An innovative epigenetic strategy for retinoblastoma treatment
Every day, in every way,
I’m getting better and better

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An innovative epigenetic strategy for retinoblastoma treatment

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1 INTRODUCTION

1.1 RETINOBLASTOMA

1.1.1 Clinical aspects

1.1.1.1 Introduction, origin and route of spread. Retinoblastoma (Rb) is an aggressive and potentially fatal tumor of the eye, rapidly developed from the immature cells of retina, which is the specialized light-sensitive inner layer of nerve tissue at the back of the eye that detects light and color. The eye is essentially an opaque eyeball filled with a water-like fluid. Its structure can be divided into two main parts: the anterior segment, that occupies approximately one-third of the volume, includes cornea, conjunctiva, aqueous humor, iris, ciliary body and lens; whereas the back of the eye or posterior segment is the remaining portion and involves sclera, choroid, retinal pigment epithelium, neural retina, optic nerve and vitreous humor[1]. During the early development in the womb, the eye cells called retinoblasts divide into new cells until they fill the retina. Normally at this point, cells stop dividing and develop into mature retinal cells. Rarely however, instead of maturing into special cells that detect light, some retinoblasts continue dividing and grow out of control, forming a cancer known as retinoblastoma. Rb is a relatively rare cancer, although represents the most frequent ocular malignancy of childhood, with different incidences around the world[2]. It can occur in heritable and non-heritable (sporadic) forms and its fundamental diversity in the pathogenesis represents the basic distinction between two main different clinical retinoblastoma phenotypes[3].

Unilateral retinoblastoma occurs if only one eye is affected and represents the great majority of the cases. The term "unilateral" roughly translates to "one side". The tumor is usually unifocal and retinoblasts are involved. In many unilateral cases, cancer mass is large and it is not possible to determine if a single tumor is present. Some individuals have instead multifocal tumors in one eye (unilateral multifocal Rb). In most patients with unilateral retinoblastoma without a family history, inactivation of both alleles of the Retinoblastoma suppressor gene (RB1) that initiated tumor development is a somatic event, and none of them is present in DNA from constitutional cells[4].
Unilateral tumors are therefore mostly sporadic and non-hereditary, however about 10-15% of all occurs in patients who have inherited or de novo germline mutations\textsuperscript{[5]}. 

\textbf{Bilateral retinoblastoma} occurs if both eyes are affected and represents about 35% of the cases\textsuperscript{[6]}. The term "bilateral" equates to "two sides". In many children with this tumor, both eyes are affected at the time of initial diagnosis and they usually carry an inherited mutation from either affected or unaffected parents\textsuperscript{[5]}. Almost all children with bilateral eye involvement have the hereditary form of retinoblastoma, in which the aberrant alteration might be inherited via the germ cells from affected parents. Other small percentage of patients with bilateral Rb has instead sporadic disease with no familial transmission, that arises through de novo mutation of \textit{RB1} gene either in the germ line (from unaffected parents) or embryo\textsuperscript{[7]}. In individuals with bilateral Rb both eyes may show multiple tumors with different sizes\textsuperscript{[8]}. Bilaterality usually happens at the same time, although some children, who are initially diagnosed with unilateral Rb, may develop a tumor in the contralateral unaffected eye few months or even years later and patients could remain at an increased risk of subsequent cancers.

Unilateral cases are more common than bilateral cases in a ratio of 2.7:1. Diagnosed patients' age correlated significantly with laterality; young onset is associated with bilateral, while increasing age with unilateral cases. Bilateral retinoblastoma in early and advanced stages of intraocular diseases also showed significant presence at a younger onset\textsuperscript{[9]}.

Retinoblastoma arises from primitive unidentified retinal stem cells or cone precursor cells in nucleated layers of the eye. Rb can spread through three ways in the body: the tumor can extend from where it began by growing into nearby areas in tissue, or by getting into the lymph system or into the blood, and then travels through the lymph or the blood vessels, respectively, to other parts of the body. Its growth is usually under the retina and the tumor can locally spread either posteriorly, seeding the subretinal space, or anteriorly, toward the vitreous and aqueous\textsuperscript{[10]} (\textit{Fig.1.1}). Involvement of the ocular coats and optic nerve occurs as a sequence of events as the tumor progresses. Invasion of the choroid is common, although occurrence of massive invasion is usually limited to advanced disease. Following invasion of the choroid, the tumor gains access to systemic
circulation via central retinal vessels and creates the potential for distant metastases, such as skull, bones and lymph nodes. Further progression through the ocular coats leads to invasion of the sclera and the orbital soft tissue. The route of spread through the optic nerve and past the lamina cribrosa may lead the subarachnoid space invasion and it increases the risk of systemic and central nervous system (CNS) dissemination\textsuperscript{[11]}. Anteriorly, Rb can spread to conjunctiva, eyelids and extraocular tissue and the diffusion of tumor seeding in the area of the vitreous base and anterior chamber may gain access to lymphatic dissemination and to systemic circulation through the canal of Schlemm\textsuperscript{[12]}.

\textbf{Fig. 1.1:} Healthy eye (left) vs intraocular retinoblastoma (right)\textsuperscript{[2]}.

\begin{enumerate}
\item \textbf{1.1.1.2 Historical perspectives.} The first description of a tumor resembling retinoblastoma was provided on 1597 by Pieter Pawius, who described a malignancy invading the orbit, the temporal region, and the cranium, filled with a "substance similar to brain tissue mixed with thick blood and like crushed stone"\textsuperscript{[13-14]}. After these observations, only in 1767 other cases bearing true resemblance to Rb were found in the literature and Hayes described the tumor like a soft cancer, publishing a description of a neuroepiblastic tumor of the retina\textsuperscript{[14-15]}. In 1805, William Hey coined the term \textit{fungus haematodes} to describe a fungating mass affecting the eye and destroying its internal organization. However, Hey thought that this tumor might affect other regions of the body, including the limbs and breast\textsuperscript{[16]}. In 1809, James Wardrop, a Scottish surgeon, provided the first description of retinoblastoma as a clinical entity. He stated that the tumor arose from the retina, based on gross morphologic studies. Wardrop documented extension of the tumor to the optic nerve and brain and later he described metastasis of this tumor to different parts of the body. He also concluded that because the ocular form of this disease so often involved children, it must be distinguished from the general classification of \textit{fungus haematodes} or soft cancer. Furthermore, he was the first to suggest that enucleation should be the primary mode of management for
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retinoblastoma\textsuperscript{[17]}. In 1836, Langenbech, Robin and Nystin confirmed by microscopic studies that the tumor definitely arose from retina\textsuperscript{[18]}. Hermann Von Helmholtz invented the ophthalmoscope in 1851 and facilitated the recognition of clinical features of Rb, with an earlier diagnosis before extraocular extension occurred. In 1864, Virchow described the tumor as glioma retinae, supporting glial cell origin within the retina\textsuperscript{[19]}. Both Flexner and Wintersteiner, in 1891 and 1897 respectively, histologically described the rods and cones of the classical rosettes found in many retinoblastomas and they indicated the tumor as a neuroephitelioma, suggesting the photoreceptor cell-layer origin\textsuperscript{[20-21]}. In 1922, Verhoeff believed that the tumor arose from retinoblasts and proposed the name "retinoblastoma", the term that was officially adopted by the American Ophthalmological Society in 1926 as a general term of this entity\textsuperscript{[22]}. In 1956, Steward found in literature fifteen cases of spontaneous remission of retinoblastomas, in which the evidence both for the diagnosis and for the regression appeared satisfactory\textsuperscript{[23]}. In 1970, Ts'o and colleagues established that retinoblastoma arises by way of photoreceptor differentiation, the most advanced degree of retinal differentiation\textsuperscript{[24-25]}. Most notably, in 1971, Alfred Knudson, by reviewing a series of clinical cases, formulated what is now known as his "two-hit" hypothesis, a mechanism that explains the subsequent genetic events that are believed to give rise to tumorigenesis in retinoblastoma. For the first time, he hypothesized that Rb may be determined by the loss (or inactivation) of both copies of a single gene. This view opened the field of investigation for the study of genetic and/or epigenetic pathogenesis in Rb and several other cancers.

Most interestingly, some authors reported the occurrence of spontaneously regressed retinoblastomas that seemed to undergo a new malignant transformation\textsuperscript{[26]}. In 1982, Gallie and coworkers suggested that non-progressive retinal lesions observed in patients known to carry genetic impairments, and previously thought to represent all examples of spontaneous regression, were indeed benign manifestations of the disease and they proposed to call them as retinoma\textsuperscript{[27]}. One year later, Margo reported analogous benign retinal tumor composed entirely of photoreceptor differentiation, which was called retinocytoma, before assuming lesions were the same. These benign variants are highly differentiated tumors with basically no growth potential\textsuperscript{[28]}. In 1984, Kyritsis described Rb as a cancer stemming from the overproduction of immature retinal cells resulting in a heterogeneous tumor cell population\textsuperscript{[29]}. More recently, both Gallie and Dimaras suggested that retinoma/retinocytoma is a precursor of retinoblastoma, a pre-cancerous lesion, and there are well-documented cases
of malignant transformation of benign lesions into Rb\textsuperscript{[30]}.

1.1.1.3 Epidemiology. Retinoblastoma is a relatively rare cancer, although it accounts for about 3\% of all pediatric malignancies from birth to 14 years old\textsuperscript{[31]}, with the average age at diagnosis at 18 months\textsuperscript{[32]} and with 90\% diagnosed before five years of age. Affected patients will develop Rb before they are born or very early in life because it originates from cells that differentiate. Only few cases have been reported in adults\textsuperscript{[32]}, when Rb is thought to arise from previously existing retinoma/retinocytoma. Rb is the second most common solid pediatric tumor after brain/nervous system tumors and lymphomas in several countries\textsuperscript{[33]}.

Prior to the 20\textsuperscript{th} century, retinoblastoma was a uniformly fatal disease. If left untreated, the mortality rate of retinoblastoma is still about 99\%\textsuperscript{[34]}. The major risk of fatality factor is whether the tumor is confined to the eye. If retinoblastoma spreads beyond the orbit or reaches the cut end of the optic nerve, the mortality rate increases markedly. Fatalities from retinoblastoma are also increased in trilateral cases\textsuperscript{[35]}, second malignant neoplasms such as sarcomas and especially in systemic metastasis\textsuperscript{[36]}.

In the early 1900s, the positive prognosis for children with retinoblastoma was a mere 5\%\textsuperscript{[37]}. Major advances, such as the understanding of the tumor molecular biology and the development of targeted therapy, have dramatically improved the survival rates of patients with retinoblastoma over the last three decades and today it is higher than 90\% in industrialized countries\textsuperscript{[38]}. However, late presentation and delayed diagnosis are still a drawback to positive prognosis in the developing world, resulting in lower survival rates\textsuperscript{[39]}. In fact, only approximately 50\% of affected children survive worldwide\textsuperscript{[40]}. This evident discrepancy is largely due to efficient earlier detection in medically developed nations when the tumor is confined to the eye, whereas in underdeveloped regions retinoblastoma is often noticed after it has invaded the orbit or brain. The high mortality is thus associated with late presentation and poor facilities for detection and treatments\textsuperscript{[41]}.

Retinoblastoma has a worldwide incidence of one case per 15-20k live births, which corresponds to about 9000 new cases every year. The disorder has no validated geographic hotspots, both males and females are equally affected\textsuperscript{[42]} and there is no predilection for any race\textsuperscript{[43]}. However, a higher incidence is noted in developing countries and this has been implicated to lower socio-economic status\textsuperscript{[7]}. Rb has not been related to smoking, alcohol assumption or any maternal problem during pregnancy. Nevertheless, it has been shown that a poor diet and infectious diseases are presently
considered risk factors for Rb development in less affluent populations throughout the world, demonstrating the crucial role of environmental factors in the tumorigenesis of retinoblastoma\textsuperscript{[44]}. Moreover, a low folate intake may play a role in the risk of occurrence\textsuperscript{[45]}. Retinoblastoma can develop in a healthy child despite a normal or premature birth\textsuperscript{[46]}. The overall incidence of childhood cancer has been slowly increasing since 1975\textsuperscript{[47]} and a rise in number of Rb cases also been detected, partly due to the environmental pollution\textsuperscript{[48]} and the exposure to chemical and physical agents\textsuperscript{[3]}. Survival and the chance of saving vision depend on severity of disease at presentation and the mortality from retinoblastoma is about 70\% in countries of low and middle income, where most of the affected children live\textsuperscript{[49]}. However, the overall incidence of retinoblastoma decreases with advancing age. About 70-80\% of patients have only one impaired eye, with a mean age of diagnosis of 24 months, while the remaining 20-30\% of affected children with multifocal retinoblastoma that affects both eyes is presented at mean age 15 months\textsuperscript{[50]}. Finally, the diffuse anterior retinoblastoma represents a rare variant of retinoblastoma and affected patients are older than those with the classical types, with the mean age being 6.1 years\textsuperscript{[12]}.  

\textbf{1.1.1.4 Clinical presentation}. The clinical presentation of retinoblastoma varies according to several aspects, like its presence within or spread beyond the affected eye, the cell morphology and the type of tumor growth and duration, degree of vascularization and the presence of calcifications, vitreous seeding, retinal detachment or hemorrhage. Doctors divide the \textit{extended tumors} into two main groups, according to the fact if the cancer is still within or it has spread beyond the eye. This distinction is made for practical purposes mostly to decide on the best treatment options. 

\textbf{Intraocular retinoblastoma} occurs in one or both eyes and may be confined to the retina or may also be found in other parts of the eye such as the choroid, ciliary body, or part of the optic nerve, but it has not distributed into surrounding tissues around the outside the eye. 

\textbf{Extraocular retinoblastoma} has instead spread to tissues beyond the eye or to other parts of the body: orbital retinoblastoma may be found in tissues around the eye; however, extraocular Rb may have spread to the CNS (brain and spinal cord) or to the liver, bones,
bone marrow, or lymph nodes. When retinoblastoma remains confined to the eye, it has one of the best survival rates of all the childhood cancers, but once the spread outside the globe, the prognosis is dismal\cite{51}. The clinical scenario of a patient being treated for intraocular disease, where survival is not in significant jeopardy, is completely different from the cases with metastatic disease for which there is a life-threatening occurrence\cite{52}.

Retinoblastoma cells are small and when stained with hematoxylin and eosin (H&E) staining they acquire a characteristic color blue. In fact, tumor is composed of small basophilic cells (retinoblasts) with large hyperchromatin nuclei and scanty cytoplasm. In many retinoblastoma specimens from patients, most of the cells appear undifferentiated; however, a sort of differentiated feature could be characterized by the formation of rosettes: round assemblage of cells consisting in a spoke circle, a halo collection surrounding a central or acellular lumen. Most of the rosettes are found in tumors of the nervous system and their detection help in the diagnosis of different cancers\cite{53}. Different rosette formations such as Flexner-Wintersteiner (FW) rosettes, Homer Wright (HW) rosettes and pseudorosettes have been described in retinoblastoma\cite{54}.

FW rosette is a feature of retinoblastoma and represents early attempt at retinal differentiation. These rosettes consist of a ring of cuboidal tumor cells neighboring the central empty lumen, which corresponds to subretinal space and it stains with alcian blue containing cytoplasmic extensions from the tumor cells. The cells surrounding the lumen are joined near the apices by intracellular connections (zonulae adherents), which is analogous to the external limiting membrane of retina. FW rosettes are not pathognomonic because they also occur in malignant medulloepitheliomas and some pineal tumors. On electron microscopy, they resemble primordial photoreceptor cells.

HW rosettes are typically seen in neuroblastoma, medulloblastoma, primitive neuro-ectodermal tumors and retinoblastoma. They lack a central lumen and their constituent cells encompass a central tangle of neural filaments. Their presence indicates neuroblastic differentiation\cite{55}.

Both these rosettes are characteristic but not sufficient to make a diagnosis of retinoblastoma and they are more common in eyes removed from younger patients.

Pseudorosettes, a ring of cells with an eosinophilic fibrillary center, are also commonly present in retinoblastomas.

About 15-20% of retinoblastomas harbor very well differentiated foci of actual photoreceptor differentiation that might be derived from retinoma precursor lesions. Such areas contain aggregates of neoplastic photoreceptors called fleurettes, which have
undergone photoreceptor differentiation. They are typically found in retinoblastomas on low magnification microscopy of H&E stained sections and are paucicellular compared to adjacent undifferentiated areas of viable tumors that appear relatively eosinophilic\(^5\). Recent studies reported of a new type of rosettes in retinoblastoma, consisting of clear lumens and collections of basophilic cells inside. This type of new rosette is larger than the FW or HW ones\(^6\). It needs to be stressed that histological assays can only be performed after the eye has been surgical removed at late stage of cancer development and after the therapies have begun. This investigation lays fundamentals for the following therapeutic steps as certain macroscopic and microscopic features contribute to determine tumor stage that leads to the prognosis and future therapeutic outcomes\(^7\).

Three retinal types are distinguished after the morphological examination of the retina, adjacent to the main tumor. Retina type 1 contains a single tumor that is sharply demarcated from surrounding normal tissue and it correlated significantly with early surgical removal of the whole eye both in hereditary and non-hereditary cases. In retina type 2, large parts of the retina are affected and the main tumor mass gradually blended with the adjacent pathological retina. The progressing tumor may release growth factors in the intraocular space that stimulate the cells of the adjacent retina and lead to multiple new primary tumors in the adjacent retinal area. Retina type 3 is characterized by a retina almost entirely affected by diffuse tumor growth and it is only present in non-hereditary cases with late surgical removal of the whole eye (at 2-5 years)\(^8\).

The degree of tumor differentiation is inversely proportional to the age in months when the eye is removed and this relationship is statistically significant. On average, retinoblastomas taken from older children tend to be poorly differentiated\(^9\).

The initial *growth pattern* and tumor cell divisions may occur in different internal retinal layers; a tumor developing on the retina surface and into the vitreous cavity has an endophytic growth, while a tumor developing from the external layers, invading the sub-retinal space and causing a retinal detachment presents an exophytic growth pattern.

**Endophytic retinoblastoma** is characterized by a tumor, which bursts through the internal limiting membrane and appears as a white-to-cream mass. It presents as one or more isolated or coalesced tumors of variable size, round or oval-shaped, yellowish-white (calcifications) or pinkish (vascularization) in color with its own, often turgescent and tortuous, vascular network. Endophytic Rb tends to increase inward into tissues in finger-
like projections from a superficial site of origin and it arises from inner layers of retinal surface toward the vitreous as a friable mass, frequently associated with fine blood vessels on its surface. Vitreous seeding also may be commonly present in endophytic Rb and anteriorly it reaches aqueous venous channels and it may permeate through lymphatic channels.

**Exophytic retinoblastoma** develops from outer layers of retina towards the choroid and is frequently associated with progressive retinal detachment, that masks to a greater or lesser degree the details of the underlying mass or masses and it is caused by subretinal fluid accumulation. It spreads outward beyond the surface epithelium from which it originates and it may become a multi-lobulated tumor. There is the presence of vitreous hemorrhage and a choroidal invasion through Bruch's membrane\[59\].

The most common growth pattern is mixed between endophytic and exophytic growths\[34\]. Both forms are distinctively characterized by vitreous and subretinal seeding and by the common presence of calcifications, in the form of white patches within the tumor mass. Necrosis is also very frequent and occurs when the tumor outgrows its vascular supply. Necrotic cells appear pink on H&E staining.

Rarely, the tumor may develop in an insidious manner within the retinal layers and subretinal space, with no elevated mass and no calcifications, growing slowly along the retinal axis towards the anterior segment where late manifestations of the disease occur in the form of pseudoinflammatory complications\[59\]. This is the diffuse infiltrating or plaque-like form retinoblastoma, first suggested by Norman Ashton in 1958 to describe the unusual nature of a flat neoplasm that seldom formed tumor masses in retina\[60\]. It is characterized by a relatively flat infiltration of the retina by tumor cells without an obvious mass. The diffuse anterior retinoblastoma presents itself as a grayish retinal coating with no elevated mass and no calcifications\[61\].

**1.1.1.5 Signs and symptoms.** Retinoblastomas nearly always occur in young children. They are often found when a parent or doctor notices a child's eye looks unusual, with typical signs and symptoms. In industrial countries, patients mainly exhibit signs rather than symptoms, presenting intraocular tumors without local extension, meanwhile in developing countries the diagnosis is frequently done only after an enlarged eye or gross orbital extension occurs.
Leukocoria, a white or light pink abnormal reflection from the retina, also known as white pupillary reflex or "amaurotic cat's eye", is the most common (60%) initial sign of retinoblastoma\cite{1}, occurring when the tumor is still contained within the vitreous and it is associated with the disease in almost half of all infants presenting with a white pupil\cite{2}. The term leukocoria literally means "white pupil" from the Greek "leucos" (white) and "korê" (pupil) and is created by the reflection of incident light off the retinal lesion within the pupillary area when the fundus is directly illuminated\cite{3}. This life-threatening white tumor then reflects light and blocks view of the red retina. Leukocoria is occasionally first noticed by close family members or after a flash photograph is taken\cite{4}.

Strabismus, also known as lazy eye, is the second most frequent manifestation, occurring in about 20% of retinoblastoma cases and it is due to loss of central vision following retinal detachment, poor visual tracking, vitreous hemorrhage, glaucoma or optic nerve involvement singly or in combination. Both exotropia (eye turned outward, temporal) or esotropia (eye turned inward, nasal) can be presenting signs for Rb\cite{5}. Strabismus usually correlates with macular involvement and it is an invaluable early sign carrying an excellent life prognosis and every chance of preserving the globe. It is an affection thus not to be taken lightly and requiring immediate ophthalmological examination comprising full fundus examination with a dilated pupil, under anesthesia if necessary\cite{3}.

