley, A.A., K.L. Douglass, and D.J. Zahniser, *Improved technology for cytology specimen preparation.* Am Clin Lab, 1991. **10**(3): p. 20-2.
494. Stoler, M.H., *Advances in cervical screening technology.* Mod Pathol, 2000. **13**(3): p. 275-84.
495. Cox, J.T., *Liquid-based cytology: evaluation of effectiveness, cost-effectiveness, and application to present practice.* J Natl Compr Canc Netw, 2004. **2**(6): p. 597-611.
496. Olms, C., N. Hix, H. Neumann, M. Yahiaoui-Doktor, and T.W. Remmerbach, *Clinical comparison of liquid-based and conventional cytology of oral brush biopsies: a randomized controlled trial.* Head Face Med, 2018. **14**(1): p. 9.
497. Klinkhamer, P.J., W.J. Meerding, P.F. Rosier, and A.G. Hanselaar, *Liquid-based cervical cytology.* Cancer, 2003. **99**(5): p. 263-71.
498. Chen, X., B. Zheng, and H. Liu, *Optical and digital microscopic imaging techniques and applications in pathology.* Anal Cell Pathol (Amst), 2011. **34**(1-2): p. 5-18.
499. Hartig, S.M., *Basic image analysis and manipulation in ImageJ.* Curr Protoc Mol Biol, 2013. **Chapter 14**: p. Unit14 15.
500. Tseleni, S., N. Kavantzas, D. Yova, E. Alexandratou, M. Ioannou-Lambrouli, H. Paraskevakou, et al., *Findings of computerized nuclear morphometry of papillary thyroid carcinoma in correlation with the age of the patients.* Gen Diagn Pathol, 1997. **143**(1): p. 23-7.
501. Lejeune, M., J. Jaen, L. Pons, C. Lopez, M.T. Salvado, R. Bosch, et al., *Quantification of diverse subcellular immunohistochemical markers with clinicobiological relevancies: validation of a new computer-assisted image analysis procedure.* J Anat, 2008. **212**(6): p. 868-78.
502. Stack, E.C., C. Wang, K.A. Roman, and C.C. Hoyt, *Multiplexed immunohistochemistry, imaging, and quantitation: a review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis.* Methods, 2014. **70**(1): p. 46-58.
503. Fuqua, T., J. Jordan, A. Halavatyi, C. Tischer, K. Richter, and J. Crocker, *An open-source semi-automated robotics pipeline for embryo immunohistochemistry.* Sci Rep, 2021. **11**(1): p. 10314.
504. Herrmann, M.D., D.A. Clunie, A. Fedorov, S.W. Doyle, S. Pieper, V. Klepeis, et al., *Implementing the DICOM Standard for Digital Pathology.* J Pathol Inform, 2018. **9**: p. 37.
505. Goto, M., Y. Nagatomo, K. Hasui, H. Yamanaka, S. Murashima, and E. Sato, *Chromaticity analysis of immunostained tumor specimens.* Pathol Res Pract, 1992. **188**(4-5): p. 433-7.
506. Matkowskyj, K.A., D. Schonfeld, and R.V. Benya, *Quantitative immunohistochemistry by measuring cumulative signal strength using commercially available software photoshop and matlab.* J Histochem Cytochem, 2000. **48**(2): p. 303-12.
507. Matos, L.L., E. Stabenow, M.R. Tavares, A.R. Ferraz, V.L. Capelozzi, and M.A. Pinhal, *Immunohistochemistry quantification by a digital computer-assisted method compared to semiquantitative analysis.* Clinics (Sao Paulo), 2006. **61**(5): p. 417-24.

508. Zhou, W.P., Y.F. Zhu, B. Zhang, W.Y. Qiu, and Y.F. Yao, *The role of ultraviolet radiation in the pathogenesis of pterygia (Review).* Mol Med Rep, 2016. **14**(1): p. 3-15.
509. Jee, D., E.C. Kim, E. Cho, and J.G. Arroyo, *Positive Association between Blood 25-Hydroxyvitamin D Levels and Pterygium after Control for Sunlight Exposure.* PLoS One, 2016. **11**(6): p. e0157501.