Proptosis, the anterior protrusion of the eye from the orbit, is because of tumor growth with displacement of normal tissues or seeding into the tissues and consequent enlargement of the tissues. It follows orbital invasion and secondary microbial infections are often present, with mucopurulent or fungating ocular mass referable to neglect or mismanagement. This is indeed a common type of presentation in most developing countries\cite{6}, due mainly to socioeconomic and cultural limitations resulting in delayed presentation\cite{7}.

Atypical signs are seen both in some retinoblastomas and certain pseudoretinoblastomas, they are often inflammatory or hypertensive and can cause major problems in diagnosis\cite{8}. Atypical changes in eye appearance are very late pathological manifestations carrying a far more reserved vital and functional prognosis and they include painful red eye, uveitis, and multifocal iris invasion associated with hyphema and iris neovascularization, heterochromia, ruberosis iridis, phthisis bulbi, secondary
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Glaucoma. All of them are commonly found in particular as ultimate complications of a very advanced and unchecked disease. Decrease in visual acuity (e.g. not fixing or following in infants, clumsiness in ambulatory children)\cite{64} and orbital inflammation after toxin release from tissue necrosis are also associated with tumor development.

**Metastases** can be considered as sign of a primary retinoblastoma, and can occur as the tumor progresses thought the orbit, the optic nerve and then reaching the brain. Other distant spread may involve the abdominal organs (such as liver, spleen, and lung), bones (especially skull and long bones), bone marrow and lymph nodes.

### 1.1.1.6 Diagnosis

There are no widely recommended screening tests to look for retinoblastoma in children without symptoms. A comprehensive diagnosis is made from history, physical, histological and radiological examinations; blood chemistry, cerebrospinal fluid and marrow aspiration analyses are also performed to better diagnose patients.

It is strictly recommended that all infants should have a red reflex examination before discharge from the neonatal nursery and at all subsequent routine health supervision visits\cite{68}. The red reflex test is performed in a dimly lit or dark room with a direct ophthalmoscope or a retinoscope and children with an abnormal red reflex need immediate referral to an ophthalmologist skilled in pediatric examinations.

Many tumors are early discovered by parents, relatives, or when a child is brought to a doctor because his/her eye does not look normal. Therefore, early detection is key to a successful outcome and an immediate referral of children with Rb increases the possibility of saving lives and eyes, and preserving useful vision. Late diagnosis rather delays treatment, retinoblastoma spreads from the eye, and the chances of survival decrease\cite{49}.

During a regular physical examination and history, doctors routinely check general signs of health and especially both eyes, including possible changes in how they appear and move, lumps or anything else that seems unusual. Vision screening in babies and children at appropriate ages and intervals may identify tumors at earlier stages, when more treatment options are available and the chance of cure is high\cite{69}. Furthermore, health care costs are reduced, resources are redirected to high-risk patients, and unnecessary clinic visits and worry for unaffected family members are eliminated. Some
patients diagnosed initially with possible retinoblastoma prove, on referral to ocular oncologists and radiologists, to have one of pseudoretinoblastoma forms\textsuperscript{[59]}. The ocular oncologist will then collect a complete medical history of the patient's health habits, past illnesses and treatments, that is important when deciding if more tests and exams are needed, mainly in patients with a family history of the disorder, who may continue to develop new tumors for a few years after diagnosis and treatment and, for this reason, they need to be examined frequently. Moreover, molecular genetic testing for early identification of asymptomatic at-risk children in a family reduces the need for costly and invasive screening procedures. Tumor and blood samples can be tested to identify possible genetic changes and eventually other family members can be directly screened.

A proportion of children who present with unilateral retinoblastoma will develop disease in the opposite eye and periodic examinations of the unaffected eye will be performed for future years.

Unlike as for many other types of cancer, biopsies are not usually done to diagnose Rb because trying to collect a tumor specimen from retina can often damage the tissue and it would risk of spread cancer cells beyond the eye, endangering the life of patient.

If a child has typical clinical manifestations of retinoblastoma, the ophthalmologist or optometrist will then closely perform the \textit{eye examination with dilated pupil}, that is so wide opened with medicated eye drops to allow the doctor to look through the lens and pupil to the retina, usually performed under general anesthesia. The inside of the eye is then examined with a light. The diagnosis is generally established after the fundoscopy with an indirect ophthalmoscopy, using a small magnifying lens and a light\textsuperscript{[40]}, and the lesion appears as a white tumor with angiomatous dilatation of the vessels. Two other types of eye exams with the dilated pupil can be achieved: the slit-lamp biomicroscopy is performed to check the retina, optic nerve, and other parts of the eye using a strong beam of light and a microscope, whereas fluorescein angiography involves an orange fluorescent dye to look at vessels and the blood flow inside the eye. The inspection of the ocular fundus is carried out every three to four weeks until age six months, then less frequently until age three years\textsuperscript{[70]}. The interval between exams is based on the stability of the disease and age of the child (i.e., less frequent visits as the child ages).

Clinical examinations with cooperative children are then performed every three to six months until age seven years and yearly and eventually biannually for life. These early
surveillance methods are required to visualize, confirm and classify the condition and they determine the unilateral or bilateral nature of the lesion, the number of tumors, their position in the retina (posterior pole and anterior retina), the tumor size (diameter and thickness), the subretinal fluid and tumor seeds, the vitreous seeding, as well the anatomical relations with the optic disc and macula\textsuperscript{[71]}

Imaging tests using \textit{ultrasound examination of the eye} will be first done to determine size, location and extent of the tumor. High-energy sound waves are bounced off the internal tissues of the eye to make echoes. A small probe that sends and receives sound waves is placed gently on the surface of the eye. The echoes make a picture of the inside of the eye and the distance from the cornea to the retina is measured. The picture called a sonogram, shows on the screen of the ultrasound monitor and can be printed to be looked at later.

Three-dimensional (3D) ultrasonography can be used to perform retinal and tumor mapping and to diagnose calcification and retinal detachment. This technique allows for new oblique and coronal views of the tumor and optic nerve; however, it is not useful for describing the extraocular spread of tumors\textsuperscript{[72]}

A novel \textit{handheld spectral-domain optical coherence tomography} (HHSD OCT) could be also exploited to identify and define the intraretinal location of a small retinoblastoma that was not detectable by the indirect ophthalmoscopy. Hence, the HHSD OCT technique can aid the ocular oncologist in the identification of very small retinoblastomas before they are visible to the eye, which allows for earlier and potentially vision-sparing treatment of these lesions\textsuperscript{[73-74]}

\textit{Color doppler} imaging reveals slightly vascularized tumor areas and can depict blood flow of retrobulbar vessels and inside the tumor\textsuperscript{[75]}

Cranial/orbital \textit{computed tomography} (CT) scan can detect intraocular tumor and its calcifications and determine how far Rb may have spread within the eye\textsuperscript{[76]}. Furthermore, tumor is seen as a mass predominantly located in the posterior ocular pole and it may present distinct contours and an inhomogeneous structure. CT scan can make a series of detailed pictures of the eye, taken from different angles. CT scanning has high sensitivity and helps in staging of intraocular tumors, the detection of extra bulbar growth, and the determination of further treatment approaches. CT scanning can be used to follow up
tumors to determine the effect of treatment and to establish a timely diagnosis of malignant tumor relapses\textsuperscript{[77]}. 

\textit{Magnetic resonance imaging} (MRI) of the orbit is the most sensitive mean of evaluating extraocular extension into the optic canal. This technique based on the elaboration of images collected by exciting the tissue using radio frequencies under a magnetic field provides detailed pictures of the eye. It has become a benchmark technique for detecting retinal detachment and intracranial tumors\textsuperscript{[78-79]}. Children with a family history of retinoblastoma are recommended for a MRI scan of the brain at regular intervals for up to 5 years to check for a trilateral retinoblastoma. Visible extension to the retrolaminar optic nerve must be investigated, especially in case of optic disc involvement, as it determines the medical approach for future treatments.

\textit{Distant staging} can be performed by different imaging procedures for identifying metastasis all over the body outside the eye. It may be performed when enucleation is necessary and histopathological risk factors have been identified. Scintigraphy involves a minimal amount of radioactive material is injected into a vein enriching into the rapidly dividing cells constituting the metastatic lesion that bright up in the picture. Moreover, if the cancer invades the brain, a lumbar puncture can reach the cerebrospinal fluid (CSF) to collect sampling for cancer cell checking. Bone marrow aspiration and blood analysis may also provide microscopic material for investigation of signs of cancer\textsuperscript{[40]}.

1.1.1.7 Classifications. After retinoblastoma has been diagnosed, tests are done to find out if cancer cells have spread within the eye or to other parts of the body. There is the need to evaluate both the extent of disease in the eye and if it has spread outside, to determine the most appropriate care of the child. Moreover, knowing the tumor stage and classify it is essential to decide the best treatment and to predict its outcome. Retinoblastoma will be classified and described at different stages, depending on where the cancer is located, if or where it has spread, and whether it is affecting other parts of the body. Tumor is then classified based on the results of eye exams, imaging tests, and any biopsies that were done. A staging system is a standard way to sum up how far a cancer has spread. Several detailed guidelines and codes, called staging systems, can be used to stage retinoblastoma, and there are two main classifications for grouping Rb presently in use: the Reese-Ellsworth and the International classifications.
Reese-Ellsworth classification is an old model of staging developed in the 1960s and it has been originally used to predict visual prognosis of affected eyes and globe salvage after external beam radiotherapy. At that time, in fact, radiotherapy was the most common first line therapeutic approach widely administered to the majority of patients. This presurgical classification therefore was in use until 1980, when the preferred line of treatment shifted from radiotherapy to chemotherapy. It is divided into 5 groups, ranging from Group I with "very favorable" cases for saving the eye, to Group V with "very unfavorable" cases that are unlikely to be controlled with chemotherapy or radiation. Each group is then divided into two sub-Groups (A and B), depending on the disc diameter in size of the tumor lesions.

International classification is currently used to quantify retinoblastoma and its associated features without need to refer to complex qualification criteria. This staging system was designed to simplify grouping, to assist in choosing the appropriate treatment and predicting its outcomes and allowing the highest chance of success especially after chemoreduction[80]. Clinicians found this new classification more reliable as it better predicts the chances of cure for intraocular retinoblastoma without the need for enucleation or radiation treatment. It is based on tumor size, location, and associated seeding. This classification is divided into 5 groups, ranging from Group A "small tumor" that is still only in the retina and up to 3mm in size, to Group E "extensive retinoblastoma", bleeding or causing glaucoma with no chances to salvage the eye; proceeding from the lowest to the highest grouping means the worsening of ocular prognosis[81-82].

At the International Symposium of Retinoblastoma held in Paris in May 2003, a committee of experts from centers worldwide drafted yet another staging system. This staging system was designed to be used in conjunction with the new intraocular grouping system and it combines clinical and pathological staging and has a single end point, the survival of Rb patients, who are classified according to extent of disease, the presence of microscopic or overt extraocular extension and metastatic extension[83]. This combined system included the use of the International Retinoblastoma Staging System (IRSS). The latter classification is in fact applied for classifying subjects after surgery to ascertain how much cancer has remained and whether the cancer has spread. The IRSS different stages span from Stage 0 "intraocular tumor only" to Stage IV "metastatic disease"[84].
It needs to be said that doctors operating in developing countries may use other classification systems that include both intraocular and extraocular retinoblastomas. In these regions, at time of presentation, cancers are more likely to have widely spread. It is therefore worthy to mention the other systems that characterize advanced stage of the disease. 

The **AJCC TNM staging system**, developed by the American Joint Committee on Cancer, can be used to describe the extent of disease progression in cancer patients in detail, particularly for those that have spread outside the eye. It utilizes in part the TNM scoring system and it is based on size, numbers and location of the primary tumor, the lymph node involvement and presence of metastasis[^85].

The **St. Jude's Children's Research Hospital staging system** includes ophthalmologist data and embraces the whole spectrum of Rb, that is classified into four stages, ranging from Stage I, in which the tumor is confined to the retina, to Stage IV, where Rb has spread through the optic nerve to the brain, or through the blood to soft tissues, bone, or lymph nodes. Each stage is then divided into sub-groups[^86].

In **Grabowski-Abramson staging system**, patients with CNS invasion are seldom curable and categorized as Stage III, while patients with systemic metastases are classified as Stage IV and they can often be rescued with high-dose chemotherapy and bone marrow rescue[^84].

The flourish of so many different attempts to characterize the various stages and severity of the disease can be ascribed to the various causes of tumorigenesis, different forms of the disease and the biodiversity of the affected subjects. Moreover, this shows the urge to find a selective and specific form of treatment more suitable for each case, closely leading to personalized medicine.

**1.1.1.8 Treatments.** The primary goal of management of retinoblastoma is to save the patient's life. Salvage of the organ (eye) and function (vision) are the secondary and tertiary goals, respectively[^87-88]. Additional priorities in Rb management include the elimination of the tumor while concurrently minimizing collateral injury to other tissues, preventing metastasis and reducing the risk of long-term secondary tumors[^89]. Only one hundred year ago, retinoblastoma was almost universally fatal but its overall...
management has dramatically evolved in the past few decades and, at present, it has the highest cure rate of any pediatric solid tumors in industrial areas\(^{63}\), although it still remains a medical issue in developing countries: where access to healthcare is limited, retinoblastoma can still cause blindness and death\(^{89}\). In the last ten to fifteen years, outcome, in terms of survival and preservation of eyes and vision, steadily improved, together with quality of patients' life. Thanks to early diagnosis and aggressive, multimodal treatment strategies, near-complete cure rates are possible in developed countries, and many patients retain functional vision in at least one eye.

The management of retinoblastoma needs a multidisciplinary team of specialists, involving pediatric oncologists, ocular oncologists, radiation oncologists, ocular pathologists, and geneticists to optimize treatment outcome\(^{89}\). The cure strategy and prognosis depend on tumor stage, number or tumor foci, localization and size of the tumor(s) within the eye(s), presence of vitreous seeding, the potential for useful vision, the extent and kind of extraocular extension and the resources available. Treatment options are also highly individualized and based on several considerations including age at presentation, systemic condition, family and societal perception, the overall prognosis and cost-effectiveness of treatment in a given economic situation.

Early diagnosis and appropriate management are crucial to reduce mortality and morbidity and increase longevity, save the eye and possibly save the remaining vision.

The management of intraocular retinoblastoma has evolved to a more risk-adapted approach that aims at minimizing systemic exposure to drugs, optimizing ocular drug delivery, and preserving useful vision. Depending on the route of administration, the possible treatments are named as focal therapy, mainly reserved for subjects presenting with small tumors, local and systemic therapies, used instead to treat advanced retinoblastoma.

For patients presenting with extraocular retinoblastoma, treatment with intensive chemotherapy is required, including consolidation with high-dose chemotherapy and autologous hematopoietic stem cell rescue. This combined treatment is the way of giving high doses of chemotherapy and replacing blood-forming cells destroyed by the cancer treatment. Stem cell rescue consists in removing stem cells from the blood or bone marrow of the patient, freeze and store them. These stem cells are reinfused after chemo to grow and restore the body's blood cells. While most patients with orbital disease and a large proportion of patients with systemic extra-central nervous system metastases can be cured, the prognosis for patients with intracranial disease is dismal.
The pattern of development and risk for these tumors are heavily influenced by the methods of treatment for retinoblastoma, which may involve surgical and/or conservative procedures.

*Enucleation* is the first line of intervention in case of Rb completely filling the globe with no hope of visual salvage due to damage to entire retina, tumor invasion in optic nerve, choroid or orbit. It continues to be the treatment of choice for advanced intraocular retinoblastoma unresponsive to other forms of conservative treatments, presenting secondary painful glaucoma, with neovascularization of iris, anterior chamber tumor invasion, necrotic tumors with secondary orbital inflammation, and tumors associated with vitreous hemorrhage. In this cases in fact the tumor morphological characteristics cannot be visualized, especially when only one eye is involved\(^90\).

Enucleation is still performed removing the whole eye leaving behind lids and extraocular muscles but removing the longest possible segment of optic nerve in continuity with the globe, usually under general anesthesia. Care during surgical procedure should be taken to avoid perforation of the globe to prevent seeding. Post-surgical investigation will reveal if the cancer is likely to have spread to other parts of the body. This protocol includes the identification of histopathologic high-risk features together with the need of adjuvant therapy and leads to a substantial reduction in the incidence of systemic metastasis\(^87\). Adjuvant chemotherapy is thus administrated to prevent or at least minimize\(^91\) the risk of metastasis in patients with unilateral sporadic retinoblastoma who underwent primary enucleation. Nevertheless, its utility remains controversial\(^92\) due to the disagreement over the interpretation of histopathologic prognostic factors to define “high-risk” for developing metastasis\(^93\). Adjuvant orbital external beam radiotherapy following enucleation is recommended in patients with tumor invading the optic nerve transection, scleral and extra-scleral extension, spontaneous or accidental ocular perforation, and intraocular surgery for unrecognized retinoblastoma.

Either during the same operation or few weeks after enucleation, an orbital implant is usually put in place of the eyeball. The implant is made out of silicone or hydroxyapatite and it is attached to the muscles that moved the eye, so it should coherently move. After several weeks, an ocularist will create an artificial eye for the affected child. This is a thin shell that fits over the orbital implant and under the eyelids, like a big contact lens. It will match the size and color of the remaining eye. Once it is in place, it will be very hard to tell it apart from the healthy eye.

Removing the eye also can affect the future growth of bone and other tissues around the
eye socket, which can make the area looking somewhat sunken. Using an orbital implant can sometimes lessen this effect. However, if there is any chance of saving useful vision in one or both eyes, other types of treatment should be preferred.

**Plaque brachytherapy**, also known as internal or episcleral plaque radiotherapy, is a focal therapy limited to small tumors and it involves the placement of a radioactive implant on the sclera for a trans-scleral irradiation of the tumor, using high energy X-rays or particles to kill cancer cells.

In the 1930s, Henry Stallard introduced small metal disks, called plaques, that allow radiation to be delivered to only a portion of the eye without irradiating the whole organ\(^94\text{-}^95\). Radioactive seeds are attached to one side of a thin piece of metal plaque (usually gold) and placed directly on the outside wall of the eye near the tumor. The plaque helps protect other nearby tissue from the radiation and keeps radiation from damaging healthy tissue. Tissue absorption of ionizing radiation may cause DNA damage and cell death. Because retinoblastoma has a high rate of proliferating cells, it is quite radiosensitive. Brachytherapy is used for solitary, medium-sized tumors (6-15mm and bigger than 3mm from the optic disc or fovea) and, if little subretinal fluid is present, plaque radiotherapy can generally be used to achieve tumor control\(^89\). Iodine 125 and Ruthenium 106 radioisotopes are the most common source of radiation currently used in brachytherapy; as well Cobalt 60 and Iridium 192 are also used. Plaque brachytherapy requires precise tumor localization and basal dimensions, used for dosimetry on a 3D tumor model. The plaque is left in situ for the duration of exposure, generally 3 to 7 days and radiation travels a much-defined trajectory that focused only on the tumor. The plaque is then removed at the end of treatment during a second operation while the child is under general anesthesia.

The advantages of plaque brachytherapy are focal delivery of radiation with minimal damage to the surrounding normal tissues, reduced risk of second malignant neoplasm and short duration of treatment. The common complications instead are radiation papillopathy and radiation retinopathy\(^96\), damage to the optic nerve that can affect vision in the long term\(^97\).

Brachytherapy has not been linked to an increased risk of developing a second cancer.

**External beam radiotherapy** has traditionally been done with conventional radiotherapy techniques that resulted in high dose delivery to the surrounding healthy tissues. It is a cancer treatment that uses high-energy X-rays or other types of radiation to kill cancer
cells or keeping them from growing. Radiation therapy focuses radiation beams from a source outside the body on the cancer and it has been a useful treatment tool of moderately advanced retinoblastoma up to the 1940s\(^9\). Until the late 1980s, radiotherapy represented the preferred form of management of retinoblastoma; accordingly the survival rate in affected children in the Western world exceeded 90%. Compared with surgery, it has in fact the advantage of possibly saving vision in the eye. However, in the long term, these young patients often presented midfacial hypoplasia\(^1\) and developed second non-ocular cancers in the irradiated field\(^1\). Because these secondary neoplasms occurred at a rate of 0.5 to 1% per year, and because half of the children who developed these other tumors died, by the 1990s, one of the most common cause of death in retinoblastoma patients was the secondary cancers related to the their radiation treatment, rather than the lesion itself\(^2\). Therefore, the risk of radiotherapy-related secondary cancers in children has led to dramatically reduce the use of external beam radiotherapy in Rb. Currently radiotherapy is still considered necessary only for patients with large tumors who are not candidates for chemosurgery but who have visual potentials. Furthermore, external beam radiotherapy can be used to treat eyes unresponsive to other treatments, those with a tumor located at the optic nerve resection margin and extraocular retinoblastoma extending through the sclera, orbit, or intracranial\(^3\).

Radiation is given 5 days a week for several weeks and the treatment for each day takes only a few minutes, usually under anesthesia. Before treatments start, the radiation team takes careful measurements with imaging tests such as MRI scans to determine the correct angles for aiming the radiation beams and delivering the proper dose of radiation. The lowest possible radiation dose combined with systemic or local chemotherapy and focal surgery may yield the best clinical outcomes in terms of local control and treatment-related toxicity.

Nowadays, many specialized centers use newer types of external low dose radiation therapy, called intensity modulated radiation therapy (IMRT), a type of 3D radiation therapy that uses a computer to make pictures of the size and shape of the tumor. IMRT allows doctors to shape the radiation beams and aim them at the tumor from several angles, as well as adjust the intensity (strength) of the beams to limit the dose reaching the nearby normal tissues\(^4\). Among those low doses radiation therapies, stereotactic radiation therapy uses special equipment to position the patient and precisely deliver radiation to a tumor. A rigid head frame is attached to the skull to keep the head still...
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during the radiation treatment. Proton beam radiotherapy is another new type of high-energy external radiation therapy and it can be used for treatment of Rb, reducing the radiation dose delivered to the adjacent orbital bone while maintaining an adequate dose to the tumor\textsuperscript{[103]}. Early results with proton beam therapy are promising, because reduces the risks of second malignancy and cosmetic and functional sequelae\textsuperscript{[104]}. Long-term follow-up of retinoblastoma patients demonstrates that proton beam radiotherapy can reach high local control rates, even in advanced cases, and many patients retain useful vision in the treated eye. Treatment-related ocular side effects are uncommon, and no radiation-associated malignancies have been observed\textsuperscript{[105]}.

\textit{Laser photocoagulation} was introduced in the 1950s by Meyer-Schwickerath\textsuperscript{[106]}, using a white laser beam aimed through the dilated pupil to destroy small tumors. This type of therapy is performed under general anesthesia and it uses a 520nm argon laser to coagulate the blood supply of the tumor by generating heat with temperatures in excess of 65°C within the treatment spot. Direct photocoagulation to the tumor must be avoided. Indirect ophthalmoscope laser photocoagulation is an effective conservative method to manage only selected small primary or recurrent posterior retinoblastomas up to 4.5mm in basal diameter and 2.5mm in thickness\textsuperscript{[90]}. Hence, the focal treatment is directed to delimit the tumor and coagulate the blood supply to the tumor by surrounding it with two rows of overlapping argon/diode or xeron laser burns. The related increase of temperature generated by the laser spot constitutes a second anti proliferative physical mechanism. Moreover, in this region, it is possible to create a synergism with the use of chemotherapy (carboplatin), given 24 hours before the beam treatment. Small posterior tumors without seeding respond well to laser photocoagulation. Most tumors require 2 to 3 sessions to be cured, with about a month between treatments. On the other hand, photocoagulation is not recommended for tumors located at or near macula or pupillary area, for those arising from a vitreous base and for mushroom shaped lesions. It is not even used for tumors impinging on the fovea, because of the risk of compromising a patient's central vision.

Complications after laser therapy include transient serous retinal detachment, vascular occlusion and preretinal fibrosis and vitreous seeding if the laser power is too high.

\textit{Cryotherapy} introduced in the 1960s by Lincoff, utilizes a frozen metal probe for destroying abnormal tissue, tumor cells and preserving vision abilities\textsuperscript{[107]}. It is a focal treatment performed under general anesthesia for small primary or recurrent retinal
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tumors anteriorly located to the equator of the globe, measuring up to 3.5mm in basal diameter and 2mm in thickness\[^{90}\]. Cryotherapy involves the use of a pencil-like probe by which liquid nitrogen is delivered and directly applied to the outer surface of the sclera adjacent to the tumor, directly behind the intraocular focus of the lesion. The cryoprobe is then frozen and thawed several times. Rapid freezing causes intracellular crystal formation, which breaks tumor cells and originates vascular occlusion. Cryosurgery is usually given 2 or 3 times for each treatment and it is applied at 4-6 week intervals until complete tumor regression. Cryotherapy can produce a large scar and cause the eye and eyelid to swell for a few days. Besides, it can damage the retina, which leads to blind spots or can temporarily cause retinal tears and detachment. Further complications include vitreous hemorrhage, choroidal effusion and localized periretinal fibrosis, proliferative vitreoretinopathy and chorioretinal atrophy. Cryosurgery, if administered 2-3 hours prior to chemotherapy, can increase the delivery of chemotherapeutic agents across the blood retinal barrier inducing a synergistic effect.

*Thermotherapy*, also called transpupillary thermal therapy, employs the heat to destroy cancer cells. It may be given using a laser beam aimed through the dilated pupil or onto the outside of the eyeball. Thermotherapy can be used alone for local control and for small tumors posteriorly located to the equator of the globe and outside retinal arcade up to 4.5mm basal diameter and 2.5mm thickness, without vitreous or subretinal seeds. It is a focal therapy and suitable for lesions adjacent to fovea and optic nerve in which plaque therapy or laser photocoagulation would possibly induce more profound visual loss. For larger tumors, it can be used along with chemotherapy (called thermo-chemotherapy) or with radiation therapy (called thermo-radiotherapy) as the heat benefits the efficacy of these other treatments. Thermotherapy is performed under general anesthesia and this type of focal therapy uses a focused heat generated by infrared light, using an 810nm diode laser, to heat and kill the tumor cells up to sub-coagulation. The temperatures, ranging from 42 to 60°C, are not quite as high as those used in photocoagulation therapy, so some of the blood vessels on the retina may be spared. It is noteworthy that laser is applied to eye tissue at sub-photocoagulation levels. Its mechanism of action is related to cell membrane and chromosomal damage, protein denaturation and ischemic necrosis of the tumor\[^{108}\]. The standard treatment for transpupillary thermotherapy is given for about 10 minutes at a time and it involves radiation from a semiconductor diode laser delivered with an indirect ophthalmoscope, in order to produce a grey white scar without photocoagulate retinal vessels. Alternatively, trans pupillary delivery can be performed
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through an operating microscope or via a trans-scleral route with a diopexy probe\textsuperscript{[87]}. Complete tumor regression can be achieved in over 85% of tumors using 3-4 monthly sessions of thermotherapy. When used as part of thermo-chemotherapy, the heat is usually applied at a lower temperature over a slightly longer period, starting within a few hours after chemotherapy. The common complications are focal iris atrophy and focal paraxial lens opacity, retinal damage retinal fibrosis, tumor seeding into the vitreous.

\textit{Chemotherapy} is the use of anti-cancer drugs to stop the growth of retinoblastoma cells, either by killing the cells or by stopping them from dividing. The way the chemotherapy is delivered depends on the stage of and the localization of the cancer in the body. Depending on the route of administration, it is classified as systemic and local.

\textit{Systemic chemotherapy} was first described by Kupfer\textsuperscript{[109]} in the 1950s, but the real interest for chemoreduction as a management option for retinoblastoma grew only in the mid-1990s, when the use of radiation-derived therapeutics was recognized as a potential cause of secondary malignancies and following preliminary observations that systemic chemotherapy delivered prior to external beam radiotherapy contributed to tumor control and ocular salvage\textsuperscript{[110]}. A subsequent study on eyes treated with systemic chemoreduction combined with focal treatments demonstrated tumor regression and decreased need for additional external beam radiotherapy and enucleation. This was a major advancement in management because it was evident that satisfactory tumor control could be attained with systemic chemotherapy while saving the eye and avoiding the adverse events of external beam radiotherapy\textsuperscript{[111]}. Using the International Classification of Rb, treatment success was found in 100% of group A, linear decreasing until nearly 50% in group D eyes. This launched the so-called "systemic chemotherapy era", from the 1990s to 2006; however, with the advent of novel local routes of administration, the use of systemic chemotherapy has decreased in recent years\textsuperscript{[89]}. In most cases, a combination of 2 to 4 chemical compounds is injected into a vein through a catheter, into muscle or given by mouth. These drugs enter the bloodstream and reach throughout the body. Systemic chemotherapy has recently become the standard of care for the management of moderately advanced intraocular retinoblastoma, even though it fails in more than two-thirds of eyes with advanced stage disease, requiring more invasive treatments such as radiotherapy or enucleation. It is mainly used to achieve the reduction of tumor size, but it is also a mean to aim at decreasing second malignancies and the risk of local and systemic relapse in advanced stage, resulting in a
good survival rate after treatment\textsuperscript{[87]}. Chemoreduction is now commonly used as the first treatment to shrink tumors that have not spread beyond the eye and it is indicated as initial therapy for bilateral advanced disease in which attempts are made to salvage both eyes. Unfortunately, chemotherapy alone rarely cured the whole of the tumor, but it can benefit size reduction and the effect of other additional local methods, such as laser, cryotherapy or plaques. With these adjunctive treatments, the success rate of systemic chemotherapy was comparable to radiation but without the radiation-related side effects\textsuperscript{[63]}. The combination of chemoreduction and aggressive focal therapies has indeed formed the new standard approach to most patients with advanced intraocular Rb. However, seeding of the vitreous cavity continues to be the primary reason for treatment failure in these patients\textsuperscript{[112]}. On the other hand, the drug administration after local treatments is under investigation because it might help prevent the recurrence of retinoblastoma, especially outside the eye. Chemotherapy may be also a postoperative adjuvant for histopathologic high-risk features and for the reduction of systemic metastasis in patients with unilateral sporadic retinoblastoma who underwent primary enucleation. It is in fact utilized quite often when the eye has already been removed, but the tumor was found to have spread into some adjacent areas in the eye. More relevantly, the systemic chemotherapy could be used to treat children whose retinoblastoma has extended beyond the eye, in patients with optic nerve invasion posterior to the lamina cribrosa, massive choroidal invasion, or any combination of optic nerve and choroidal invasion (for which local treatments will bring limited improvements). If the cancer has spread to the brain, chemical compounds may also be given directly into the cerebrospinal fluid that surrounds it, using a technique called intrathecal chemotherapy. Systemic chemotherapy is administered in cycles, with each period of treatment followed by a rest period to give the body time to recover. Each cycle typically lasts for a few weeks and the total length of treatment is often several months\textsuperscript{[113]}. The Food and Drug Administration (FDA) approved a list of generic and brand names of cyclophosphamide for retinoblastoma. Three classes of agents are also commonly employed: DNA-crosslinking agents (carboplatin, cisplatin), DNA topoisomerase 2 inhibitors (etoposide, topotecan, teniposide) and Vinca alkaloids (vincristine). Regimen and doses are managed for intraocular retinoblastoma and the most commonly used combination to shrink the tumor is vincristine/etoposide/carboplatin\textsuperscript{[88]}. Doxorubicin and
other chemotherapeutic drugs might be used if the standard combined therapy is not effective.

For instance, three cycles of carboplatin-based chemotherapy over 3 months can reduce the size of tumors by almost 50%\(^{[114]}\).

Unfortunately, when given into the bloodstream chemotherapy can cause adverse effects in different parts of the body. Children tend to have less severe side effects from chemotherapy and to recover from these more quickly than adults do. For this reason doctors might even decide to give them a high dose of drug needed to eradicate the tumor. These side effects depend on the type of drugs, the doses used and for how long they are given. Possible side effects might be temporally or permanent hearing loss\(^{[115]}\), mouth sores, loss of appetite, nausea and vomiting, diarrhea or constipation, increased chance of infections, bruising or bleeding and fatigue. Other adverse events, such as neurotoxicity, hyponatremia, nephrotoxicity, ototoxicity and febrile neutropenia, can also occur. Treatment failure in the form of persistent vitreous and subretinal seeds and intraretinal tumors following therapy have been attributed to the inability of the treatment drugs to reach the tumor\(^{[116-117]}\). Tumor resistance or unresponsiveness to chemotherapy is thought to occur more often in well-differentiated tumors, presumably because cells are not cycling, so they are less likely to respond to treatment modalities affecting cell division\(^{[118]}\). Secondary acute myelocytic anemia, a virulent form of leukemia that is difficult to treat and has a high mortality rate, has been reported after chemotherapy in some children with retinoblastoma\(^{[119]}\) and it is now the most common second cancer in some countries. In addition, frequent complications of chemotherapy include the need for transfusions and ports\(^{[120]}\).

The occurrence of several serious side effects limits the doses of systemic drug that can be given to treat retinoblastoma. Newer techniques help keeping the drug concentrated in the areas around the tumors and they are exploited with the hopes of both getting higher doses of chemo exclusively to the target tumors and reducing the morbidities and side effects associated with systemic therapy.

Therefore local chemotherapy delivery methods, including periocular injections, intra-ophthalmic artery infusions, and intravitreal injections, have been developed in the hope of controlling disease in advanced cases\(^{[112]}\).

**Periocular chemotherapy** is involved in the treatment of some advanced intraocular cancers with vitreous seeds and it has been used for retinoblastoma control as an adjunct to systemic chemotherapy and local treatment in order to avoid enucleation, to treat
tumor recurrence and whenever high doses of chemo are needed directly inside the eye, either as subconjunctival or subtenon delivery. Along with systemic chemotherapy in fact, one of the drugs may be injected through the membrane covering the muscles and nerves in the tissues around the eye, where it slowly diffuses into the back of the eyeball, achieving high levels within the vitreous\textsuperscript{121}. Periocular injection is performed usually under general anesthesia and it enables transcleral drug delivery, using the large surface area of the sclera and its high permeability to small molecules without the hazard of puncturing the globe. Either carboplatin or topotecan can be employed and complications of periocular chemotherapy include redness, orbital and eyelid edema, ecchymosis, orbital fat atrophy, muscle fibrosis leading to strabismus, and optic atrophy. Because of its significant toxic effects, it is rarely used in current practice\textsuperscript{122}.

**Intra-arterial chemotherapy** represents a new approach for the management of retinoblastoma and it consists in injecting drugs directly into the ophthalmic artery, the main vessel that supplies blood to the eye. During the last decade, Suzuki and colleagues started treating patients with intracarotid artery melphalan\textsuperscript{123}, developing a technique to safely and effectively cannulating the ophthalmic artery for chemotherapy infusion. According to this technique, usually performed under general anesthesia, a long and very thin catheter is inserted into a large artery on the inner thigh and slowly threaded through the blood vessels all the way up into the ophthalmic artery, where drugs, such as melphalan, topotecan or carboplatin are then infused. The treatment is slowly delivered for 30 minutes in a pulsatile fashion taking care not to occlude the artery and to minimize reflux into the internal carotid artery. This process may then be repeated every few weeks, depending on how much the tumor shrinks, and most patients receive 3 monthly sessions. This technique seems to elicit promising results obtaining good tumor control and minimal side effects, mainly due to the low amount of drug occurred for treatment. Most notably, enucleation or external-beam radiotherapy could be avoided in two-thirds of affected eyes with advanced intraocular retinoblastoma managed primarily with intra-arterial chemotherapy.

To treat tumors that are widespread within the eye and not responsive to other treatments or have come back after treatment, **intra-vitreal chemotherapy** can be used, injecting the therapeutic agent directly into the vitreous cavity of the eyeball through the pars plana under aseptic precautions. The main concern with this technique is that placing the needle into the eye to give the drug might open a small hole that could allow tumor cells
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to spread outside, so medical specialists are required. A recent improvement added to the
technique is to create a zone of transient intraocular lower pressure so to minimize cell
spreading during administration of a small-volume dose of melphalan, topotecan, or a
combination of both injected into the eye using a fine frozen needle\(^{[124]}\). The first-line
indication for intravitreal chemotherapy includes vitreous seeds refractory to standard
therapy and recurrent vitreous seeds after previous therapy\(^{[125]}\).

Children with retinoblastoma that has spread outside the eye and who are unlikely to be
cured with conventional treatments need multiple-agent, high-dose chemotherapy and
autologous hematopoietic stem cell transplantation as rescue therapy for the bone
marrow\(^{[126]}\), although this is a complex treatment that can cause life-threatening side
effects. Regimens with chemical compounds proven to be effective include vincristine,
cyclophosphamide, doxorubicin, and platinum as well as epipodophyllotoxin-based
drugs\(^{[127]}\).

1.1.1.9 Recurrence. During and after treatment for retinoblastoma, the main interest for
patients and physicians is about the tumor itself, the short- and long-term tumor effects
and its treatment.
Follow-up care is a central part of the process once the treatment is finished and it offers
children the best chance for recovery and long-term survival. Cancer recurrence is in fact
a possible side effect of certain treatments.
Orbital recurrence of retinoblastoma following successful treatment of intraocular disease
is rare, however, Rb may regrow after a period of time during which the tumor could not
be detected. Following chemoreduction and focal consolidation, tumor relapse is found in
almost one fifth of total Rb at 7 years and increasing of tumor thickness is the most
important predictive factor of recurrence\(^{[128]}\). Cancer may recur into the eye in the same
place as the original (primary) tumor, in tissues around the eye, or in other places in the
body.
Recurrent retinoblastoma in the only remaining eye represents a worse scenario and after
the failure of conventional treatments, enucleation is the only option left\(^{[129]}\).
The potential for recurrence of Rb during treatment and active follow-up raises the
possibility of complex and challenging treatment. The relapse of tumor is crucial source
of concern following intraocular surgical procedures and aggressive laser treatment. As
well, risk related to medical procedures concern extraocular extension of Rb or tumor
seeding in the needle tracks, following the violation of the sclera or aspiration of
intraocular retinoblastoma, respectively\textsuperscript{[130]}. Furthermore, high-energy focal treatment can disrupt the internal limiting membrane, causing dispersion of malignant cells from the tumor\textsuperscript{[131]}. Repeated, aggressive laser treatment may cause a focal thinning of the sclera within the atrophic scar, easing a portal of entry for active tumor cells to seed the orbit\textsuperscript{[132]}. Although external beam radiotherapy represents the most reliable method to treat retinoblastoma with vitreous seeding, tumor recurrence after this procedure can occur generating the need for other types of treatments to preserve the eyeballs\textsuperscript{[133]}. In children with hereditary retinoblastoma, formation of new tumors is common up to 3 or 4 years given to the generic nature of the disease.

1.1.1.10 Non-ocular tumors associated with Rb. Effort to ensure survival rate and saving of the eyes and vision steadily increased success during last decades: due to the early diagnosis and the improved treatment techniques, the 10-year survival rate of primary retinoblastoma has been among the highest of all childhood cancers at greater than 90% in developed countries\textsuperscript{[134]}. Before the 1970s, the majority of new malignancies in survivors of retinoblastoma arose in the prior radiation fields, and could be labeled as radiation-induced neoplasias\textsuperscript{[135]}. Secondary tumors arising from non-irradiated areas were initially reported by Jensen and colleagues in 1971\textsuperscript{[136]}. Few years later, Abramson demonstrated the association of the risk of non-ocular cancers with heritable retinoblastoma\textsuperscript{[137]}. Bader and colleagues firstly identified the trilateral retinoblastoma in addition to having hereditary retinoblastoma\textsuperscript{[138]}. Retinoblastoma survivors and their offspring increased during the mid-twentieth century as diagnostics and treatments improved. However, as treatment methods for retinoblastoma continue to evolve, the type and distribution of second non-ocular malignancies will continue to change. Second primary tumors are new primary lesions that occur in a person who had cancer in the past, they are unrelated to the first cancer and may occur months or even years after the initial tumor was diagnosed and treated. Currently, secondary malignancies represent an increasingly significant topic, becoming the leading cause of death of germinal retinoblastoma survivors, who become susceptible to developing second non-ocular cancers\textsuperscript{[139]}. Hence, long-term survivors of hereditary retinoblastoma have a significantly high risk for secondary malignancies and they are at an increased 20-fold risk of developing and dying from subsequent non-ocular cancers, especially bone and soft tissue sarcomas that account for approximately 40% to 60% of all second cancers\textsuperscript{[120]}. 

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Other second primary tumors among retinoblastoma survivors include trilateral Rb, malignant melanomas and neoplasms of brain and meninges. These tumors usually persist decades after the retinoblastoma diagnosis and manifest in childhood, adolescence or even adulthood.

This predisposition to pediatric second primary sarcomas and other tumors has been attributed to genetic susceptibility as well as past invasive therapies\(^{140}\). The pattern of development and risk for these tumors are so heavily influenced by the initial methods of treatment for retinoblastoma. There is convincing epidemiologic evidence linking past radiotherapy with sarcomas in hereditary patients. In these view, the specific extraocular new neoplasms might be caused by treatments with radiations. Their incidence is in fact increased to more than 50% in individuals with Rb who have received external beam radiation therapy\(^{141}\). In addition to radiotherapy, chemotherapy, specifically alkylating agents, have been associated with the risk of bone cancer after Rb\(^{142}\), but less so for soft tissue sarcomas\(^{143}\).

Survivors of heritable retinoblastoma who are not exposed to high-dose radiotherapy have a high lifetime risk of developing a late-onset cancer\(^{144}\).

Another risk factor for the development of second malignancies is retinoma/retinocytoma, a rare intraocular malignancy that appears to be either an inactive form indicative of spontaneous remission or a benign variant of retinoblastoma and it is characterized by homogenous, translucent greyish masses, calcifications, with pigment migration and proliferation bordering the tumor\(^{27}\). These lesions display genetic inactivation and represent a step towards Rb development\(^{145-146}\).

The majority of bone and soft tissue sarcomas among hereditary Rb survivors occur in the head, within the radiation field, but they also take place outside. Sarcomas account for almost half of the second primary cancers in hereditary Rb survivors, arising at ages similar to the pattern of occurrence in the general population.

There has been a trend over the past two decades to replace radiotherapy with chemotherapy and other focal therapies, in order to reduce the incidence of second cancers in Rb survivors. Screening for second primary malignancy is an important part of pediatric oncological follow-up in patients with heritable retinoblastoma. Given the excellent survival of most Rb patients treated in the past, it is important for survivors, their families and health care providers to be aware of the heightened risk for sarcomas in hereditary patients and they should then undergo regular medical surveillance in adult years\(^{140}\).
Survivors of non-hereditary Rb do not present genetic changes in all of their cells, so they are at much lower risk of a subsequent primary cancer, similar to the risk in the general population\[^{147}\], making second primary sarcomas very rare in these patients. Still, their risk of some cancers might be higher from getting chemotherapy and/or radiation therapy.