510. Pajic, B., I. Vastardis, P. Rajkovic, B. Pajic-Eggspuehler, D.M. Aebersold, and Z. Cvejic, *A mathematical approach to human pterygium shape.* Clin Ophthalmol, 2016. **10**: p. 1343-9.
511. Shirzadeh, E., M. Najafi, M. Nazarzadeh, G. Fazli, F. Falanji, L.S. Aldaghi, et al., *Expression of Pluripotency Markers, SOX2 and OCT4, in Pterygium Development.* Crit Rev Eukaryot Gene Expr, 2018. **28**(2): p. 155-162.
512. Tan, D.T., W.Y. Tang, Y.P. Liu, H.S. Goh, and D.R. Smith, *Apoptosis and apoptosis related gene expression in normal conjunctiva and pterygium.* Br J Ophthalmol, 2000. **84**(2): p. 212-6.
513. Kurt, A., R. Kilic, M. Tad, and O.A. Polat, *YKL-40 expression in pterygium: a potential role in the pathogenesis.* Int Ophthalmol, 2019. **39**(7): p. 1445-1450.
514. Liu, T., Y. Liu, L. Xie, X. He, and J. Bai, *Progress in the pathogenesis of pterygium.* Curr Eye Res, 2013. **38**(12): p. 1191-7.
515. Witmer, A.N., G.F. Vrensen, C.J. Van Noorden, and R.O. Schlingemann, *Vascular endothelial growth factors and angiogenesis in eye disease.* Prog Retin Eye Res, 2003. **22**(1): p. 1-29.
516. Gumus, K., S. Karakucuk, G.E. Mirza, H. Akgun, H. Arda, and A.O. Oner, *Overexpression of vascular endothelial growth factor receptor 2 in pterygia may have a predictive value for a higher postoperative recurrence rate.* Br J Ophthalmol, 2014. **98**(6): p. 796-800.
517. Ling, S., Q. Li, H. Lin, W. Li, T. Wang, H. Ye, et al., *Comparative evaluation of lymphatic vessels in primary versus recurrent pterygium.* Eye (Lond), 2012. **26**(11): p. 1451-8.
518. Dong, Y., S. Kase, Z. Dong, J. Fukuhara, Y. Tagawa, E.T. Ishizuka, et al., *Regulation of vascular endothelial growth factor-C by tumor necrosis factor-alpha in the conjunctiva and pterygium.* Int J Mol Med, 2016. **38**(2): p. 545-50.
519. Zhong, M.S., W. Fu, Q. Zhang, YA., *Quantitative detection of the expression level of transforming growth factor-beta and its receptors in pterygium with RT-PCR.* Int. J. Ophthalmol, 2009. **2**(4): p. 4.
520. Solomon, A., M. Grueterich, D.Q. Li, D. Meller, S.B. Lee, and S.C. Tseng, *Overexpression of Insulin-like growth factor-binding protein-2 in pterygium body fibroblasts.* Invest Ophthalmol Vis Sci, 2003. **44**(2): p. 573-80.
521. Wong, Y.W., J. Chew, H. Yang, D.T. Tan, and R. Beuerman, *Expression of insulin-like growth factor binding protein-3 in pterygium tissue.* Br J Ophthalmol, 2006. **90**(6): p. 769-72.
522. Yu, H. and T. Rohan, *Role of the insulin-like growth factor family in cancer development and progression.* J Natl Cancer Inst, 2000. **92**(18): p. 1472-89.

523. van Setten GB, A.M., Blalock TD, Grotendorst G, Schultz GS., *Detection of connective tissue growth factor (CTGF) in pterygium*, in *ARVO annual meeting* 2003.
524. Tong, L., *Expression of p27(KIP1) and cyclin D1, and cell proliferation in human pterygium.* Br J Ophthalmol, 2008. **92**(1): p. 157.
525. Scholzen, T. and J. Gerdes, *The Ki-67 protein: from the known and the unknown.* J Cell Physiol, 2000. **182**(3): p. 311-22.
526. Ljubojevic, V., R. Gajanin, L. Amidzic, and Z. Vujkovic, *The expression and significance of p53 protein and Ki-67 protein in pterygium.* Vojnosanit Pregl, 2016. **73**(1): p. 16-20.