*Second tumors* after retinoblastoma are frequent in children with hereditary form, due to their genetic impairments in each cell. They have a much higher risk for developing other types of cancer throughout their lives and this is even higher in any parts of the body that received radiation during treatment for Rb. Younger children treated with radiation therapy are more likely than older children to develop side effects such as second cancers or problems with bone growth in the irradiated area. Chemotherapy with certain drugs can also increase the risk of some cancers.

Survivors of retinoblastoma, particularly patients carrying a germinal mutations or who have a retinoma, should undergo lifelong surveillance for second primary tumors. They should also avoid smoking, damaging exposure to sunlight, and ionizing radiation when possible.

Most of second tumors are very treatable if detected early, and so it is very important that these children are followed closely throughout life. The entire body must be examined carefully to avoid missing these second tumors and they are classified in four main types depending on their general features\[^{139}\].

*Benign tumors*, especially lipomas, are frequently reported in patients with hereditary disease\[^{148}\]. These lipomas are preferentially located on the face, neck, shoulders, and upper chest\[^{149}\]. Additionally, many patients with both hereditary retinoblastoma and lipomas easily develop other secondary malignancies and these results suggest that the presence of benign tumors may indicate an elevated second cancer risk. Certain germline mutations may also predispose the patient to both lipomas and secondary tumors and this finding may have future implications on follow up and screening of retinoblastoma survivors for second malignancies\[^{150}\].

*Second malignancies* appear to be closely related to the initial treatment procedure. As trends in treatment continue to change and the length of follow-up increases, the rate of bone and soft tissue cancer development may decline while the rate of epithelial cancers are likely to increase. It is thus described an incidence rate of approximately 2% per year.
from the time of diagnosis of the second malignancy and the latency period decreases as each additional cancer is diagnosed. It has also been reported a predictable pattern for third, fourth, and fifth malignancy development based on location of the second tumor[151].

Risk of bone and soft tissue sarcomas begins within 10 years of treatment for hereditary Rb and continues throughout adulthood. Bone sarcomas are one of the most common second primary cancers occurring after hereditary retinoblastoma, accounting for 25-30% of all second primary cancers[152]. They are typically diagnosed in Rb survivors between 10 and 20 years of age, similar to the incidence pattern in the general population. The majority of second primary osteosarcomas occurred within the radiation field in the head region, but up to 40% was diagnosed outside the treatment field, primarily in the lower legs[153], and their clinical outcomes in Rb survivors may be more favorable than those of conventional osteosarcoma[154].

Soft tissue sarcomas are also one of the most common subsequent cancers following hereditary Rb accounting for 12% up to 32% of all second cancers[152]. An increased risk for soft tissue sarcomas was observed within 10 years of Rb diagnosis and continued through adult life up to 50 years after Rb, with the specific subtypes leiomyosarcomas and rhabdomyosarcomas, both occurring at similar ages as in the general population[155].

**Trilateral retinoblastoma** is a well-recognized syndrome that consists of unilateral or bilateral retinoblastoma associated with an intracranial neuroblastic tumor, commonly in the region of the pineal gland (pinealoblastoma), developing also a primitive neuroendocrine tumor in the brain[156-157]. The vital prognosis for these cerebral tumors is very poor[59]. The intracranial mass is often located in the pineal region, but may also be a suprasellar or parasellar tumor, presenting variable incidence of 0.5% to 6% among Rb patients[158].

It has historically been a highly fatal malignant tumor and a specific subset of patients are more likely to develop these lesions including those with a family history of retinoblastoma, bilateral disease, diagnosis within the first 6 months of life, and prior treatment with external beam radiation[35]. In the pre-chemoreduction era, pineoblastoma was found in 6% of bilateral retinoblastoma patients and 10% of patients with hereditary retinoblastoma[127]. It is an important cause of mortality in retinoblastoma patients during the first 5 years of life[160].

Trilateral Rb incidence has decreased recently, but the underlying cause for this shift
remains disputable. In the past, these tumors demonstrated a poor prognosis and they are still the most frequent cause of death in retinoblastoma survivors between the ages of 5 to 10 years\textsuperscript{[161]}. It is important to note that the classification of pineoblastoma as a second malignancy as opposed to a variant of the primary tumor is controversial. It often cannot be differentiated from retinoblastoma histologically and has occasionally been documented to occur prior to the development of ocular manifestations in some patients\textsuperscript{[162]}. For these reasons, some studies have not included trilateral retinoblastoma as a second malignancy, but the classification has varied over the years causing some discrepancy in the literature.

An independent second non-ocular retinoblastoma located in the ovary of a bilateral ophthalmic Rb survivor has been reported in literature. Eighteen years after radiation of the right eye and enucleation of the left eye, the patient was found to have a large left ovarian tumor involving the fallopian tube, mesentery, and lymph nodes. Histologically, the concurrent presence of Homer Wright and Flexner-Wintersteiner rosettes confirmed the identification as retinoblastoma. Additionally, molecular analysis demonstrated genetic alterations in different pattern in the patient and this difference suggests that the ovarian tumor was of a separate clonal origin from the original eye tumor\textsuperscript{[163]}.  

1.1.1.11 Psychosocial and financial care. Retinoblastoma emerges in infancy or very early in life and presents unique psychosocial and financial challenges to patients and caregivers. Support for Rb families is important at the time of initial diagnosis, during active treatment and throughout long-term follow-up. Relatives affected by a childhood cancer experience present anxiety and distress for a long period, remaining after treatment is complete and the child is considered cured\textsuperscript{[164]}. Rb diagnosis induces short-term emotional distress for parents and long-term concerns in affected children because they are all forced to suddenly change their lives. Psychosocial effects could manifest as increased levels of depression, anxiety, and concerns about mortality\textsuperscript{[165]}. An integrated multidisciplinary team of pediatric cancer specialists is then important in the management of retinoblastoma; it incorporates the skills of the following health care professionals and others to ensure that children receive treatment, supportive and rehabilitation that will achieve optimal survival and quality of life\textsuperscript{[166]}. Pediatric psychosocial oncology particularly provides emotional assistance and palliative care for families and children, including sibling and spiritual support, anticipatory grief
counseling, and bereavement follow-up\textsuperscript{[167]}, facing challenging experiences during hospitalization. Access to care is one of the most difficult issues that Rb families must overcome. Not all patients in fact receive optimal treatments yet, due to inadequate knowledge of Rb in primary care settings and wide variations in access to care. Specialists in retinoblastoma are in fact available in only a few centers of excellence, so a patient's province of residence significantly affects access to high-quality Rb care and cures are not still accessible to all. Most patients in developing countries are required to costly traveling great distances to receive appropriate and high-quality care\textsuperscript{[64]}. Hence, for many families treatment will imply moving to another city and separation from precious social support networks. Moving may impose changing terms of employment for at least one parent and significant financial burdens on the family\textsuperscript{[168]}. Many children and families need constant assistance during the school years and some of those need support as they enter adulthood, when both the disease and high-stressed treatment have major implications\textsuperscript{[168]}. Growing children report medically related phobias during or after treatment and they become aware of vision loss or impairment, focusing on the ocular prosthesis and the asymmetrical facial structure, especially after teasing or bullying episodes at school. Parents commonly also express anxiety over the integration process and the need for classroom support\textsuperscript{[164]}. Adult Rb survivors face new and different challenges. Rb has been associated with reduced levels of education attainment, social integration, employment, and income\textsuperscript{[170]}. Re-educating Rb survivors about their diagnosis and treatment as they get older is extremely important and it is done by easy access and open communication with medical experts\textsuperscript{[120]}. As young adults then consider having children of their own, they need to understand the communicating risk to future offspring when heritable Rb is present. Some affected patients have in fact a family history of the disease and they need genetic counseling\textsuperscript{[171]}, providing individuals and families with information on the nature, inheritance, future health risks and implications of genetic disorders. This will help them make informed medical and personal decisions, risks of developing Rb, screening protocols and reproductive options. The genetic cancer risk assessment is important for identifying at-risk individuals and the molecular predictive and/or prenatal testings are useful to identify individuals with heritable retinoblastoma and clarify the genetic status for all family members\textsuperscript{[172]}. Retinoblastoma is a severe disease that carries a heavy burden in term of personal, familiar and social commitments. For this, researchers and clinicians, together with psychologists and policy makers should convey a huge effort toward patients' life improvement. In this view, there is an urgent need for innovative therapeutic approaches.
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1.1.2 Molecular basis

1.1.2.1 Knudson's two-hit hypothesis. Retinoblastoma was the first tumor to draw attention to the genetic etiology of cancer. The chain of events inside cells that leads to retinoblastoma is complex and it has been known that there are variable components to this condition and Rb is mainly brought about by the biallelic loss or inactivation of the recessive human retinoblastoma susceptibility tumor-suppressor gene $RB1/p105^{[173]}$. It is located on sub-band 13q14.2 and consists of 27 exons, spanning approximately 180 kb of genomic DNA$^{[174]}$. The gene usually encodes the ubiquitously expressed 110 kD nuclear RB protein with a regulatory function in the cellular growth cycle at the G1 checkpoint$^{[63]}$. Cell cycle progression is dependent on a series of molecular regulation after cells are stimulated by growth factors that bind to corresponding membrane receptors and relay the signals through protein phosphorylation and trigger gene expression$^{[175]}$.

In the '60s, first Henry Stallard and then Kusum Lele investigated cytogenetically peculiarities in cases of retinoblastoma. They both identified germline deletions of chromosome 13 in rare patients with bilateral retinoblastoma and presenting mental retardation and a dysmorphic syndrome suggested that the retinoblastoma susceptibility gene was localized in this chromosomal region$^{[176]}$. In 1971, Alfred Knudson advanced understanding of cancer when he analyzed the long-known fact that children unilaterally affected by this tumor are commonly diagnosed at an older age than are bilaterally affected children, and formulated the hypothesis that two events must be rate limiting for the Rb development$^{[49]}$. Knudson described the "two-hit" mechanism to explain the genetic events that give rise to tumorigenesis in patients with retinoblastoma: two active copies of the $RB1$ gene are normally carried in human cells and inactivation of both alleles in the developing retina is then required for tumor
initiation\textsuperscript{[178]}. David Comings then expanded the notion to include malignancy-suppressing loci, recognizing that Knudson's hits might be either mutations or gene silencing inactivating both copies of the retina-specific gene\textsuperscript{[179]}. In 1986, Friend characterized a gene localized in the region of interest and constituting a site of inactivating germline mutations in children with bilateral retinoblastoma, confirmed that this gene corresponds to the retinoblastoma susceptibility gene, then called \textit{RB1}\textsuperscript{[174]}. Its identification proved the complementary hypotheses of Knudson and Comings, opened the way to cancer susceptibility gene testing and allowed definition of the risk of retinoblastoma within particular families.

This malignancy results from loss-of-function of both \textit{RB1} alleles, although other genes might be involved in tumor development\textsuperscript{[180]}. The theoretical model proposed by Knudson was accepted and used worldwide to explain some of the most important features concerning the different genetic, clinical, and epidemiological aspects of retinoblastoma, although the model itself has been considered controversial.

Steward and colleagues observed some cases of spontaneously regressed retinoblastoma more than a decade before the formulation of the mutational "two-hit" model\textsuperscript{[23]}, and it still represents a theoretical challenge to it. In fact, Knudson's hypothesis gives no clear-cut explanation of how a structurally modified DNA could lead to a whole array of cancer phenotypes, including the spontaneous remission of retinoblastomas, unless the tumor presents high penetrance (90\%)\textsuperscript{[181]}. Penetrance, however, is an undefined concept, which does not correspond to any known biochemical/molecular mechanisms, and is presently viewed as a pure stochastic fluctuation in gene expression\textsuperscript{[44]}. The mutational "two-hit" model has been recently challenged by evidences showing that aneuploidy and genetic instability play an essential role in the genesis of cancer\textsuperscript{[182]}. Nevertheless, neither the mutational nor the aneuploidy models seem to exhaustively explain the variegated phenotypic expression of retinoblastoma\textsuperscript{[3]}.

In addition, imprinting is a process by which human genes are functionally inactivated and its detection in retinoblastoma represents another argument against the mutational model, which assumes that gene expression can be altered only in the presence of structural DNA modifications, and in favor of the epigenetic one\textsuperscript{[44]}. Depending on when and where the change in the \textit{RB1} gene occurs, two different types of retinoblastoma can arise based on whether or not the child inherited the condition from the genetics of one or both parents.
**Hereditary retinoblastoma** occurs if one *RB1* allele is mutated or deleted in germline cells\(^{[183]}\), whereas the other allele is inactivated at the cellular level, in a somatic cell of developing retina. The abnormality is congenital, that is present at birth, and it is in all the cells of the body\(^{[184]}\). Affected patients started with heterozygous alleles (*RB1/RB1\(^{+}\)*) or inherited germline *RB1* pathogenic variant\(^{[185]}\) and hereditary Rb is then characterized by early onset and presence of multifocal tumors in both the eyes, due to the more probable loss of heterozygosity (LOH) in rapidly dividing cells, and multiple tumors occur.

More than 2500 nucleotide variants have been observed in patients with hereditary Rb\(^{[186]}\); the majority of *RB1* pathogenic variants result in a premature termination codon, usually through single base substitutions, small length mutations\(^{[187]}\), frame-shift variants, out-of-frame exon skipping caused by splice site variants. Pathogenic variants have been found scattered throughout exon 1 to exon 25 of *RB1* and its promoter region (5′-end). Recurrent pathogenic variants are observed at methylated CpG dinucleotides that are part of CGA codons or the splice donor site of intron 12. Other important types of pathogenic variants in Rb are complex rearrangements and small deletions, that are identified in about 10% of patients with bilateral or familial retinoblastoma\(^{[188-189]}\), resulting in the inactivation of one *RB1* allele. The loss or inactivation of the remaining normal *RB1* allele might then occur as the second event, leading to loss of function and Rb tumorigenesis\(^{[190]}\). In most of affected children, there is no family history of this cancer\(^{[19]}\). The defective *RB1* gene can be inherited from either parents (in about 25% of Rb cases) or the gene change first occurs *de novo* in parental germline cells before conception or in utero during early embryonic development. About 5-10% of patients have familial Rb, in which the disease is searched in the subject known as proband, a person serving as the starting point for the genetic study that will uncover more than one affected member in the family\(^{[7]}\).

In case of hereditary retinoblastoma, only one more "hit" is required to produce disease and it occurs in any retinal cell. During early childhood, these patients are predisposed to retinoblastoma because a mutation in the wild type allele will lead to biallelic inactivation of the *RB1* gene and thereby initiate the development of a tumor focus.

Hereditary Rb cases represent only approximately 1/3 of total retinoblastoma and tend to have bilateral and multifocal tumors. They also have a significantly increased risk for second primary neoplasms\(^{[19]}\) and the high incidence among patients suggests that this tumor-suppressor gene plays a key role in the etiology of several other primary malignancies\(^{[190]}\).
Their offspring will have a 50% chance of inheriting the pathogenic variant and a higher incidence of developing retinoblastoma, as this trait is transmitted in an autosomal dominant fashion with high penetrance (90%)\(^\text{[181]}\). The penetrance of the \(RB1\) mutation (laterality, age at diagnosis and number of tumors) is probably dependent on concurrent genetic modifiers such as \(MDM2\) and \(MDM4\) genes, whose polymorphisms may influence development and/or survival in \(Rb\)\(^\text{[180]}\).

**Non-hereditary (sporadic) retinoblastoma** occurs if both the \(RB1\) alleles are inactivated at the cellular level, in a somatic cell after conception in uterus or in early childhood in developing retina\(^\text{[191]}\) and they represent approximately 2/3 of total retinoblastoma cases. Affected patients started with two wild type alleles and both of these must become non-functional to produce the disease. The probability of both events occurring in the same cell in the eye is low and hence most of sporadic Rb tumors are unilateral and occur as a single tumor with late onset\(^\text{[7]}\). In the majority of cases, there is no other member of the family affected with the tumor and children with this type of retinoblastoma do not have the same increased risk of other cancers as children with congenital retinoblastoma.

The biallelic \(RB1\) gene inactivation in patients occurs through LOH at polymorphic loci located on chromosome 13\(^\text{[4]}\). LOH can result from deletions and several chromosomal mechanisms such as mitotic recombination and non-disjunction\(^\text{[192]}\). The first "hit" of sporadic Rb is due to a mutation or a deletion, whereas the second somatic event could be either another oncogenetic event or an epigenetic modification.

Therefore, the absence of detectable \(RB1\) mutations in some patients suggests that alternative genetic and/or epigenetic mechanisms may underlie the development of non-heritable \(Rb\)\(^\text{[193]}\).

Gene silencing after epigenetic variations is then observed in about 10% of unilateral sporadic retinoblastoma\(^\text{[194]}\). Approximately 3% of unilateral, non-familiar retinoblastomas have no somatic \(RB1\) alterations and in one-half of these cases, the amplification of \(MYCN\) oncogene might initiate the disorder in presence of non-mutated \(RB1\) genes. This gene encodes for N-Myc, a transcription factor controlling the expression of cell cycle genes involved in promoting cell proliferation and regulating in particular the global chromatin structure through histone acetyltransferases\(^\text{[195]}\), both in gene-rich regions and at sites far from any known gene\(^\text{[196]}\).

These \(MYCN\)-involved tumors are larger and more invasive than \(RB/RB\), characterized by distinct histological features and diagnosed at a significantly younger median age\(^\text{[197-198]}\). In another small subset of tumors without detectable somatic \(RB1\) mutations, an
acute genomic event concerned chromosomal "shattering" is rarely occurring focally on chromosome 13 spanning the \( RB1 \) locus. This chromothripsis is responsible for inactivating the \( RB1 \) gene, leading to loss of protein expression and can be considered as an additional mechanism for retinoblastoma initiation and tumorigenesis.

Aside from \( RB1 \), recurrent gene mutations were very rare. Other genomic changes that occur in a small minority of patients include \( BCOR \) or \( CREBBP \) mutation/deletion and \( OTX2 \) amplification \[^{199}\].

Although it is not expressed in normal human retinas, the proto-oncogene \( SYK \) was found to be upregulated in retinoblastoma and is required for Rb cell survival \[^{200}\].

Both amplification of the \( MDMX \), a gene related to \( MDM2 \), and increased expression of its protein are strongly selected during tumor progression as a mechanism to suppress the \( p53 \) response and inactivate the \( p53 \) pathway in \( RB1 \)-deficient retinal cells \[^{201}\]. \( MDMX \) promotes Rb progression in mice and human patients, even though it can be blocked by Nutlin-3, a small-molecule inhibitor of the \( MDMX-P53 \) interaction that efficiently kills retinoblastoma cells \[^{202}\].

Among other identified oncogenes and tumor suppressors, some have become targets motivating the search for novel therapeutic solutions. Candidate driver oncogenes that were recently emphasized in retinoblastoma studies include the following genes: \( MDM4 \), \( KIF14 \), \( DER \) (chromatic remodeling factor), \( E2F3 \), a transcription factor and a tumor suppressor, \( CDH11 \) (cadherin) \[^{195}\].

Genetic test may ascertain hereditary or non-hereditary form of retinoblastoma in subjects. Moreover, \( RB1 \) genetic investigation provides important information for other family members. After identifying the genetic alteration in the affected member, target test may be performed screening other members for the same genetic alteration.

DNA is isolated and the two copies of the \( RB1 \) gene are evaluated by direct DNA sequencing from available eye tumor tissue from affected individual. DNA from peripheral blood is then screened for the presence of one of the two gene alterations that was found in the tumor. If one of the \( RB1 \) pathogenic variant identified in the tumor sample was also present in the affected individual's blood sample, this would strongly support a diagnosis of hereditary retinoblastoma. The absence of any retinoblastoma gene abnormalities in DNA from the blood sample typically confirms a diagnosis of sporadic disease. In addition, there are several options for an affected parent to preserve the offspring from transmitting familial \( RB1 \) mutation. These include both prenatal and preimplantation genetic diagnoses.
1.1.2.2 Rb family members. RB1, p107 and RB2/p130 tumor suppressor genes are collectively called Rb family members. RB1 is the central gene involved in Rb tumorigenesis, although it is not the only one interested in tumor initiation and dysfunction of the other retinoblastoma-related genes is indeed central for Rb development\[203\]. RB1 is fundamental for regulating cell growth and cell cycle progression and its "loss of function" initiates retinoma and causes genomic instability\[145-146\], but is insufficient to cause retinoblastoma. Failure of both cell cycle control and genomic stability leads to changes in other tumor-suppressor genes, differently involved in cancer progression\[204\]. Some events that directly or not, via alteration of upstream components, inhibit the three Rb family members activities can contribute to cancer development, inducing uncontrolled cellular proliferation\[205-206\]. Genetic and/or epigenetic events can lead to the failure of these tumor-suppressor gene functions, contributing in transformation to cancer phenotype\[207\]. These two complementary mechanisms are implicated in every step of carcinogenesis, from the responses to carcinogen exposures to the progression into malignancy\[208\]. Either oncogenetic or epigenetic alterations in addition to RB1 inactivation and/or silencing are therefore commonly found in Rb\[191\].

RB1 and the other retinoblastoma-related genes normally encode for "pocket proteins"\[209\], with a highly conserved region domain and their expression is differently regulated throughout the cell cycle\[210\].

The three members of the Rb gene family have a crucial role as negative regulators of cell cycle progression through modulation of transcription factors\[211\]. RB protein controls the expression of genes that mediate cell fate through a critical phase of its growth cycle\[212\].

Rb family members negatively interact with various E2F transcription factor proteins, thereby blocking different subsets of gene promoters, through phosphorylation by cyclin-dependent kinases (CDKs) regulation\[213\]. The CDKs are responsible for cell cycle transition through different phases. For G1 phase progression, the G1 cyclins associated CDKs can phosphorylate and inactivate RB. Because the phosphorylation sites of the protein are multiple, they become a family of checkpoint to prevent release of E2F family transcription factors under a stress condition, such as DNA damage. In addition, the CDKs activity and RB phosphorylation are decreased by the family of CDK inhibitors (CKIs), including INK4 and CIP/KIP family proteins\[214\]. Rb family members exert their function interfering with the coordinated regulation of the enzymatic activity of CDKs, which are indeed key regulatory factors of the cell cycle progression\[215\].
After gene dysfunction or gene silencing, the nuclear RB phospho-protein results inactivated or absent in retina cells and this is a fundamental event in retinoblastoma tumorigenesis, due to its ability to regulate the cell cycle with a central role in controlling the commitment of a cell to initiate DNA replication and cell division\textsuperscript{[216]}. Hence, the RB protein may be non-functional after phosphorylation and it appears to release E2F from the inhibitory complex, enabling it to promote the transcription necessary for cell progression into late G\textsubscript{1} and S phase\textsuperscript{[217]}. Loss of RB function deprives the cell of an important mechanism for slowing down cell proliferation through modulation of gene expression. Pathogenic variants in \textit{RB1} and in other family members lead to the expression of proteins that have lost cell cycle-regulating functions. Moreover, phosphorylation of RB controls its interaction with other proteins\textsuperscript{[218]} and if the binding activity of the pocket domain is lost, this results in the release of cellular proteins\textsuperscript{[219]}. Silencing RB activity is a frequent feature in cancer because it allows deregulated cell cycle progression and promotes tumor growth\textsuperscript{[218]}. Moreover, RB may hold additional tumor-suppressor functions, in several cancers, including alternative roles in the cell cycle, maintenance of genome stability and apoptosis\textsuperscript{[203]}. At the same time, RB protein is also involved in control of termination of cellular differentiation and in exit of the cell from the cell cycle during development, interacting with more than 100 different other proteins\textsuperscript{[220]}. 
1.2 EPIGENETICS

1.2.1 Epigenetic revolution

1.2.1.1 Epigenetic landscape. During an emerging scientific and philosophic revolution in the last century, the biologist Conrad Hal Waddington contended that classical Darwinian evolution ought to be revised by considering phenotype. In 1940, he formulated a theory called canalization, to explain reciprocal internal and external interactions between environment and phenotype on the one hand (nurture) and genotype and phenotype on the other (the nature of nature)[221]. Waddington proposed a dynamic model of evolution, introducing the concept of an "epigenetic landscape" to represent the process of cellular decision-making during development[222]; his epigenetic landscape can be seen as a metaphor for how gene regulation modulates development. At various points in this dynamic visual metaphor, a heritable phenotype (a cell or a nucleus), depicted by a ball rolling down a hillside, can take specific permitted trajectories, leading to different outcomes or cell fates[223]. Waddington portrayed this epigenetic landscape as a tilted, or rather undulated, surface, which is tilted so that points representing late states are lower than those corresponding earlier ones (Fig. 1.2). The surface presents with a cascade of branching ridges and valleys (the environment), called creodes, which in the context of cell lineage selection, represent the series of either/or fate choices made by the developing cell. Therefore, a creode represents the developmental pathway followed by a cell as it grows to form part of a specialized organ. In the case of a pathway or a creode, which is deeply carved in the hillside, external disturbance is unlikely to prevent normal development. When progression may undergo alteration by external forces, the embryo so attempts to regulate its growth and differentiation by returning to its normal developmental trajectory. Small differences in placement atop the hill can lead to dramatically different results by the time the ball reaches the bottom. This represents the tendency of neighboring regions of the early embryo to develop into different organs with radically different structures. Since intermediate structures rarely exist between organs, each ball that rolls down the hill is canalized to a region distinct from the others. Moreover, the formation of the body depends not only on its genetic makeup, but also by the different ways genes are expressed in different regions of the embryo. The more deeply creodes are carved into the epigenetic landscape, the weaker is the influence of genes over development[224].
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Waddington then envisioned that on this landscape the presence or absence of particular genes acts by determining which path should be followed from a certain point of divergence, thus providing in a single image an appealing, and influential, metaphor for the connection between genotype and phenotype. Successive generations of the same phenotype will tend to seek the same path and the phenotype will become fixed, or canalized regardless of the variability of its environment or genotype[225].

The question of whether the epigenetic landscape can be mapped out quantitatively to provide a predictive model of the directionality of the cellular differentiation remains largely unanswered. In the '70s, the mathematician René Thom tried to interpret the undulating surface of the landscape, although after several studies he considered it was impossible[226].

Wang and colleagues have then proposed a probabilistic method to quantify the epigenetic landscape for a gene network regulating cell fate, where the elevation of the surface is inversely related to the likelihood of occurrence of a particular state[227-228]. More recently, a rigorous quantification of the gene regulatory circuits that govern cell lineage choice was studied and a subsequent mapping of the epigenetic landscape was created that could potentially help identify optimal routes of cell fate reprogramming[229].

![Fig. 1.2: Epigenetic landscape][222]

1.2.1.2 Origins and definitions. The history of epigenetics is linked with the study of evolution and development. During the past 50 years, the meaning of the term "epigenetics" has itself undergone an evolution that parallels the impressive rate to which knowledge has increased to unveil the molecular mechanisms underlying regulation of gene expression in eukaryotes. Present definitions of epigenetics reflect the understanding that although the complement of DNA is essentially the same in all of an organism's somatic cells, patterns of gene expression differ may greatly differ among cell types, and these patterns can be clonally inherited[230]. It is now widely accepted that one of the most effective and investigated epigenetic
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changes, among others, is methylation of cytosine residues; however this was a colossal step to take at the beginning of the epigenetic studies. Historically in fact, DNA methylation was described in mammals as early as DNA, although his biological meaning was not immediately evident\textsuperscript{[231-232]}. Later, Hotchkiss first described two different bands in a preparation of calf thymus using paper chromatography: one for cytosine and a band for "modified cytosine"\textsuperscript{[233]}. He reconciled this evidence by hypothesizing that this second band was 5-methylcytosine, as it separated from cytosine band in a manner that was similar to how thymine separated from uracil, and he further suggested that this modified cytosine naturally existed in DNA. At this time, however the term epigenetic modification was still not in use.

In 1942, Waddington introduced the term "epigenetics" as a refinement of his conception of the "epigenetic landscape", to denote a phenomenon that conventional genetics could not explain\textsuperscript{[234]}. The prefix "epi-" is derived from the Greek preposition ἐπι, meaning above, on, or over. This word derived from the Aristotelian word "epigenesis" and it was used to describe events that could not be explained by genetic principles and literally means "in addition to changes in genetic sequence", in order to study the processes by which genotypes give rise to phenotypes. Waddington coined this term defining epigenetics as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being" and it involves understanding chromatin structure and its impact on gene function\textsuperscript{[235]}. The term epigenetics was originally used to denote the poorly understood processes by which a fertilized zygote developed into a mature, complex organism, and in fact, the Waddington's definition was rooted exclusively in embryology and developmental biology\textsuperscript{[236]}. Until the 1950s, the word "epigenetics" was used more broadly and less precisely to categorize all of the developmental events leading from the fertilized zygote to the mature organism, that is, all of the regulated processes that, beginning with the genetic material, shape the final product\textsuperscript{[237]}.

In 1958, David Nanney, revisiting Waddington's theory, suggested the hypothesis about a cellular epigenetic control system. This system has been involved in determining cellular characteristics and the term epigenetic was chosen to emphasize the reliance of these systems on the genetic systems and to underscore their significance in developmental processes. Certain patterns of expression, although specifically induced, may be perpetuated in the absence of the inducing conditions. Some epigenetic systems show a wide range of stability characteristics and the cellular memory may be heritable. For this
reason, cells with the same genotype may not only manifest different phenotypes, but these differences' in expressed potentialities may persist indefinitely during cellular division. Nanney also hypothesized epigenetic devices may be localized in the nucleus and on the chromosomes, like the genetic system\[^{238}\].

Two years later, Salvador Luria firstly defined epigenetics in cellular manner. The most important distinction is between genetic and epigenetic mechanisms of cellular alteration\[^{239}\]. Luria in fact wrote: "A change is defined as genetic (or nucleic) if it alters the genetic materials of the cell, that is, the structure, size, or number of the coded macromolecules -nucleic acids- that carry large amounts of detailed information usable for coding other molecular species. Epigenetic (or epinucleic) changes are changes in the expression of genetic potentialities, such as activations, inhibitions, or competitive interactions, whether exerted at the level of primary action of genetic elements or at other levels of cellular metabolism\[^{240}\]."

In 1969, Griffith and Mahler suggested that modifications of DNA bases may modulate gene expression and that DNA methylation may be important in long-term memory function\[^{241}\], and although many researchers then proposed DNA methylation as a regulation mechanism, it was not until the 1980s that several studies showed that this epigenetic alteration was involved in gene regulation and cell differentiation\[^{242}\].

At the time of Waddington's experiments, genetics was an infant field, and little knowledge of the genome and its biology existed. The field of epigenetics has grown during the last decades, surely motivated by the increasing knowledge on DNA molecular biology, gene regulation, and many related aspects. Thus, over the following years, with the rapid growth of genetics, the meaning of the word epigenetics has gradually narrowed and been refined. Hence, Holliday and Plough proposed, among others, the methylation of cytosine-guanine (CpG) dinucleotide rich regions of the DNA\[^{243}\], as the biochemical basis of epigenetic regulation of gene expression, showing that gene expression can be either entirely stopped or increased in total absence of evident or detectable changes (mutations) of the basic DNA structure of the genes. In 1987, Holliday then demonstrated the inheritance of epigenetic defects in a malignant cell and explained the "epimutation" concept, to describe a mutation that alters the DNA activity without changing its chemical sequence. Now it is known that the control of gene expression in higher organisms is related to the methylation of cytosine in DNA and requires that the patterns of methylation become inherited. Loss of methylation, which can result from DNA damage, will lead to heritable abnormalities in gene expression, and these may be important in oncogenesis and aging. Transformed permanent lines often
lose gene activity through *de novo* methylation. Epigenetic defects in germline cells due to loss of methylation can also be repaired by recombination at meiosis but some are transmitted to offspring\(^{244}\). In the attempt to refine further the concept, epigenetics has been defined and it was generally accepted as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence"\(^{245}\). Most of the heritable epigenetic changes are established during differentiation and are stably maintained through multiple cycles of cell division and reverberate on multiple generations\(^{246}\). This enables the daughter cells to have distinct identities while containing the same genetic information. This heritability of gene expression patterns mediated by epigenetic modifications is collectively referred to as the "epigenome". It provides a mechanism for cellular diversity by regulating what genetic information can be accessed by cellular machinery. Failure of the proper maintenance of heritable epigenetic marks can result in inappropriate activation or inhibition of various signaling pathways and lead to disease states such as cancer\(^{247}\).

The discovery that epigenetic (or functional) modulation of gene expression is dependent on the environment, can give rise to is stable changes, and can these might be transmitted from one generation to the next, has opened a completely new perspective in the study of the interactions between environment and human genome. This appears as the way to ultimately clarify how gene-environment interaction leads to the development of many different human diseases, including cancer. Epigenetics investigates the causative mechanisms through which genes bring about their phenotypic effects that necessarily involve adaptive interaction with the environment. One of the most recent definitions of epigenetics quotes: "an emerging branch of investigation in cancer research (but also in other fields of clinical pathology) which studies the interactions between environment and genome in determining disease"\(^{248}\).

More recently, the term has been used to refer to any potentially stable and heritable change in gene expression or cellular phenotype that occurs without changes in Watson-Crick base pairing of DNA\(^{223}\). In a broad sense, epigenetics is considered a bridge between genotype and phenotype; a phenomenon that changes the outcome of a locus or chromosome without changing the underlying DNA sequence. It is clear therefore that the definition of epigenetics has evolved over time following the flight of new evidences that would implicate it in a wide variety of biological processes and it has temporarily landed to indicate the study of cellular and physiological phenotypic trait variations that result from external or environmental factors that switch genes on and off and affect how cells express genes\(^{249}\).
Epigenetics has morphed from a phenomenon to a branch of science whose molecular underpinnings are now reasonably well understood. The current state of knowledge of epigenetics has in fact evolved to include the understanding of DNA methylation, chromatin modifications, non-coding RNA and their effects on gene expression[234]. The current definition of epigenetics is indeed "the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence"[250].

Even though the vast majority of cells in a multicellular organism share an identical genotype, organismal development generates a diversity of cell types with disparate, yet stable, profiles of gene expression and distinct cellular functions. It is noteworthy therefore that even cellular differentiation may be considered an epigenetic phenomenon, largely governed by epigenetic changes rather than alterations in genetic inheritance. For this, over the years, numerous biological phenomena have been lumped into the category of epigenetics. These include seemingly unrelated processes, such as paramutation in maize, type switching in yeast, position effect variegation in the fruit fly Drosophila and the discordant course of disease in identical twins.

1.2.2 Epigenetic mechanisms

1.2.2.1 Chromatin structure and function. Chromatin is the complex of conserved histone proteins and genomic DNA that makes up chromosomes and it is tightly bundled to fit into the nucleus. Namely, it is made of repeating units of nucleosomes, which consist of 146-147 base pairs of DNA wrapped around a histone octamer formed by four histone partners, an H3-H4 tetramer and two H2A-H2B dimers proteins[251]. Each octamer contains two units of each principal or variant histone[252]. Histones are small basic proteins rich in amino-acids lysine and arginine. The four nucleosomic histones involve two domains: the C-terminal domain, which is located inside the nucleosome core, and the N-terminal domain with lysine residues extending out of the nucleosome. Among the four amino-terminal tails extending from the globular region of the histones, H3 and H4 become accessible and they are targeted for various post-translational modifications, such as acetylation, methylation, phosphorylation, and ubiquitination[253-254]. Linker DNA connecting nucleosomes associates with the main form or variants of the linker histone H1.

Chromatin structure defines the state in which DNA information is organized within a
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cell. Histone modification can in fact subsequently affect DNA processes, such as transcription, DNA repair and replication, and chromosomal organization. This arrangement of the genome into a precise compact structure greatly influences the abilities of genes to be activated or silenced\textsuperscript{[250]}. Epigenetic modifications can modulate gene expression in physiological mammalian development, occurring not just in the womb, but also over the full course of a human life span. These modifications are heritable and reversible, and determine cell-type and tissue specificity during cell growth and the development of an organism. Critical examples of epigenetic regulation in eukaryotic biology are indeed the process of cellular differentiation and development of germ cells.

Epigenetic changes work together to regulate the functioning of the genome by altering the local structural dynamics of chromatin, primarily regulating its accessibility and compactness. The interplay of these modifications creates an epigenetic landscape that regulates the way the mammalian genome manifests itself in different cell types, developmental stages and diseases\textsuperscript{[255]}.

During morphogenesis, cells start in a pluripotent state, from which they can fully differentiate into many cell types, and progressively falls into a state of narrower potential, by activating some genes while inhibiting the expression of others. Their gene-expression programs become more defined, restricted and, potentially, "locked in"\textsuperscript{[256]}. Therefore, epigenetic changes regulate gene expression so defining cellular identity. These modifications form the basis of cellular differentiation, imposing memories upon the cells that determine both lineage specification and potential for production of specific cell types\textsuperscript{[257]}.

As a consequence it is not difficult to accept how epigenetic changes could be strongly implicated a wide variety of major pathological conditions, such as several kind of tumors, schizophrenia and reproductive, autoimmune, or neuro-behavioral illnesses, mental retardation\textsuperscript{[258]}, and other health threatening conditions, such as alcoholism, obesity and cognitive dysfunctions. The abnormal gene expression profile in fact alters the cellular identity and contributes to the switch into a malignant phenotype.

Recognized or suspected drivers behind epigenetic processes include many agents, such as heavy metals pollution, exposure to pesticides, diesel exhaust, tobacco smoke, polycyclic aromatic hydrocarbons, radioactivity, viruses, bacteria and solicitations from hormones basic nutrients\textsuperscript{[251]}. 

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Epigenetic mechanisms that modify chromatin structure can be divided into some main categories: 1) DNA hyper-/hypo-methylation, 2) covalent histone modifications, like acetylation and methylation, 3) non-covalent histone mechanisms, such as incorporation of histone variants and nucleosome remodeling and 4) non-coding RNAs, including miRNAs.

The interactions between these mechanisms, especially DNA methylation machinery and histone modifying enzymes, determine the complexity of epigenetic regulation of gene expression, chromatin organization and cellular identity\(^{259}\).

### 1.2.2.2 DNA methylation

DNA methylation is perhaps the best-characterized chemical stable and heritable modification of chromatin. It results from the transfer of a methyl group (–CH\(_3\)) from a methyl donor substrate, namely S-adenosyl-L-methionine (AdoMet), to a cytosine in a CpG context. This addition of the methyl group is almost exclusively found on the 5′- position of the pyrimidine ring of cytosines (5mC) adjacent to a guanine, predominantly where these bases occur consecutively. Although methylation of cytosine bases in mammalian DNA has been primarily described in the context of CpG dinucleotides\(^{260}\), evidence suggested that cytosines in non-CpG sequences are also frequently methylated, especially in embryonic stem cells\(^{261}\).

Other rare modifications in mammalian DNA are methylation of adenine and guanine bases\(^{262}\).

DNA methylation is a covalent modification of nucleic bases that can confer stable silencing and, thus, transcriptional repression\(^{263}\) of repetitive elements, imprinted genes, X chromosome in females\(^{264}\). It is noteworthy to mention that these alterations are mediated by a group of highly conserved enzyme called DNA methyltransferases (DNMTs).

Roughly 70% of cytosine residues of all CpG dinucleotides undergo covalent methylation in mammals\(^{265}\) and their patterns occur in gene regulation and chromatin organization during embryogenesis and gametogenesis. Most of the CpG dinucleotides sites in mammalian genome, especially the repetitive DNA elements, transposons, CpG poor intergenic regions and imprinted gene promoters, are scattered throughout bulk chromatin\(^{266}\). They are generally heavily methylated, accompanied by repressive histone marks, which together determine a silent chromatin state\(^{267}\) that contributes to the transcriptional repression. In many organisms, the formation of heterochromatin, the
tightly packed and inactivated form of DNA, is therefore mediated in part by methylated DNA and the proteins bound to it, in combination with RNA and histone modifications. Most genomes with high levels of DNA methylation are depleted of CpGs due to the frequent deamination of 5-methyleytosine into thymidine. This generates mCpG:TpG mismatches which, if unrepaired, are further stabilized by DNA replication\cite{268}.

However, CpG dinucleotides are not evenly distributed across the human genome, but are concentrated in short CpG-rich DNA stretches and regions of large repetitive sequences\cite{269}. These regions have a high density of unmethylated dinucleotide CpGs and they are referred to as "CpG islands". Approximately 15% of the CpG dinucleotides are in CpG islands, often found in the promoters of both "housekeeping" genes and tissue specific genes\cite{265}. Apart from the inactivated X chromosome and imprinted genes, CpG islands are normally unmethylated, at least in germ cells, and perhaps throughout the developing and adult organism\cite{270} for most CpG islands. Therefore, most mammalian gene promoters are embedded within CpG islands that remain unmethylated during normal development and in differentiated tissues\cite{271}. When active, as in the case of tumor suppressor genes, they are accompanied by active histone marks allowing for a transcriptionally active open chromatin structure.

Tissue specific genes with non-CpG island promoters have been shown to exhibit tissue specific methylation\cite{272}. CpG islands can become methylated in normal adult tissues and this phenomenon may be related to age\cite{273}.

The decreased occurrence of CpGs is best explained by the fact that methylated cytosines are mutational hotspots\cite{274} leading to CpG depletion during evolution.

The first large-scale computational analysis of CpG islands using vertebrate sequences in GenBank was performed in the '80s by Gardiner-Garden, who defined a CpG island as being a 200 bp-5kb regions of DNA with a high GC content (greater than 50%) and an observed to expected ratio (ObsCpG/ExpCpG) of CpG greater or equal to 0.6\cite{275}. The exact definition of what constitutes a CpG island is somewhat arbitrary because the cutoffs for the parameters used to describe them can make significant differences to what sequences are included within the definition\cite{269}.

Embryonic stem cells, the pluripotent stem cells derived from the blastocyst of early mammalian embryos, undergo bimodal distribution pattern of DNA methylation: they present a global CpG methylation, whereas their CpG islands and pluripotency gene promoters are mostly unmethylated.

An extensive epigenetic reprogramming, mainly due to DNA demethylation, leads to the conversion of germ cells into immortal cells that can pass on the genome to the next
generation during early embryonic development. In the absence of germline-specific reprogramming, germ cells would preserve the old, parental epigenetic memory, which would prevent the transfer of heritable information to the offspring.

Furthermore, a large number of experiments have shown that methylation of promoter CpG islands is involved in many cellular processes during development, which results in long-term transcriptional silencing, including autosomal imprinting and X chromosome inactivation in female mammals\cite{276}. These are classic examples of such naturally occurring CpG island methylation during development\cite{277} and all of them seem to be stably maintained.

Taken together, DNA methylation of CpG clusters, located in the promoter regions of genes, gives a stable, heritable, and critical component of epigenetic regulation\cite{223}, correlating with transcriptional repression\cite{278}. It so provides a stable gene silencing mechanism that plays an important role in regulating gene expression and chromatin architecture, in association with histone modifications and other chromatin associated proteins.

Somatic cells show tissue-specific methylation of some CpG islands, occurring at developmentally important genes\cite{279}. Most non-CpG island promoters are also methylated together with pluripotency gene promoters, however these cannot reset epigenetic information by preserving the full methylation pattern on imprinting genes.