527. Dieckman, L.M., B.D. Freudenthal, and M.T. Washington, *PCNA structure and function: insights from structures of PCNA complexes and post-translationally modified PCNA.* Subcell Biochem, 2012. **62**: p. 281-99.
528. Liang, K., Z. Jiang, B.Q. Ding, P. Cheng, D.K. Huang, and L.M. Tao, *Expression of cell proliferation and apoptosis biomarkers in pterygia and normal conjunctiva.* Mol Vis, 2011. **17**: p. 1687-93.
529. Yang, K., M. Hitomi, and D.W. Stacey, *Variations in cyclin D1 levels through the cell cycle determine the proliferative fate of a cell.* Cell Div, 2006. **1**: p. 32.
530. Karadag, R., N. Bayram, S. Oguztuzun, B. Bozer, H. Bayramlar, G.G. Simsek, et al., *Investigation of Glutathione S-Transferase Isoenzyme Protein Expression in Patients With Pterygium.* Cornea, 2016. **35**(8): p. 1089-92.
531. Laborde, E., *Glutathione transferases as mediators of signaling pathways involved in cell proliferation and cell death.* Cell Death Differ, 2010. **17**(9): p. 1373-80.
532. Reed, J.C., *Proapoptotic multidomain Bcl-2/Bax-family proteins: mechanisms, physiological roles, and therapeutic opportunities.* Cell Death Differ, 2006. **13**(8): p. 1378-86.
533. Dushku, N. and T.W. Reid, *P53 expression in altered limbal basal cells of pingueculae, pterygia, and limbal tumors.* Curr Eye Res, 1997. **16**(12): p. 1179-92.
534. Tamm, I., Y. Wang, E. Sausville, D.A. Scudiero, N. Vigna, T. Oltersdorf, et al., *IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs.* Cancer Res, 1998. **58**(23): p. 5315-20.
535. Guha, M. and D.C. Altieri, *Survivin as a global target of intrinsic tumor suppression networks.* Cell Cycle, 2009. **8**(17): p. 2708-10.
536. Ambrosini, G., C. Adida, and D.C. Altieri, *A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma.* Nat Med, 1997. **3**(8): p. 917-21.
537. Altieri, D.C., *The molecular basis and potential role of survivin in cancer diagnosis and therapy.* Trends Mol Med, 2001. **7**(12): p. 542-7.
538. Cheung, C.H., C.C. Huang, F.Y. Tsai, J.Y. Lee, S.M. Cheng, Y.C. Chang, et al., *Survivin - biology and potential as a therapeutic target in oncology.* Onco Targets Ther, 2013. **6**: p. 1453-62.

539. Eckelman, B.P., G.S. Salvesen, and F.L. Scott, *Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family.* EMBO Rep, 2006. **7**(10): p. 988-94.
540. Johnson, M.E. and E.W. Howerth, *Survivin: a bifunctional inhibitor of apoptosis protein.* Vet Pathol, 2004. **41**(6): p. 599-607.
541. Wheatley, S.P. and D.C. Altieri, *Survivin at a glance.* J Cell Sci, 2019. **132**(7).
542. Uren, A.G., L. Wong, M. Pakusch, K.J. Fowler, F.J. Burrows, D.L. Vaux, et al., *Survivin and the inner centromere protein INCENP show similar cell-cycle localization and gene knockout phenotype.* Curr Biol, 2000. **10**(21): p. 1319-28.
543. Fukuda, S., J. Hoggatt, P. Singh, M. Abe, J.M. Speth, P. Hu, et al., *Survivin modulates genes with divergent molecular functions and regulates proliferation of hematopoietic stem cells through Evi-1.* Leukemia, 2015. **29**(2): p. 433-40.
544. Okada, H., C. Bakal, A. Shahinian, A. Elia, A. Wakeham, W.K. Suh, et al., *Survivin loss in thymocytes triggers p53-mediated growth arrest and p53-independent cell death.* J Exp Med, 2004. **199**(3): p. 399-410.
545. Leung, C.G., Y. Xu, B. Mularski, H. Liu, S. Gurbuxani, and J.D. Crispino, *Requirements for survivin in terminal differentiation of erythroid cells and maintenance of hematopoietic stem and progenitor cells.* J Exp Med, 2007. **204**(7): p. 1603-11.