On the other hand, several in vivo studies showed that germline undergoes extensive epigenetic regulation, and thus genomic reprogramming\cite{280}. In contrast, the repetitive genomic sequences that are scattered all over the human genome are heavily methylated, which prevents chromosomal instability by silencing non-coding DNA and transposable DNA elements\cite{267}. DNA methylation can lead to gene silencing by either preventing or promoting the recruitment of regulatory proteins to DNA. Alternatively, it can provide binding sites for methyl-binding domain proteins, which can mediate gene repression through interactions with histone deacetylases\cite{281}.

Pluripotent stem cells express genes that encode a set of core transcription factors, while genes that are required later in development are repressed by histone marks: this mechanism confers short-term, and therefore flexible, epigenetic silencing. By contrast, the methylation of DNA confers long-term epigenetic silencing of particular sequences in somatic cells. Long-term silencing can be reprogrammed by demethylation of DNA, and this process might involve DNA repair\cite{256}.
DNA methylation may exert its repressive effect on gene transcription in two ways: on one hand, methylation of CpG in the promoter region impedes the binding sites of specific transcription factors and directly interferes with gene activation\textsuperscript{[282]}, while on the other hand, methylated cytosines attract mediators of chromatin remodeling, such as histone-modifying enzymes or other repressors of gene expression. CpG islands serve as docking sites for proteins that specifically recognize these binding sites and repress transcription indirectly, via recruitment of co-repressors that modify chromatin\textsuperscript{[283]}. It is evident therefore that the inhibitory effect of CpG island methylation on gene expression can reverberate throughout the involvement of proteins with high affinity for methylated CpGs towards additional proteins that eventually contribute to changing the chromatin structure. A pivotal role in gene modulation is therefore played by cellular machinery that regulates the methylated state of CpG residues: the enzymatic family of DNA methyltransferases and the methyl CpG-binding proteins.

**DNA methyltransferases** (DNMTs) is a family of nuclear enzymes and they catalyze DNA methylation\textsuperscript{[284-286]} (Fig 1.3).

The precise DNA methylation patterns found in the mammalian genome are generated and heritably maintained by the cooperative activity of the maintenance or de novo methyltransferases, playing a role in gene regulation and chromatin organization. Maintenance methyltransferases add methyl groups to hemi-methylated DNA during its replication, whereas de novo DNMTs act after DNA replication. Changes in DNMTs function may result in a gain or loss of DNA methylation, often found in altered patterns associated with cancer. In general, the mammalian DNMTs (DNMT1, DNMT2, DNMT3A and DNMT3B), in conjunction with accessory proteins (DNMT3L), are responsible for DNA methylation during gametogenesis, embryogenesis and somatic tissue development\textsuperscript{[287]}. DNMT1 is the most abundant DNA methyltransferase and is considered the key maintenance hemi-methyltransferase in mammals\textsuperscript{[288]}, which acts residing at the replication fork and copying DNA methylation patterns at hemi-methylated DNA onto the newly synthesized strand\textsuperscript{[289]}. It can ensure the mitotic inheritance of methylated DNA bases throughout the life of the organism. Moreover, its inactivation did not affect global methylation in cancer and it may be responsible for both de novo and maintenance methylation of tumor suppressor genes.

In contrast, DNMT3A and DNMT3B are responsible for the de novo methylation of unmethylated DNA sites\textsuperscript{[290]}; they are associated with nucleosomes and expressed mainly in early embryonic development, for imprinting and X-chromosome inactivation. They
act independent of replication, methylating newly replicated DNA, but they may also assist in maintaining methylation patterning at highly methylated genomic regions and interact with HDACs, targeting them to heterochromatin. Various studies have shown that DNMT3A and DNMT3B target different sites for methylation depending on the cell type and the stage of development. De novo methyltransferases may be directly targeted to specific DNA sequences, may necessitate the interaction with other DNA binding proteins or may be guided by RNA interference (RNAi) in a process called RNA-directed DNA methylation (RdDM). Inhibition of DNMT3B reduces overall global methylation, with demethylation being markedly potentiated when both DNMT1 and DNMT3B are simultaneously deleted.

DNMT3L has no enzyme activity and is not a methyltransferase, but it has a similar expression pattern to DNMT3A and DNMT3B.

**Fig. 1.3:** DNMT enzyme

*Methyl CpG-binding proteins* exert their function as transcriptional repressors via chromatin modification and they are often part of large repressor complexes as NuRD, NoRC, mSin3A and SWI-SNF. They recognize methylcytosine residues and a subset may biochemically recruit transcriptional repressor complexes, including histone deacetylase enzymes, suggesting an indirect repressive effect of DNA methylation. These proteins present a highly conserved DNA interaction surface, termed the methyl CpG-binding domain or MBD.

Currently two major families of methyl-CpG binding proteins are known in vertebrates: MBDs and Kaiso-like proteins, that both recognize more complex sequences than a single methylated CpG, thus favoring a gene or locus specific role for each member.

MBDs family proteins are classified as MeCP2, MBD1, MBD2, MBD3 and MBD4 and they are highly conserved in all vertebrates. MeCP2 was discovered at first and
represents a 53 kDa protein containing an N-terminal methyl-CpG binding domain (MBD) and a C-terminal transcriptional repression domain (TRD)\[281\]. It is noteworthy how MeCP2 is now widely known to be strictly associated with Rett syndrome, a girl suffering mental retardation arising after birth in the child phase of development. This syndrome is one of the epigenetic diseases, such as X-fragile syndrome in which the cause is a malfunction of methylation machinery\[301\].

All MBD proteins, except MBD3, specifically recognize and bind to methylated DNA in vitro and in vivo\[283\].

Kaiso-like family was discovered a DNA binding factor involved in non-canonical Wnt signaling\[302\] and as a protein that binds to methylated DNA\[286\]. Kaiso and the two recently identified Kaiso-like proteins ZBTB4 and ZBTB38 contain a conserved POZ domain involved in protein-protein interactions and three C2H2 zinc finger motifs, two of which are essential for binding to methylated DNA\[303\]. In addition, in some circumstances these proteins may bind to even unmethylated DNA\[304\].

1.2.2.3 Chromatin Variations. In addition to methylation at CpG islands in DNA, another significant epigenetic processes occurring during embryonic development and in somatic cells contribute to chromatin modification. The chromatin complex can be modified by introducing acetyl- groups (acetylation), enzymatic proteins and other nucleic acid in forms of RNAs. These changes alter the way chromatin is arranged, which, in turn, can determine whether the associated chromosomal DNA will be transcribed, influencing gene expression. In general, tightly folded chromatin, also known as condensed or hetero- chromatin, tends to be shut down, and remain not expressed, while more open chromatin is functional and is associated DNA that can be transcribed\[305\]. Histone variations work by either changing the accessibility of chromatin or by recruiting and/or occluding non-histone effector proteins, which decode the message encoded by the modification patterns. Their specific patterns are present within distinct cell types and are proposed to play a key role in determining cellular identity\[306\].

Chromatin plays a key role in regulating transcriptional activity and any modification in these core histone proteins can have an impact in the activation and/or repression of the transcriptional process\[287\]. Such modifications product highly positively charged histone tails that can strongly interact with the negatively charged DNA making it inaccessible for the transcriptional machinery\[307\]. Alternatively, modified histone tails can create an interface for the recruitment of transcription factors.
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There is an apparent interdependence between DNA methylation and histone modifications (i.e. methylation, acetylation etc.), that regulates genome-wide changes in chromatin structure and transcriptional regulation\cite{308} acting as a key mechanisms in cell fate, including malignant transformation\cite{223}.

To date, the best-characterized modifications are acetylations and methylations of lysine residues on histones H3 and H4\cite{276}. In particular, histone H3 modifications are notorious for affecting transcriptional activity\cite{309} and they can be considered as epigenetic biomarkers, and can be further exploited as powerful clinical tools in cancer diagnosis, prognosis and risk assessment\cite{310}.

Chromatin modifications can be divided into two main groups, depending on the chemical interactions, and these covalent and non-covalent mechanisms could be mechanistic linked each other.

*Covalent modifications* affect histone proteins that contain a globular C-terminal domain and an unstructured N-terminal tail\cite{311}. Histone can receive a variety of posttranslational histone modifications (PHMs), including acetylation, methylation, ubiquitylation, sumoylation and phosphorylation on specific amino acid residues on histones. Therefore, histone tails may undergo a number of covalent modifications, and it is thought that the conserved transfer of these posttranslational modifications between cell generations is essential for epigenetic inheritance.

An important histone modification is the acetylation of lysine residues at the N-terminal region of histones (mainly H3), which regulates whether chromatin is in the open or closed formation. This chemical process adds an acetyl- group to the amino acid lysine and is usually associated with active de-condensed chromatin and correlates with transcriptional activation\cite{252}. Acetylated lysines provide in fact an open chromatin conformation, whereas deacetylated lysines are generally associated with heterochromatin and inhibit transcription\cite{312}.

Histone methylation is involved in several biological processes, including DNA repair, cell cycle, stress responses, development, differentiation, and aging. A prominent histone modification is the transfer of one, two, or three methyl groups to lysine or arginine residues of histone H3\cite{313}. These variations in charge due to these modifications can directly alter the physical properties of the chromatin fiber, leading to changes in higher-
order structures; specific distribution patterns of histone marks in fact contribute to chromatin organization. Any change in histone methylation can alter any one of these biological functions, resulting in the development of disease\textsuperscript{314}.

During embryonic development, stem cells present a bivalent domain structure, consisting of the active and repressive methylation state and thus showing a coexistence of active and repressive marks at site of promoters of developmentally important genes and a plastic genome. On the contrary, somatic cells in normal conditions present a restricted epigenome and tissue-specific domains. Methylation of lysine (K) or arginine (R) in histone proteins alters the compaction or relaxation of chromatin depending on the position of amino-acidic residues and the number of methyl groups\textsuperscript{315}. For instance, active promoters are enriched in trimethylated histone H3 at K4 and active enhancers are enriched in monomethylated H3 at K4 and acetylated H3 at K27. In contrast, repressed promoters are enriched in methylated H3 at K27 (H3K27me) and K9, that inhibits gene transcription, marks silent DNA and it is widely distributed throughout heterochromatin\textsuperscript{316}. This is the type of epigenetic change that, together with DNA hypermethylation, is responsible for the inactivated X chromosome of females. These histone modifications regulate key cellular processes such as transcription, replication and repair\textsuperscript{252}.

Covalent histone modifications are dynamically regulated by enzymes that add and remove chemical groups to histone proteins. This variety of histone-modifying enzymes is then responsible for a multiplicity of posttranslational modifications on the specific amino-acid residues on histones\textsuperscript{317}. These modifications are almost reversible reactions and play a significant role in the regulation of transcription\textsuperscript{318}.

Histone acetyltransferases (HATs) are enzymes that acetylate lysine amino acids on histones by transferring an acetyl group from acetyl-CoA to form acetyl-lysine. There are many forms of these enzymes, such as HAT p300, CREB-binding protein (CBP), P300/CBP-associated factor (PCAF), and general control of amino acid synthesis protein 5 (GCN5). They all play a potential pathological role in asthma, chronic obstructive pulmonary disorder (COPD), and different types of cancers as well as learning and memory deficits\textsuperscript{319-320}. It is thought that HAT p300 and CBP regulate expression of tumor suppressor and promoter genes, and PCAF and GCNF perform global acetylations and non-histone acetylations. GCNF is also crucial in cell-cycle progression, which is significant in cancer therapies\textsuperscript{320}. HATs p300 and PCAF often induce increased gene
transcription in airway inflammation disorders such as asthma and COPD, leading to excessive expression of inflammatory genes.

Histone deacetylases (HDACs) are enzymes that remove acetyl- groups from an acetyl-lysine that resides within the NH$_2$-terminal tail of core histones, allowing the histones to wrap the DNA more tightly (Fig. 1.4). To achieve proper development and function, it is essential to not only acetylate and open certain regions of chromatin, but also to deacetylate and close other regions of the genome. HDACs are involved in the regulation of integrated cellular functions in the nucleus and cytoplasm. Hence, HDACs lead to heterochromatin and gene silencing and work in concert with co-activators, corepressors, transcription factors and HATs to change the structure of histones and modulate transcription of genes$^{[322]}$.

So far, eighteen HDACs have been identified in humans, which are subdivided into four structurally and functionally different phylogenetic classes.

Class I HDACs are zinc-dependent proteases, mostly localized within the nucleus and they involve in cellular growth and development, especially playing a role in cell survival and proliferation$^{[323]}$. They remove acetyl groups from specific lysine residues on histones in the nucleosome. This modification affects how tightly DNA is wound around the histone structure, thus HDAC-mediated modifications can lead to induction of gene silencing$^{[324]}$. Class I HDACs are also implicated in regulation by a process involving the methylation of CpG residues in upstream promoter regions of silenced genes. When these CpG residues are methylated, methyl domain-binding proteins (MDBP) actively recruit HDACs (mainly HDAC I, II, and III) to the histone, further inhibiting gene transcription$^{[325]}$.

Classes II HDACs are as well zinc-dependent proteases, shuttle between nucleus and cytoplasm and they are involved in cellular growth and development, in particular with tissue-specific roles$^{[326]}$. They are able to shuttle back and forth between the nucleus and cytoplasm and to remove acetyl groups from proteins other than histones$^{[327]}$.

Class III HDACs, also known as sirtuins, are mainly involved in metabolic processes and require the cofactor NAD$^+$ for their deacetylase function. They and are not targeted by the currently available HDAC inhibitors$^{[328]}$. Additionally, several sirtuins are known to regulate ATP production while others are known to protect against tumorigenesis during oxidative stress.

Class IV is a zinc-dependent protease, comparatively new and less investigated.
Together HATs and HDACs define the pattern of histone acetylation. It has been proposed that histone modifications, acting alone, sequentially, or in combination, represent a "code" that can be recognized by non-histone proteins, which form complexes that are important for regulation of gene transcription. These enzymes do not bind to DNA directly, but rather interact with DNA through multiprotein complexes that include corepressors and coactivators. Class I and class II HDACs form multiprotein complexes containing transcription factors with diverse functions.

Other two kind of histone-modifying enzymes are histone methyltransferases (HMTs) and histone demethylases (HDMs) that add or remove methyl groups, respectively.

These histone-modifying enzymes interact with each other as well as other DNA regulatory mechanisms to tightly link chromatin state and transcription. They may be targeted to specific DNA sequences directly or necessitate the interaction of intermediate group proteins and/or RNAi. In contrast to DNA methylation, it is still unclear how and if histone modifications are correctly replicated during mitosis, and as a result, it should be questioned whether covalent histone modifications are epigenetic marks.

Non-covalent mechanisms, such as nucleosome remodeling and replacement of canonical histone proteins with specialized histone variants, are labile epigenetic regulatory mechanisms and provide the cell with additional tools for introducing variations into the chromatin structure.

In addition to serving as the basic modules for DNA packaging within a cell,
nucleosomes act as ATP-mediated chromatin-remodeling complexes. Both the sliding of already existing and incorporation of new nucleosomes may regulate gene expression by altering the accessibility of regulatory DNA sequences to transcription factors\textsuperscript{[255]}. Precise positioning of nucleosomes around gene promoters\textsuperscript{[335]} correlates with either gene silencing or gene activation by modulating chromatin accessibility\textsuperscript{[336]}. The interaction of nucleosome remodeling machinery with methyl CpG-binding protein MeCP2 and histone methyltransferases plays a crucial role in establishing global gene expression patterns and chromatin architecture\textsuperscript{[337]}. In addition to physical alterations in nucleosomal positioning via nucleosome remodelers, the incorporation of histone variants, e.g. H3.3 and H2A.Z, into nucleosomes also influences nucleosome occupancy and thus gene activity\textsuperscript{[338]}. Unlike the major histone subtypes whose synthesis and incorporation is coupled to DNA replication in S phase, these variants are synthesized and incorporated into chromatin throughout the cell cycle\textsuperscript{[339]}. H3.3 and H2A.Z are preferentially enriched at promoters of active genes or genes poised for activation and can mediate gene activation by altering the stability of nucleosomes\textsuperscript{[340]}. H2A.Z incorporation may also contribute to gene activation by protecting genes against DNA methylation\textsuperscript{[341]}. Acetylated H2A.Z associates with euchromatin and ubiquitylated H2A.Z with facultative heterochromatin. Like canonical histones, histone variants undergo various posttranslational modifications, which determine their nuclear localization and function\textsuperscript{[250]}.  

1.2.2.4 Non-coding RNAs. It is debatable whether the role of non-coding (ncRNAs) constitutes an epigenetic phenomenon. Some researchers will claim that non-coding RNAs are a fundamental part of nature coding and do not satisfy the definition of epigenetics. However, the majority of them feel that since ncRNAs do indeed affect regulation of genes, they are a \textit{bona fide} mechanism of epigenetic change\textsuperscript{[315]}. The family of ncRNAs is diverse and complex. It can be divided into eight groups: ribosomal (rRNAs), transfer (tRNAs), micro (miRNAs), long non-coding (lncRNAs), small nucleolar (snRNAs), small interfering (siRNAs), small nuclear RNAs (snRNAs) and piwi-interacting (piRNAs). In general, ncRNAs are important epigenetic regulators in development and disease, especially miRNAs and lncRNAs. DNA information is transcribed into RNA molecules, of which only about 3% are translated into proteins through with messenger RNA (mRNA); the remaining is considered ncRNAs. Some of these ncRNAs are functional molecules, such as rRNAs and tRNAs, which play different roles in mRNAs translation, and snRNAs, which are
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involved in RNA splicing. Other ncRNAs are instead implicated in gene regulation, by tampering with different phases of the full process, including mRNA expression, maturation, degradation and translation\textsuperscript{[342]}. Regulatory ncRNAs might affect gene expression by altering chromatin conformation\textsuperscript{[343]}, or by triggering histone modifications and DNA methylation\textsuperscript{[258]} and thus, they are involved in controlling multiple epigenetic phenomena, in almost all eukaryotes.

**miRNAs** are short non-coding RNA sequences (21-25 single-stranded nucleotides) that regulate gene expression by targeting specific mRNAs for degradation. In particular, they act binding to complementary sequences in the 30 UTR of multiple target mRNAs, leading to translational repression (imperfect sequence match) or mRNA cleavage (perfect match)\textsuperscript{[313]}. It has been recently discovered that they can also recruit chromatin-modifying factors, altering chromatin conformation\textsuperscript{[344]}. Since the first miRNA was characterized in 1993, an increasing number of other miRNAs have been identified. Altered expression profiles of miRNAs in patients revealed their crucial role in cellular events and the development of diseases\textsuperscript{[345]}. They are expressed in a tissue-specific manner and control a wide array of biological processes including cell proliferation, apoptosis and differentiation. miRNAs show an extensive role in maintaining global gene expression patterns\textsuperscript{[346]}. Like normal genes, the expression of miRNAs can be regulated by epigenetic mechanisms\textsuperscript{[347]}. In addition, miRNAs can also modulate epigenetic regulatory mechanisms inside a cell by targeting enzymes responsible for DNA methylation and histone modifications\textsuperscript{[348]}.

**lncRNAs** are functional ncRNAs, each exceeding 200 nucleotides in length and lacking functionally open reading frames. They regulate gene expression through different molecular mechanisms. lncRNAs can mediate the activity of proteins involved in chromatin remodeling and histone modification, or act as an RNA decoy or sponge for miRNAs. They can also bind to specific protein partners to modulate the activity of that particular protein\textsuperscript{[349]}. Recent advancements in technology to identify lncRNAs using microarrays provide a great bulk of novel data from genomewide studies, and have revealed potential use of ncRNAs as diagnostic and prognostic biomarkers in various human disorders including skin diseases\textsuperscript{[350]}. A classic example of lncRNA involved in gene silencing by heterochromatin formation is \textit{Xist}, which has a key role in the X chromosome inactivation process\textsuperscript{[351]}. However,
beyond *Xist* also other lncRNAs have been demonstrated to have a role in altering chromatin structure[352].

Non-coding RNAs often act in concert with various components of the cell's chromatin and DNA methylation machinery to achieve stable silencing. Several of these RNAs, and particularly the repeat-associated siRNAs, are clearly epigenetic, as they can induce long-term silencing effects that can be inherited through cell division[353]. Such interaction among the various components of the epigenetic machinery re-emphasizes the integrated nature of epigenetic mechanisms involved in the maintenance of global gene expression patterns.

### 1.2.3 Epigenetics in cancer

Epigenetic mechanisms are essential for normal development and maintenance of tissue-specific gene expression patterns in mammals and, therefore, their disruptions can lead to altered gene function and malignant cellular transformation. The initiation and progression of cancer, traditionally seen as a genetic disease, is now involved in epigenetic abnormalities along with genetic alterations[250]. After several studies, it is well known that the precise epigenomic landscape found in normal cells may undergo extended distortion in tumors and its global change is thus a hallmark of cancer. Moreover, the transition to transformed cells during tumorigenesis involves an extensive reconfiguration of the genome's expression program. Therefore, malignant cells present aberrant somatic reprogramming leading to gene silencing through formation of a compact repressive chromatin structure that results in reduced cellular plasticity. The epigenetic modifications, along with widespread genetic alterations[354], play an important role in tumor initiation and progression[247]. Both alterations interact at all stages of cancer development, working together to promote its development[355].

The cancer epigenome is characterized by global changes in DNA methylation and histone modification patterns as well as altered expression profiles of chromatin-modifying enzymes. DNA methylation (especially *de novo* hypermethylation) and histone modifications (mostly methylated histone H3 at K9) work independently and in concert to alter gene expression during tumorigenesis. Hence, the epigenetic changes result in global dysregulation of gene expression profiles leading to the development and progression of disease states[258]. Epi-changes also can lead to silencing of tumor
suppressor genes independently and in conjunction with deleterious genetic mutations or deletions; thus, serving as the second hit required for cancer initiation according to the "two-hit" model proposed by Knudson[356]. In addition to inactivating tumor suppressors, epigenetic variations can also promote tumorigenesis by activating oncogenes. Nevertheless, since epigenetic alterations, like genetic mutations, are mitotically heritable, they are selected for in a rapidly growing cancer cell population.

Epigenetic regulation was first confirmed to occur in human cancer in 1983 by Feinberg and Vogelstein, using primary human tumor tissues[357], and it has since been observed in many other illnesses and health conditions[351]. Researchers found that diseased tissue from patients with colorectal cancer had less DNA methylation than normal tissue from the same patients. Furthermore, Ushijima and colleagues recently proposed to consider epigenetic mechanisms as one of the five most important causal issues in the cancer field, accounting for one-third to one-half of known alterations[358], indicating that epigenetic changes may be more common in human cancer than DNA sequence mutations[305].

1.2.3.1 DNA methylation aberrations. The intricate organization of DNA methylation and chromatin conformation, known to regulate the normal cellular homeostasis of gene expression patterns, somehow becomes unrecognizable in almost all types of cancers, leading to aberrant gene expression profiles[359]. Environmental exposure to nutritional, chemical, and physical factors may stably modify gene expression through aberrant methylation of CpG islands, such as at the promoter regions of "housekeeping" genes, transposable elements adjacent to genes with metastable epi-alleles, and regulatory elements of imprinted genes[3]. Most human cancers have been found to host global DNA hypomethylation, including hypomethylation of repetitive elements, coupled with an increased de novo methylation, and subsequent hypermethylation of CpG islands, at specific tumor suppressor gene promoters[360]. A tumor epigenome is thus marked by genome-wide hypomethylation and site-specific CpG island promoter hypermethylation[247] leading to transcriptional repression and then, the abnormal increase in de novo methylation can be critical factors in cell transformation[361]. Furthermore, promoter hypermethylation of CpG islands plays a causal role in the initiation and the progression of cancer, through the transcriptional silencing of critical growth regulators and tumor suppressor genes[362], including RB1, p16INK4a, MLH1 and BRCA1[363], and genes controlling the immune response and drug sensitivity[364]. This abnormality is the trademark epigenetic change and happens early in
the development of cancer\textsuperscript{[258]}. Therefore, pathological patterns of DNA methylation are consistently found in human tumors, including both widespread genomic hypomethylation and regions of abnormally increased methylation. Because methylated genes are typically turned off, loss of DNA methylation is thought to cause abnormally high oncogene activation by altering the arrangement of chromatin, whereas hypermethylation is proposed to silence protective tumor suppressor genes\textsuperscript{[365]}.

Under normal conditions, CpG islands are unmethylated and their hypermethylation, during carcinogenesis, is hypothesized to have profound effects in mammalian gene expression\textsuperscript{[359]}. In fact, it has been estimated that aberrant methylation of cytosine residues, within these CpG islands, is the single most common lesion in cancer cells even when compared to the overall rate of both mutations and cytogenetic abnormalities\textsuperscript{[366]}. Although about 60\% of all genes in humans are associated with unique CpG islands\textsuperscript{[362]}, aberrant methylation is initiated at approximately 1.4\% of 45000 CpG islands in the human genome, and may continue to accumulate to as many as 10\% of these islands during tumor development\textsuperscript{[367]}. The aberrant hypermethylation indeed leads to gene silencing because promoters become embedded in heterochromatin and they are surrounded by tightly compacted nucleosomes, which contain deacetylated histones impeding transcription.

In Rb, CpG island hypermethylation occurs early and correlates with loss of transcription\textsuperscript{[368]}. For these reasons, an over-expression of all DNMTs was described in several cancer types, suggesting that methyltransferase cooperativity (specifically between DNMT1 and DNMT3B) maintains DNA methylation and gene silencing in human cancer cells\textsuperscript{[362]}.

In addition to direct inactivation of tumor suppressor genes, DNA hypermethylation can also indirectly silence additional classes of genes by silencing transcription factors and DNA repair genes, leading to inactivation of their downstream targets and allowing cells to accumulate further genetic lesions, respectively. Furthermore, gene silencing may also be in part mediated by elevated occupancy of methyl-DNA-binding proteins\textsuperscript{[369]} that recruit in turn transcriptional repressors and render histones hypoacetylated at promoter regions.

Other examples of repression mediated by hypermethylation include silencing of miRNA that in turn results in upregulation of proteins regulated by miRNA or deregulation of non-coding RNA\textsuperscript{[370]}.

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While evidence has strongly linked tumor-specific CpG island hypermethylation to cancer development, via silencing tumor suppressor genes, there is as well a strong correlation between global genomic hypomethylation and carcinogenesis. DNA hypomethylation leads to aberrant activation of genes and non-coding regions through a variety of mechanisms that contributes to cancer development and progression, increasing abnormal chromosomal instability and activating transcriptional regulation of proto-oncogenes\cite{371}. Therefore, global DNA hypomethylation plays a significant role in tumorigenesis and occurs at various genomic sequences and large chromosomal domains located in lamin-associated regions\cite{372}, including repetitive elements, retrotransposons, CpG poor promoters, introns and gene deserts\cite{373}. In tumors, a general reduction of approximately 10% of the 5-methylcytosines (5-mC), also known as the "fifth base", was described: a high 5-mC content of CpG islands in tumor suppressor genes as well as an increased global DNA hypomethylation status both contribute to the carcinogenic process\cite{293}. It has been estimated that malignant cells have 20-60% less 5-mC content than their normal counterparts, with approximately 70-80% of those 5-mC residues occurring within rich CpG sequences\cite{359}. The presence of such 5-mC amount has been strongly implicated in mutagenicity, partly because of its ability to promote deamination of cytosine bases to uracil ones. In fact, 5-mCs are estimated to deaminate at a rate of two to four times more rapidly than their cytosine equivalent\cite{374}. Recently, another modified form of cytosine, 5-hydroxymethylcytosine (5-hmC), has been identified and is now recognized as the "sixth base" in the mammalian genome\cite{375}. This novel independent epigenetic marker is derived from 5mC in a process catalyzed by ten-eleven translocation (TET) enzymes\cite{376} and it acts not only as an intermediate in the DNA demethylation process, but also playing an important role in the regulation of gene expression in severe diseases, such as Rett syndrome and cancer\cite{377}. Loss of DNA methylation at repeat sequences accounts for about 20-30% in the human genome\cite{378} and it leads to increased genomic instability by promoting chromosomal rearrangements\cite{379}, activation of retrotransposons and translocation to other genomic regions\cite{380}. One of the most well established epigenetic markers is the hypomethylation-induced activation of proto-oncogenes, including \textit{c-Myc} and \textit{H-ras}, which has been strongly linked to an increased risk of many cancers\cite{359}. A frequently occurring consequence of abnormal DNA methylation, both hyper- and hypo-, is an aberrant expression of enzymes involved in these processes. Genetic mutations of enzymes involved in DNA methylation has been recently been reported in
human cancer, thus emphasizing the synergic contribution of genetic changes and epigenetic alterations to the development of the disease[313].

1.2.3.2 Changes in histone modifications. In higher eukaryotes, modifications in DNA methylation patterns, although important, are not sufficient to induce cellular transformation and subsequent induction of tumor. Recent advances in high-throughput sequencing have enabled genome-wide mapping of chromatin changes occurring during tumorigenesis. These studies have revealed a global loss of histone acetylation, which is mediated by HDACs, resulting in gene repression[381]. Similarly to what seen for methylation enzymes, HDACs are often found overexpressed in various types of cancer[382] and HATs, which work in concert to maintain histone acetylation levels, can also be altered. HDAC activity is recruited by co-repressor proteins to certain regions of the chromatin and the consequent aberrant histone acetylation is in fact responsible for the pathogenesis of certain cancers[383].

In addition, tumor cells also display widespread changes in lysine and arginine histone methylation patterns and they are associated with aberrant silencing of tumor suppressor genes[384]. Both HMTs and lysine specific-demethylases work in coordination to maintain global histone methylation patterns and their dysregulation results in altered distribution of these marks in cancer and leads to cancer progression.

1.2.3.3 Other epigenetic mechanisms. The global epigenetic alteration in tumor involves other epigenetic mechanisms, like nucleosome remodeling and histone variants, miRNAs deregulation and epigenetic switching, that work together with DNA methylation and histone modifications in the changes of chromatin structure.

*Nucleosome remodeling and histone variants* are labile epigenetic regulatory mechanisms and play a central role in both tumor-specific gene silencing and gene activation by modulating chromatin accessibility. MBD proteins may involve DNA methylation-induced silencing of tumor suppressor genes in cancer. It concerns distinct changes in nucleosome positioning, resulting in nucleosome occupancy at transcription start site. Nucleosome remodeling can also lead to aberrant gene silencing via the transmission of repressive epigenetic marks to tumor-suppressor gene promoters.

A significant proportion of methylated promoters are bound by a single MBD protein, most often MBD2[385].

For instance, the SWI/SNF complex is a multi-subunit ATP-dependent chromatin-
remodeling complex\textsuperscript{[386]}, whose aberrations are associated with cancer development\textsuperscript{[387]} and abrogation of SWI/SNF function through alterations in its various subunits is observed in around 20\% of human cancers\textsuperscript{[388]}, suggesting that this complex may have a functional role in preventing tumor development in diverse tissues.

In addition to remodeling complexes, the histone variant H2A.Z is overexpressed in several types of tumor and has been associated with the promotion of cell cycle progression\textsuperscript{[389]}.

\textit{miRNAs deregulation} is responsible for a widespread change in miRNA expression during tumorigenesis\textsuperscript{[390-391]}. Since miRNAs regulate genes involved in transcriptional regulation, cell proliferation and apoptosis, alteration in their expression can promote tumorigenesis. miRNAs can function as either tumor suppressors or oncogenes depending upon their target genes. Many tumor suppressor miRNAs that target growth-promoting genes are repressed, whereas oncogenic miRNAs, which target growth inhibitory pathways, are often upregulated in cancer\textsuperscript{[392]}.

Changes in miRNA expression can be achieved through various mechanisms including chromosomal abnormalities, transcription factor binding and epigenetic alterations\textsuperscript{[393]}.

\textit{Epigenetic switching} may be performed in tumor cells for gene silencing. The detailed mechanisms by which discrete genome regions undergo hyper- or hypomethylation are still unclear. Early evidence suggested that elevated DNMT levels might trigger hypermethylation of tumor suppressor gene promoters, which would consequentially result in cancer cell proliferation\textsuperscript{[394]}. In addition to this "selection" model, an alternative mechanism has been proposed that takes advantage of the current genome-wide epigenetic studies in stem cells. Investigators have suggested that the establishment of aberrant epigenetic profiles in cancer may undergo a process that is similar to epigenetic reprogramming during development\textsuperscript{[259]}. In embryonic stem cells, developmentally important genes are reversibly silenced by polycomb proteins that are able to remodel chromatin structure for gene silencing, through the establishment of the repressive mark. After differentiation of normal cells, these genes continue to be inhibited, due to polycomb-group proteins associated with H3K27m, that work independently of DNA methylation to aberrantly repress genes in cancer cells\textsuperscript{[395]}.

Controversially, during cancer initiation the promoters might become methylated and thereby set up for long term silencing. The reversible gene repression by polycomb mark is so replaced with long-term silencing by \textit{de novo} DNA methylation, possibly performed
through the recruitment of DNMTs via the polycomb complex\textsuperscript{[396]}. This tumor-specific "epigenetic switching" of the plastic polycomb mark with more stable DNA methylation results in the permanent silencing of key regulatory genes that may contribute to cell proliferation and tumorigenesis\textsuperscript{[397]} and it is involved in the gene silencing through formation of a compact chromatin structure and reduced cellular plasticity.

1.2.3.4 Epigenetics in retinoblastoma. Recent whole-genome sequencing of Rb uncovered a tumor that had no coding-region genetic mutations or focal chromosomal lesions other than in the \textit{RB1} gene. Consequently, the overall mutational rate and number of structural variations resulted one of the lowest seen in human cancer to date, indicating Rb to be characterized by a relatively stable genome\textsuperscript{[398]}. It becomes clear therefore that epigenetic alterations of multiple tumor-promoting pathways is required for the development of retinoblastoma and that epigenetics, rather than the classical genetic model, fits the variegated phenotypic expression of the disease, opening new outstanding scenarios in the fields of diagnosis, treatment and prevention. The epigenetic model of retinoblastoma development emphasizes the role of environment and its interaction with the genome. For instance, it is possible that certain chemical compounds or low levels radiation can initiate biological perturbations that can lead to malignancy, despite being weak mutagens or lacking mutagenic activity altogether\textsuperscript{[399]}. Environmental toxicants, including radiations, wrong diets, and infectious diseases also play a major role in conditioning the degree of DNA methylation in embryos during pregnancy, thus leading to stable, functional alterations of the genome, which can be also transmitted through generations, thus mimicking a hereditary disease\textsuperscript{[3]}.

In addition, both covalent/non-covalent modifications of DNA and histone proteins changes can influence the overall chromatin structure. Rb also may develop quickly because of the epigenetic deregulation of key cancer pathways as a direct or indirect result of \textit{RB1} loss\textsuperscript{[200]}.

\textit{Methylation} described for Rb tumorigenesis induce silencing of both alleles of tumor suppressor gene \textit{RB1} and other genes such as \textit{MLH1} and \textit{RB2/p130}\textsuperscript{[400]}, resulting in complete loss of function. Aberrant methylation of CpG island of the promoter region of the additional genes such as \textit{RASSF1A}\textsuperscript{[401-402]}, \textit{MGMT}\textsuperscript{[401,403]} and \textit{p16}\textsuperscript{INK4A} (\textit{CDKN2})\textsuperscript{[404]}, \textit{LDHA}\textsuperscript{[405]}, \textit{NEUROG1}, \textit{DAPK}, \textit{RUNX3}\textsuperscript{[406]}, \textit{APC-2}\textsuperscript{[407]} and \textit{TFF3}\textsuperscript{[408]} has also been shown in Rb. Recently, Livide and colleagues distinguished a novel set of hypermethylated genes in Rb, including \textit{MSH6}, \textit{CD44}, \textit{PAX5}, \textit{GATA5}, \textit{TP53}, \textit{VHL},
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Moreover, Zhang and colleagues identified fifteen cancer-related genes that were deferentially expressed and which also exhibited correlative histone modifications in Rb, when compared to normal tissue. In particular, downregulated genes with associated inactivating histone modifications included CTNNL1, SRY, SOX2 and ADAMTS18.

Methylation of the promoter region of RB1 has been first demonstrated in 1989 by Greger and colleagues, who identified CpG 106, an island overlapping the promoter and exon 1, to be methylated in some retinoblastomas, thus silencing gene expression. Since then, multiple CpG islands within the RB1 promoter and gene have been identified and characterized in Rb, demonstrating an epigenetic component to gene inactivation and subsequent development of the tumor. Methylation of the RB1 promoter occurs in about 10% of unilateral, sporadic retinoblastoma tumors, leading to loss of function.

RB1 is one of "housekeeping" gene, and its function is to regulate cell growth by preventing cells from dividing too fast or in an uncontrolled way. The promoter region of RB1 lies 185-206 bp upstream of the initiation codon and contains putative binding sites for the transcription factors RBF-1, Spl, ATF, and E2F. The RB1 gene has a small CpG island that encompasses the promoter, and this cluster remains unmethylated in all tissues during development. However, both in vitro and in vivo studies have suggested that partial CpG methylation decreased promoter activity to intermediate levels, whereas abnormal hypermethylation of the RB1 promoter dramatically reduces RB expression. Decreased RB level has been found particularly in sporadic retinoblastoma that, on the other hand, is the most commonly accepted form of non-hereditary disease, suggesting that Rb is an epigenetic, rather than a genetic disease. Interestingly, as a key gene in cell cycle control, RB1 has been found aberrantly methylated, alone or together with other cell cycle regulating genes in different types of cancers.

Methylation of transposable elements adjacent to the genes with metastable epialleles is a second epigenetic modification occurring during retinoblastoma development. Metastable epialleles are defined as rare gene loci that can be epigenetically modified in a variable and reversible manner, so that a distribution of phenotypes can occur from genetically identical cells. The phenotypic expression of retinoblastoma is not only highly variable, but can also be
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Epigenetics, by looking at gene expression as the result of the functional interaction between genes and the environment, admits that the resulting phenotype could be regulated and consequently it exhibits different degrees of variability and plasticity. Alterations in phenotypic expression can be also explained by the presence of transposons, repetitive transposable elements dispersed throughout the mammalian genome, that are epigenetic silenced by the methylation of their CpG islands. The epigenetic state of a subset of transposable elements is metastable; these mobile elements are variably expressed in genetically identical individuals due to epigenetic modifications occurring during the early development. In contrast with other regions of the human genome, the epigenetic changes occurring at the insertion site of transposable elements can be considered as a stochastic event, which causes individual variation. Therefore, given their role in silencing genes and their variability within the same individual, transposons are responsible for both inter- and intra-individual variations in phenotypic expression of the same genes within different cells of the same organism, thus leading to aneuploidy and/or epigenetic cellular mosaicism.

By adopting the epigenetic model, phenotypic variation in the clinical expression of retinoblastoma is easily explained by the variable exposure of the fetus to environmental toxicants that, in turn, may determine the degree of hypomethylation of different key genes. Within this conceptual framework, Rb mosaicism can be viewed as the result of the interaction between the environment and the transposable elements of the genome.

Methylation of regulatory elements of imprinted genes is another epigenetic modification occurring during retinoblastoma development. Imprinting is defined as a non-Mendelian, germline inherited epigenetic form of gene regulation involving heritable DNA methylation and histone modification. The human genome is subject to imprinting, which represents the consequence of epigenetic inactivation of different genes in either the male or the female gametes, so that in the resulting zygote they complement each other, and the normal embryo development proceeds. Because imprinted genes are epigenetically modified through the methylation of CpG islands in both the male and female gametes, the expression of different genes in the zygote, embryo, and fetus, derived from the fusion of the two, will depend on the parental environment in which both gametes have grown and differentiate.

Abnormal expression of imprinted genes during development may result in severe pediatric disorders, where epigenetic alterations have an important contributory or
causative role. Moreover, imprinted gene dysregulation can also occur in somatic cells, by either epigenetic modifications or genetic mutations, causing cancer. In Knudson's hypothesis, inheriting an imprinted \textit{RB1} gene means that one copy of the gene is already functionally inactivated, representing the first "hit", and only a single event is further requested for both copies to be inactivated. Recent data show that \textit{RB1} gene is imprinted in retinoblastoma with a shift of expression in favor of the maternal allele\cite{418}.

\textit{Gene co-expression profiling and new markers for retinoblastoma.} A new way to investigate possible new genomic markers involved in a multifactorial disease such as retinoblastoma is to analyze the microarray-based differential gene expression (DGE) reporting evidence for up- or downregulated gene sets. A recent paper analyzed microarrays data from Weri-Rb1 before and after treatment with DAC, using network analysis to design a map of gene-gene interaction of epigenetic pathways regulated by DNA methylation. Potentially epigenetically regulated biomarkers were identified and their time-related profile was studied to decipher their involvement in the pro-apoptotic effect of DAC. Selected gene methylation analysis was performed to confirm the direct regulation of the treatment on those hypermethylated genes responsible for cancer associated silencing: DAC treatment of the Weri-Rb1 cell line induced the re-expression of \textit{RB2} and its related pro-apoptotic \textit{E2F1}, \textit{p73} and \textit{p53} genes, thus highlighting a crucial role of epigenetic events\cite{195}.

This kind of newtwork integration between experimentally collected evidence and statistical inference study also hold potential for predictive analysis indicating possible co-expression gene associations that would not be predicted otherwise, to unveil relevant pathway cross talks, and to identify unexpected correlative or causal relationship because of non-canonical gene interplay\cite{419}.

1.2.4 Integrated epigenome analysis for personalized therapeutic targets

It has been described as genome and epigenome alterations may cooperate to promote oncogenic transformations. The disruption of epigenomic control is pervasive in malignant conditions. Recent innovations in chromatin immunoprecipitation technology, paired with microarrays and high-throughput sequencing, have enabled unprecedented insights into protein-DNA interactions and chromatin architecture in a wide range of
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biological models, and particularly in cancer-related ones. Recently, the development of Next Generation Sequencing (NGS) technology allowed a deep investigation on the emerging role played by epigenetics in cancer.\textsuperscript{[420]}

NGS allows drawing a precise landscape of gene expression where the epigenetic variations can be targeted and reversed, suggesting a rationale for cancer therapies aimed to achieve epigenetic reprogramming. It follows that epigenetic changes in protein-coding genes and nc RNAs activity form a sort of signature to the genetic landscape of each individual, revealing different molecular mechanisms responsible for cancer development. The identification of such expression signatures can further characterize patient groups according to diagnostic, prognostic and therapeutic factors.