546. Miletic, A.V., J. Jellusova, M.H. Cato, C.R. Lee, G.V. Baracho, E.M. Conway, et al., *Essential Role for Survivin in the Proliferative Expansion of Progenitor and Mature B Cells.* J Immunol, 2016. **196**(5): p. 2195-204.
547. Rajagopalan, S. and M.K. Balasubramanian, *Schizosaccharomyces pombe Bir1p, a nuclear protein that localizes to kinetochores and the spindle midzone, is essential for chromosome condensation and spindle elongation during mitosis.* Genetics, 2002. **160**(2): p. 445-56.
548. Velculescu, V.E., S.L. Madden, L. Zhang, A.E. Lash, J. Yu, C. Rago, et al., *Analysis of human transcriptomes.* Nat Genet, 1999. **23**(4): p. 387-8.
549. Dang, C.V., E.P. Reddy, K.M. Shokat, and L. Soucek, *Drugging the 'undruggable' cancer targets.* Nat Rev Cancer, 2017. **17**(8): p. 502-508.
550. Mita, A.C., M.M. Mita, S.T. Nawrocki, and F.J. Giles, *Survivin: key regulator of mitosis and apoptosis and novel target for cancer therapeutics.* Clin Cancer Res, 2008. **14**(16): p. 5000-5.
551. Martinez-Outschoorn, U.E., M.P. Lisanti, and F. Sotgia, *Catabolic cancer-associated fibroblasts transfer energy and biomass to anabolic cancer cells, fueling tumor growth.* Semin Cancer Biol, 2014. **25**: p. 47-60.
552. Thies, K.A., J.E. Lefler, G. Leone, and M.C. Ostrowski, *PTEN in the Stroma.* Cold Spring Harb Perspect Med, 2019. **9**(10).
553. Liu, L., L. Huang, J. He, S. Cai, Y. Weng, S. Huang, et al., *PTEN inhibits non-small cell lung cancer cell growth by promoting G0/G1 arrest and cell apoptosis.* Oncol Lett, 2019. **17**(1): p. 1333-1340.
554. Li, B., J. Zhang, Y. Su, Y. Hou, Z. Wang, L. Zhao, et al., *Overexpression of PTEN may increase the effect of pemetrexed on A549 cells via inhibition of the PI3K/AKT/mTOR pathway and carbohydrate metabolism.* Mol Med Rep, 2019. **20**(4): p. 3793-3801.

555. Wu, H., K. Wang, W. Liu, and Q. Hao, *PTEN overexpression improves cisplatin-resistance of human ovarian cancer cells through upregulating KRT10 expression.* Biochem Biophys Res Commun, 2014. **444**(2): p. 141-6.
556. Li, M.F., H. Guan, and D.D. Zhang, *Effect of overexpression of PTEN on apoptosis of liver cancer cells.* Genet Mol Res, 2016. **15**(2).
557. Bao, L., X. Li, and Z. Lin, *PTEN overexpression promotes glioblastoma death through triggering mitochondrial division and inactivating the Akt pathway.* J Recept Signal Transduct Res, 2019. **39**(3): p. 215-225.
558. de Araujo, W.M., B.K. Robbs, L.G. Bastos, W.F. de Souza, F.C. Vidal, J.P. Viola, et al., *PTEN Overexpression Cooperates With Lithium to Reduce the Malignancy and to Increase Cell Death by Apoptosis via PI3K/Akt Suppression in Colorectal Cancer Cells.* J Cell Biochem, 2016. **117**(2): p. 458-69.
559. Wang, X. and X. Jiang, *PTEN: a default gate-keeping tumor suppressor with a versatile tail.* Cell Res, 2008. **18**(8): p. 807-16.
560. Hagerling, C., A.J. Casbon, and Z. Werb, *Balancing the innate immune system in tumor development.* Trends Cell Biol, 2015. **25**(4): p. 214-20.
561. Jang, Y.J., J.H. Kim, and S. Byun, *Modulation of Autophagy for Controlling Immunity.* Cells, 2019. **8**(2).
562. Monkkonen, T. and J. Debnath, *Inflammatory signaling cascades and autophagy in cancer.* Autophagy, 2018. **14**(2): p. 190-198.
563. Shibutani, S.T., T. Saitoh, H. Nowag, C. Munz, and T. Yoshimori, *Autophagy and autophagy-related proteins in the immune system.* Nat Immunol, 2015. **16**(10): p. 1014-24.
564. Pan, H., L. Chen, Y. Xu, W. Han, F. Lou, W. Fei, et al., *Autophagy-associated immune responses and cancer immunotherapy.* Oncotarget, 2016. **7**(16): p. 21235-46.
565. Linzer, D.I. and A.J. Levine, *Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells.* Cell, 1979. **17**(1): p. 43-52.
566. Nakayama, M. and M. Oshima, *Mutant p53 in colon cancer.* J Mol Cell Biol, 2019. **11**(4): p. 267-276.
567. Valente, L.J., D.H. Gray, E.M. Michalak, J. Pinon-Hofbauer, A. Egle, C.L. Scott, et al., *p53 efficiently suppresses tumor development in the complete absence of its cell-cycle inhibitory and proapoptotic effectors p21, Puma, and Noxa.* Cell Rep, 2013. **3**(5): p. 1339-45.
568. Harris, H., O.J. Miller, G. Klein, P. Worst, and T. Tachibana, *Suppression of malignancy by cell fusion.* Nature, 1969. **223**(5204): p. 363-8.
569. Donehower, L.A., M. Harvey, B.L. Slagle, M.J. McArthur, C.A. Montgomery, Jr., J.S. Butel, et al., *Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours.* Nature, 1992. **356**(6366): p. 215-21.
570. Rashid, O.M., A.D. Cassano, and K. Takabe, *Thymic neoplasm: a rare disease with a complex clinical presentation.* J Thorac Dis, 2013. **5**(2): p. 173-83.
571. Boutelle, A.M. and L.D. Attardi, *p53 and Tumor Suppression: It Takes a Network.* Trends Cell Biol, 2021. **31**(4): p. 298-310.

572. Avery-Kiejda, K.A., N.A. Bowden, A.J. Croft, L.L. Scurr, C.F. Kairupan, K.A. Ashton, et al., *P53 in human melanoma fails to regulate target genes associated with apoptosis and the cell cycle and may contribute to proliferation.* BMC Cancer, 2011. **11**: p. 203.
573. Gartel, A.L. and A.L. Tyner, *The role of the cyclin-dependent kinase inhibitor p21 in apoptosis.* Mol Cancer Ther, 2002. **1**(8): p. 639-49.
574. Deng, C., P. Zhang, J.W. Harper, S.J. Elledge, and P. Leder, *Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control.* Cell, 1995. **82**(4): p. 675-84.
575. Sullivan, K.D., C.L. Gallant-Behm, R.E. Henry, J.L. Fraikin, and J.M. Espinosa, *The p53 circuit board.* Biochim Biophys Acta, 2012. **1825**(2): p. 229-44.
576. Liu, G., J.M. Parant, G. Lang, P. Chau, A. Chavez-Reyes, A.K. El-Naggar, et al., *Chromosome stability, in the absence of apoptosis, is critical for suppression of tumorigenesis in Trp53 mutant mice.* Nat Genet, 2004. **36**(1): p. 63-8.
577. Jiang, L., N. Kon, T. Li, S.J. Wang, T. Su, H. Hibshoosh, et al., *Ferroptosis as a p53-mediated activity during tumour suppression.* Nature, 2015. **520**(7545): p. 57-62.
578. Brady, C.A., D. Jiang, S.S. Mello, T.M. Johnson, L.A. Jarvis, M.M. Kozak, et al., *Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression.* Cell, 2011. **145**(4): p. 571-83.
579. Ventura, A., D.G. Kirsch, M.E. McLaughlin, D.A. Tuveson, J. Grimm, L. Lintault, et al., *Restoration of p53 function leads to tumour regression in vivo.* Nature, 2007. **445**(7128): p. 661-5.
580. Martins, C.P., L. Brown-Swigart, and G.I. Evan, *Modeling the therapeutic efficacy of p53 restoration in tumors.* Cell, 2006. **127**(7): p. 1323-34.
581. Messina, R.L., M. Sanfilippo, V. Vella, G. Pandini, P. Vigneri, M.L. Nicolosi, et al., *Reactivation of p53 mutants by prima-1 [corrected] in thyroid cancer cells.* Int J Cancer, 2012. **130**(10): p. 2259-70.
582. Athar, M., C.A. Elmets, and L. Kopelovich, *Pharmacological activation of p53 in cancer cells.* Curr Pharm Des, 2011. **17**(6): p. 631-9.
583. Mrakovcic, M. and L.F. Frohlich, *p53-Mediated Molecular Control of Autophagy in Tumor Cells.* Biomolecules, 2018. **8**(2).
584. White, E., *Autophagy and p53.* Cold Spring Harb Perspect Med, 2016. **6**(4): p. a026120.
585. Jung, C.H., S.H. Ro, J. Cao, N.M. Otto, and D.H. Kim, *mTOR regulation of autophagy.* FEBS Lett, 2010. **584**(7): p. 1287-95.
586. Kenzelmann Broz, D., S. Spano Mello, K.T. Bieging, D. Jiang, R.L. Dusek, C.A. Brady, et al., *Global genomic profiling reveals an extensive p53-regulated autophagy program contributing to key p53 responses.* Genes Dev, 2013. **27**(9): p. 1016-31.
587. Bang, S., S. Kaur, and M. Kurokawa, *Regulation of the p53 Family Proteins by the Ubiquitin Proteasomal Pathway.* Int J Mol Sci, 2019. **21**(1).
588. Todoric, J., L. Antonucci, G. Di Caro, N. Li, X. Wu, N.K. Lytle, et al., *Stress-Activated NRF2-MDM2 Cascade Controls Neoplastic Progression in Pancreas.* Cancer Cell, 2017. **32**(6): p. 824-839 e8.

589. Hatchi, E., G. Rodier, C. Sardet, and L. Le Cam, *E4F1 dysfunction results in autophagic cell death in myeloid leukemic cells.* Autophagy, 2011. **7**(12): p. 1566-7.
590. Duan, L., R.E. Perez, X. Lai, L. Chen, and C.G. Maki, *The histone demethylase JMJD2B is critical for p53-mediated autophagy and survival in Nutlin-treated cancer cells.* J Biol Chem, 2019. **294**(23): p. 9186-9197.
591. Garrison, S.P., J.R. Jeffers, C. Yang, J.A. Nilsson, M.A. Hall, J.E. Rehg, et al., *Selection against PUMA gene expression in Myc-driven B-cell lymphomagenesis.* Mol Cell Biol, 2008. **28**(17): p. 5391-402.
592. Gudkov, A.V. and E.A. Komarova, *Pathologies associated with the p53 response.* Cold Spring Harb Perspect Biol, 2010. **2**(7): p. a001180.
593. Ceccaldi, R., K. Parmar, E. Mouly, M. Delord, J.M. Kim, M. Regairaz, et al., *Bone marrow failure in Fanconi anemia is triggered by an exacerbated p53/p21 DNA damage response that impairs hematopoietic stem and progenitor cells.* Cell Stem Cell, 2012. **11**(1): p. 36-49.
594. Checler, F. and C. Alves da Costa, *p53 in neurodegenerative diseases and brain cancers.* Pharmacol Ther, 2014. **142**(1): p. 99-113.
595. Turan, M. and G. Turan, *Bcl-2, p53, and Ki-67 expression in pterygium and normal conjunctiva and their relationship with pterygium recurrence.* Eur J Ophthalmol, 2020. **30**(6): p. 1232-1237.
596. Ueda, Y., S. Kanazawa, T. Kitaoka, Y. Dake, A. Ohira, A.M. Ouertani, et al., *Immunohistochemical study of p53, p21 and PCNA in pterygium.* Acta Histochem, 2001. **103**(2): p. 159-65.
597. Kayat, K.V., P.E. Correa Dantas, S. Felberg, M.A. Galvao, and M.A. Saieg, *Exfoliative Cytology in the Diagnosis of Ocular Surface Squamous Neoplasms.* Cornea, 2017. **36**(1): p. 127-130.
598. Paronetto, M.P., I. Passacantilli, and C. Sette, *Alternative splicing and cell survival: from tissue homeostasis to disease.* Cell Death Differ, 2016. **23**(12): p. 1919-1929.
599. Miyashita, T. and J.C. Reed, *Tumor suppressor p53 is a direct transcriptional activator of the human bax gene.* Cell, 1995. **80**(2): p. 293-9.
600. Xu, Y.X., L.Y. Zhang, D.L. Zou, Z.S. Liu, X.M. Shang, H.P. Wu, et al., *Differential expression and function of survivin during the progress of pterygium.* Invest Ophthalmol Vis Sci, 2014. **55**(12): p. 8480-7.
601. Nubile, M., C. Curcio, M. Lanzini, R. Calienno, M. Iezzi, A. Mastropasqua, et al., *Expression of CREB in primary pterygium and correlation with cyclin D1, ki-67, MMP7, p53, p63, Survivin and Vimentin.* Ophthalmic Res, 2013. **50**(2): p. 99-107.
602. Cantrup, R., R. Dixit, E. Palmesino, S. Bonfield, T. Shaker, N. Tachibana, et al., *Cell-type specific roles for PTEN in establishing a functional retinal architecture.* PLoS One, 2012. **7**(3): p. e32795.
603. Cao, L., E.O. Graue-Hernandez, V. Tran, B. Reid, J. Pu, M.J. Mannis, et al., *Downregulation of PTEN at corneal wound sites accelerates wound healing through increased cell migration.* Invest Ophthalmol Vis Sci, 2011. **52**(5): p. 2272-8.

604. Huang, H., C.J. Potter, W. Tao, D.M. Li, W. Brogiolo, E. Hafen, et al., *PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye development.* Development, 1999. **126**(23): p. 5365-72.
605. Guha, M., J. Plescia, I. Leav, J. Li, L.R. Languino, and D.C. Altieri, *Endogenous tumor suppression mediated by PTEN involves survivin gene silencing.* Cancer Res, 2009. **69**(12): p. 4954-8.
606. Li, X., Y. Dai, and J. Xu, *MiR-21 promotes pterygium cell proliferation through the PTEN/AKT pathway.* Mol Vis, 2018. **24**: p. 485-494.
607. Stein, Y., V. Rotter, and R. Aloni-Grinstein, *Gain-of-Function Mutant p53: All the Roads Lead to Tumorigenesis.* Int J Mol Sci, 2019. **20**(24).
608. Amer, R., L. Rabinowich, G. Maftsir, I. Puxeddu, F. Levi-Schaffer, and A. Solomon, *Mycophenolic acid suppresses human pterygium and normal tenon fibroblast proliferation in vitro.* Br J Ophthalmol, 2010. **94**(10): p. 1373-7.
609. Larrayoz, I.M., A. de Luis, O. Rua, S. Velilla, J. Cabello, and A. Martinez, *Molecular effects of doxycycline treatment on pterygium as revealed by massive transcriptome sequencing.* PLoS One, 2012. **7**(6): p. e39359.
610. Tanaka, M., C.J. Rosser, and H.B. Grossman, *PTEN gene therapy induces growth inhibition and increases efficacy of chemotherapy in prostate cancer.* Cancer Detect Prev, 2005. **29**(2): p. 170-4.
611. Duffy, M.J., N.C. Synnott, S. O'Grady, and J. Crown, *Targeting p53 for the treatment of cancer.* Semin Cancer Biol, 2020.
612. Yu, J., J. Feng, Y. Jie, T. Jin, Z.Q. Pan, S.Y. Li, et al., *[Preliminary observation on the treatment of primary pterygium with modified conjunctival transplantation and amniotic membrane transplantation combined with use of interferon eye drops].* Zhonghua Yan Ke Za Zhi, 2020. **56**(10): p. 768-773.
613. Young, A.L., D. Cao, W.K. Chu, T.K. Ng, Y.W.Y. Yip, V. Jhanji, et al., *The Evolving Story of Pterygium.* Cornea, 2018. **37 Suppl 1**: p. S55-S57.