If one pushes the concept further, it should be expected therefore that each tumor type might have, for example a characteristic DNA methylation pattern. In addition, it could be uncertain whether altered DNA methylation patterns are unique for a specific tumor type or instead comparable across different types of cancers. Recently, an atlas of DNA methylation was generated across a variety of samples from several cell lines and tissues, providing insight on gene regulation aspects and disease that lead to the identification of methylation signatures in part cancer-associated and in part cell-type specific.\textsuperscript{[286]}

High-throughput techniques, including microarray and sequencing-based technologies, provide genome-scale whole methylomes, confirming the role of aberrant methylation as a cancer hallmark. This has allowed identifying novel types of biomarkers and is expected to shed light over new cancer subtyping.\textsuperscript{[420-421]}

From these studies, it emerges that although chromatin regulation and thus control of gene expression have been central to epigenetic drug developments; other epigenetic mechanisms have therapeutic potential: "Big Omics Data" analysis is destined to reveal the role for new actors in cancer, such as the usually lowly expressed lnc RNAs, which are detected in wide-spectrum transcriptome profiling.\textsuperscript{[422]} This new development will expand cancer phenotyping, through increased marker detection, deeper sub-typing and more stratified methylation. This will also provide new candidate targets, together with generating hypotheses for innovative clinical trials design.

Understanding the mechanisms of anticancer activity of epi-drugs, such as inhibitors of methyltransferase or deacetylase, is essential for drug design in targeted therapies. Knowledge of the involved patterns of activity is needed to design optimized clinical protocols as well as to develop new classes of drugs. In this view, the screening of already approved library of epigenetic active molecules could represent a cost-effective...
approach to discovery suitable candidates for alternative clinical applications\textsuperscript{423}. This new approach is used in "Disease stratification studies" that aims at identifying patterns of epigenetic variation such as DNA methylation, to correlate the expression of these altered pathways with an increased risk of cancer development, therefore pinpointing specific driver mutations. Such investigations are mostly taking place during clinical trials, at a stage when patient cohort genetic profiles might indicate shared epi-mutations to be targeted with specific drug. Editing the epigenome and its built-in players is the new frontier of drug discovery, the one shaping the road map of personalized medicine in cancer\textsuperscript{424}.

Progresses in NGS and Omics approaches have allowed systematic analysis and identification of novel epigenetic marks (top-down mapping of epigenetic changes). The most salient achievement is however to define epi-modification in space to identify landmark distribution across the genome. For this, computational analysis is used with a bottom-up epigenetic investigation to map the spatial reconstruction of localized changes on the epigenome searching for possible markers. Computational efforts are currently directed towards building integrative comparative analyses of multi-platform outsourced datasets, paving the way for the design of algorithmic pipelines aimed at detect clinically relevant biomarkers and to provide more accurate patient stratification.

\textbf{1.2.5 Epigenetics in cancer drug resistance}

In recent years, the different classes of drugs and regimens used clinically have provided an improvement in tumor management. However, cancers have the ability to develop resistance to traditional therapies and thus, treatment is often palliative for the majority of cancer patients. Tumors often are made of a mix of malignant cells, some of which are drug sensitive while others resist to drugs. Therefore, transformed cells respond poorly to chemotherapy mainly due to the development of the multi-drug resistance phenotype. Response to treatment does not generally result in complete remission and disease cure is uncommon for patients presenting with advanced stage cancer. Consequently, the remaining resistant cells make the tumor grow again, causing chemotherapy failure. The development of drug resistance is in fact one of the major challenges in cancer therapy and a limiting factor in patient survival\textsuperscript{425}.

There are several categories of mechanisms that can enable or promote direct or indirect
drug resistance in human cancer cells. These mechanisms can act independently or in combination and through various signal transduction pathways and are classified as: drug inactivation, alteration of drug targets, drug efflux, DNA damage repair, cell death inhibition, epithelial-mesenchymal transition and metastasis and cancer cell heterogeneity. Both intrinsic and acquired drug resistance in tumors occur as a result of an alteration in gene expression, which, in some cases, is due to aberrant epigenetic regulation.

Epigenetic alterations probably contribute to chemo-resistance at several steps of drug response pathways in tumors. The two coexisting processes of global hypomethylation and regional hypermethylation confer a selective advantage by turning on and off different sets of genes with opposite roles. Hypermethylation of CpG islands at specific tumor suppressor gene promoters causes the downregulation of genes whose products are required to suppress tumorigenesis, while global hypomethylation induces activation of genes with crucial roles in the transformation process. It is obvious that single gene alteration is sufficient neither for cancer development nor for drug resistance acquirement, but it is rather the concerted work of several genes that eventually determines the cell state and the drug fate inside the tumor cell[426].

Successful treatment of cancer requires a clear understanding of chemotherapeutic resistance. Therefore, one major research goal is to identify the critical genes involved in chemotherapy response to predict the best therapy option for patients, and numerous studies have been suggested a direct role for epigenetic gene inactivation in determining tumor chemosensitivity[427-430]. Likely for us, the majority of the epigenetic mechanisms are reversible, and researchers may be able to take advantage of this opportunity to develop treatments that can counteract drug resistant malignancies[431].

Recent studies suggest that epigenetic alterations, mainly due to DNA methylation, play a role in the development of acquired multidrug resistance. For instance, methylation at MDRI promoter increases drug resistance, making it an excellent target for epigenetic treatment. Moreover, the tumor-suppressor gene $p73$ is generally silenced by epigenetic alterations and it is related to poor response to therapy[432-433]. Besides, several studies have demonstrated that some pro-apoptotic genes, such as $ApaF-1$[434] and $CASP8$, are frequently inactivated by methylation[435]. Their expression can be restored by treatment with DNMTs inhibitors, which can increase chemosensitization. Demethylating agents in fact might be useful in sensitizing multidrug resistant cancer cells to other types of drugs[431], enhancing chemosensitivity.
Epigenetic mechanisms can also influence DNA damage repair. For example, inactivation of *FancF*, through promoter methylation or ncRNA, has been observed in many malignancies\(^4\). Moreover, DNA mismatch repair processes can be lost due to hypermethylation of *MLH1* gene promoter, that is also important to determine chemotherapeutics sensitivity, and this can lead to tumor development. In these cases, treatment with demethylating agents was used to decrease methylation level and to restore tumor sensitization to other drugs, including cisplatin, carboplatin, temozolomide and epirubicin\(^4\).

The DNA repair enzyme MGMT inhibits the killing of tumor cells by alkylating chemotherapy agents. Methylation of *MGMT* causes gene silencing and decreased MGMT production. Epigenetic alteration of *MGMT* expression has been associated with a modified chromatin configuration. Cells can acquire resistance to a methylating agent, by either reactivating a previously silenced *MGMT* gene, or by repressing the *hMSH6* mismatch repair gene. The number of active MGMT molecules at the time of methylation determines the capacity of a cell for MGMT repair. Treatment with chemical methylating agents alters gene expression patterns by increasing genomic DNA methylation, which ultimately leads to increased repair or tolerance of O6-methylguanine and the emergence of chemotherapy resistance\(^4\).

Epigenetically mediated forms of drug resistance are observed in several cancers. The first experimental evidences were collected in the early 2000: for example, methotrexate resistance in breast cancer cells is caused by an inherent defect in drug uptake and a lack of reduced folate carrier (RFC) expression. In one study, the treatment of these cells with a methylation inhibitor improved methotrexate uptake but also restored RFC expression, which promoted methotrexate efflux. These results suggest that the demethylating agent counteracts some methotrexate-resistance mechanisms while improving others\(^4\). Melanoma cells can also exhibit drug resistance via epigenetic mechanisms. They are notoriously unresponsive to conventional chemotherapy and can be resistant to fotemustine. This acquired resistance is associated with high MGMT activity and the *MGMT* gene appeared hypermethylated. However, these cells were effectively sensitized when treated with demethylating agent\(^4\).

As well, drug resistance in melanoma cells seems to be due, at least in part, to *Apaf-1* inactivation through epigenetic alterations. This gene encodes for a cell-death effector that binds to cytochrome C to activate Caspase-9 and induces apoptosis. Some other
studies, involving malignancies spanning from myelodysplastic syndromes to solid cancers, have demonstrated that Apaf-1 expression can be restored by treatment with DNA methylation inhibitors and that return of physiological levels of this cell-death effector enhances chemosensitivity\[440\]. Similarly, in various tumors also Caspase-8 is frequently inactivated by methylation and treatment with demethylating agents can restore its expression, increasing chemosensitization\[435\]. Additionally, some prostate cancers exhibit androgen resistance that may be due to transcriptional inactivation of the androgen receptor gene caused by DNA methylation. Cytosine DNA methyltransferase inhibitors have been found to restore androgen responsiveness in androgen-refractory tumor cells, though, and these cells are then responsive to growth inhibition by anti-androgens\[441\]. Overall, as epigenetic alterations have been increasingly recognized as a cause of drug resistance in many different kinds of cancer, thus, epigenetic therapy could be utilized as a priming therapy to sensitize drug-resistant cancer cells in conjunction with conventional and targeted chemotherapy.

1.2.6 Epigenetic therapy

Unlike specific mutations that permanently turn off gene expression, epigenetic promoter methylation and histone modifications are potentially reversible processes. Therefore, epigenetic abnormalities involved in tumor progression provide the rationale for the development of an "epigenetic therapy" as a treatment option to restore the abnormal epigenome. It has encouraged the use of DNA demethylating and chromatin-remodeling compounds, with the consequent reactivation of silenced genes\[442\]. In the recent past, many epigenetic drugs have been discovered that can effectively reverse DNA methylation and histone modification aberrations occurring in cancer\[250\].

Epigenetic therapy development represents an important part of pharmacology for human cancer and the pharmacoepigenetics is now emerging as a new branch of personalized medicine, which can individually interfere on the patient epigenomic state. In the near future, one can forecast that this customized epigenetic intervention will improve the individual response to the treatment\[443\]. However, to date clinical practice may exploit the two most prominent classes of drugs used in reactivating epigenetically silenced genes, including those that inhibit DNMTs and HDACs enzymes (Table 1.1).
### Chemical Classification of epigenetic drugs

<table>
<thead>
<tr>
<th>Nucleoside Analogue Inhibitors</th>
<th>Non-Nucleoside Analogue Inhibitors</th>
<th>Small Molecules</th>
<th>Antisense Oligonucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemical name</strong></td>
<td><strong>Other names</strong></td>
<td><strong>Chemical name</strong></td>
<td><strong>Other names</strong></td>
</tr>
<tr>
<td>5-azacytidine</td>
<td>AZA (Vidaza)</td>
<td>Procainamide</td>
<td>Procan-SR (Procanbid)</td>
</tr>
<tr>
<td>5-aza-2'-deoxycytidine</td>
<td>Deoxabicline (DAC; Dacogen)</td>
<td>Procanine hydrochloride</td>
<td>Novocan (SP01A)</td>
</tr>
<tr>
<td>5,6-dihydro-5-azacytidine</td>
<td>DHAC</td>
<td>Epigallocatechin-3-gallate</td>
<td>EGCG</td>
</tr>
<tr>
<td>5-fluoro-2'-deoxycytidine</td>
<td>FdCyd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-hydrizinyl-phthalazinylarabinosyl-5-azacytidine</td>
<td>Zebularine</td>
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<table>
<thead>
<tr>
<th><strong>Hydroxamates</strong></th>
<th><strong>Aliphatic Acids (Short-Chain Fatty Acid)</strong></th>
<th><strong>Cyclic Tetrapeptides and Analogues</strong></th>
<th><strong>Benzamides</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemical name</strong></td>
<td><strong>Other names</strong></td>
<td><strong>Chemical name</strong></td>
<td><strong>Other names</strong></td>
</tr>
<tr>
<td>Suberoylanilide hydroxamic acid</td>
<td>Vorinostat (SAHA; Zolinza)</td>
<td>sodium butyrate</td>
<td>Phenylbutyrate</td>
</tr>
<tr>
<td>suberyl-3-aminopyridinamine hydroxamic acid</td>
<td>PXD101 PX105684 Belinostat</td>
<td>Valproic acid</td>
<td>VPA</td>
</tr>
<tr>
<td>Oxamflatin</td>
<td>Metacept 3</td>
<td>AN-9</td>
<td>Pivaloyloxymethyl butyrate</td>
</tr>
<tr>
<td>LAQ824</td>
<td>Dacinostat</td>
<td>OSU-HDAC42</td>
<td>AR-42</td>
</tr>
<tr>
<td>LBH-589</td>
<td>Panobinostat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITF2357</td>
<td>Givinostat</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.1**: Chemical classification of epigenetic drugs.

Here below the most prominent categories are listed.

#### 1.2.6.1 DNMTs inhibitors.** DNMTs inhibitors are among the first epigenetic drugs proposed for use as cancer therapeutics. The remarkable discovery that the mechanism by which some cytotoxic agents lead to the inhibition of DNA methylation and the consequent induction of gene expression that caused differentiation in cultured cells led to the potential exploitation of these drugs in cancer therapy[250]. After these preliminary observations, reactivation of tumor suppressor genes by inhibiting DNMT1 has become a promising strategy for cancer therapy. Several DNA hypomethylating agents are currently being evaluated in preclinical and clinical studies. They belong to different chemical classes and nature: the commonly used DNA demethylating agents include**
cytidine or deoxycytidine analogues, but also antisense oligonucleotides, small molecules and non-nucleoside analogues. Despite their efficacy, most of the DNA hypomethylating agents show severe drawbacks that include molecule instability and relatively high toxicity due to their incorporation into DNA or into both DNA and RNA\cite{444}.

**Nucleoside analogues** are the oldest form of methylation inhibitors, and some of these compounds such as 5-Azacytidine and 5-Aza-2'-deoxycytidine have been Food and Drug Administration (FDA) approved for the treatment of certain cancers\cite{361}. These nucleoside analogs are incorporated into the DNA of rapidly growing tumor cells during replication and they inhibit DNA methylation by anchoring DNA methyltransferases onto the DNA, leading to their depletion inside the cell\cite{258}. They reduce DNA methylation, activating tumor suppressor genes aberrantly silenced in cancer and reestablishing differentiation cycle. However, the ability of these drugs to be incorporated into DNA raises concerns regarding their potential toxic effect on normal cells\cite{250}, therefore providing driving for the study of delivery system that might reduce effective dosage administration.

5-azacytidine (Aza, Vidaza) is a cytidine analogue, with a nitrogen atom in the place of carbon 5. Inside the cell, it is triphosphorylated by uridine cytidine kinase to 5-azacytidine 5'-triphosphate and gets incorporated into RNA and, to a lesser extent, into DNA during replication. It is recognized by DNMTs as a site of action, and the normal reaction involving the transfer of a methyl group begins to take place. The nitrogen group in the fifth position causes the formation of an irreversible DNMT1-Aza linkage, which triggers the degradation of the enzyme and leads to widespread reductions in methylation\cite{445}. Because Aza integrates into DNA during replication, rapidly dividing cancer cells are more susceptible to its effects. Aza has been shown to delay the onset of leukemia\cite{446} and in 2004 it was FDA approved for use in myelodysplastic syndromes (MDS)\cite{447}. However, it is relatively unstable, not available to be taken orally and it can present severe toxic side effects, especially at high doses, when it can cause neutropenia\cite{448}.

5-aza-2'-deoxycytidine (DAC, Decitabine, Dacogen®) is an analogue of deoxycytidine, with a nitrogen atom in the place of carbon 5 (Fig. 1.5). Like Aza, Sorm and co-workers synthesized this compound in 1964\cite{449}. It was initially used as antimetabolite agents in leukemia chemotherapy until its hypomethylating properties were discovered.
DAC can be considered as a prodrug that requires metabolic activation: it is transported into cells by human concentrative nucleoside transporter-1 and converted to active phosphorylated form by deoxycytidine kinase to 5-aza-2'-deoxycytidine-5'-triphosphate\(^{[450]}\). The deoxyribose analogue is incorporated into DNA strands during replication, in place of 5-methylcytosine and it is integrating into the genome of rapidly proliferating cells during the S-phase of the cell cycle\(^{[451]}\). The nitrogen atom in place of a carbon in the 5-position allows the break of interaction between DNA and the DNMT\(^{[452]}\). DNA methyltransferase results in fact inactivated due to covalent bond between the 5-azacytosine ring of DAC incorporated into DNA and the active site of the enzyme. In addition, the irreversible covalent protein adduction precludes the resolution of the complex and it compromises the functionality of DNA and triggers DNA damage signaling, resulting in the degradation of the trapped DNMTs\(^{[453]}\). The result of this process is hypomethylation of DNA and cellular differentiation or apoptosis. Further methylation of cytosine residues is so inhibited, causing the passive loss of cytosine methylation in the daughter cells after replication. Various studies indicated that the efficacy of DAC might be due to the re-expression of genes that control cell apoptosis, cell cycle arrest, cancer testis antigens, MHC molecules and co-stimulation molecules by DNA demethylation. As a result, DAC enhanced the anti-tumor immune response and inhibited tumor development\(^{[454]}\). Clinically, previous observations indicated that higher doses of DAC induce cytotoxicity, while low-dose, prolonged infusion with DAC correlates with clinical response in hematological disorders\(^{[455]}\). In 2006, FDA approved DAC for MDS and promising results have emerged from the treatment of other hematological malignancies, such as acute and chronic myeloid leukemia\(^{[456]}\), even though about half of treated patients reported granulocytopenia\(^{[452]}\) and myelosuppression\(^{[457]}\) as the major adverse effects. Over the past two decades, several other clinical trials have focused on the DAC-based injectable anti-proliferative therapy in patients with solid tumors, indicating that lower doses but high intensity, multi-day, and multi-cycle DAC administration was effective, especially for patients with ovarian cancer\(^{[458]}\), non-small cell lung cancer (NSCLC)\(^{[452,459]}\) and colorectal cancer\(^{[460]}\). Although DAC showed clinical activity as single agent, it shows a better outcome in combination therapies with cytotoxic drugs, molecular targeted agents and other epigenetic agents or immunotherapy. Nevertheless, the drug combinations might generate undesired hematological toxicity. To avoid or reduce this occurrence, the use of relatively low doses of DAC would be desirable. Interestingly, the sequential
administration regimen with DAC given as first agent produced a better outcome than that observed after concurrent administration. DAC effect could in fact reverberate on cancer cells that completed several division cycles. DAC might enhance the sensitivity of the current available agents to tumor cells, overcoming the resistance of molecular targeted therapy, inducing cell reprogramming and activating the immune responses.

DAC is characterized by poor chemical stability, which depends on temperature and pH if free in blood. It is quite unstable; however, its degradation products are thought to have pharmacological effects. These degradation compounds are four guanylurea derivatives (α-pentose, β-pentose, α-hexose, β-hexose isomers) and one formylate derivative. DAC may be classified also in α- and β- anomers and it has a short half-life of 15 to 25 minutes, due to its rapid inactivation. In particular, together with Aza, DAC is metabolized by cytidine deaminase (CDA), the enzyme that renders this drug inactive by converting them into 5-azauridine compounds. The high level of CDA in the human liver and spleen is largely responsible for shorter half-lives of both agents in vitro and in vivo.

Therefore, an increase in CDA activity may reduce efficacy by lowering drug levels and shortening the half-life times.

Furthermore, the oligo dinucleotide SGI-110 contains DAC as a half of the molecule and it has been found to be very effective in inhibiting DNA methylation, but its stability and cytotoxicity is comparable with that of Decitabine alone. This compound could be used for effective delivery and cellular uptake of nucleotide drugs, as it is resistant to cytidine deaminase.

\[ \text{Fig 1.5: 5-Aza-2'}-\text{deoxycytidine} \]

Zebularine is a ribonucleoside analogue that has a mechanism similar to Aza. Once into DNA, the fraudulent bases forms covalent bonds with DNMTs, resulting in the depletion of active enzymes and the demethylation of DNA. Although the drug is not yet FDA approved, it presents good results in mouse models and its possible clinical use has been still under investigation. Zebularine is more stable than Aza and it can inhibit DNA
methylation and induce re-expression of methylation-silenced genes\cite{467}.

Another nucleoside analogue is 5,6-dihydro-5-azacytidine (DHAC), a hydrolytically stable congener of Aza, which in past was tested showing an antileukemic activity in preclinical models\cite{468} and the possibility to restore the estrogen sensitivity in ER-breast cancer\cite{469}. Hydralazine hydrochloride (also known Apresoline) is an antihypertensive agents and a DNMT inhibitor that has been used to hypomethylate T cells in experimental systems\cite{470}.

The nucleoside-like compounds 1-β-D-arabinofuranosyl-5-azacytosine (also called Fazarabine) and 5-fluoro-2'-deoxycytidine (also called FdCyd) are other DNMT inhibitors under investigation in clinical trials\cite{471-472}.

**Antisense oligonucleotides** are also used to inhibit abnormal methylation as a treatment option. MG98 is a second-generation DNMT1 inhibitor and it is a 20-base pair antisense oligonucleotide that binds the 3' untranslated region of the enzyme, preventing transcription of the gene\cite{473}. Studies in mouse models of bladder and colon cancer showed that administration of MG98 led to re-expression of the tumor-suppressor gene p16\textsuperscript{INK4A}. Clinical trials have shown instead controversial results, but MG98 appears to have been successful in the treatment of advanced renal cell carcinoma. The most promising results came from a study in which MG98 was given in combination with Roferon-A, a chemotherapeutic drug. Decreased levels of DNMT1 were observed and tumor progression was slowed, with minimal toxicity from MG98\cite{474}. Additional clinical trials examining the most effective dosages and dosing schedules for MG98 are currently in progress.

**Small molecule.** RG108 is a relatively new DNA methylation inhibitor presenting high specificity and low toxicity. It binds to and directly inhibits the DNMT1 enzyme active site. Its in vitro use in human cancer cell lines showed significant demethylation and re-expression of the p16\textsuperscript{INK4A} and led to slow cancer cell growth. Because the RG108 mechanism of action does not involve enzyme trapping, the toxicity of the drug is reduced. In addition, it did not affect the methylation status of centromeric satellite repeats, an unexpected but advantageous trait that is likely to increase stability of hypomethylated chromatin\cite{475}.

Development of other small molecule inhibitors such as SGI-1027 represents an alternative treatment option to further reduce toxic effects\cite{476}. Either these molecules can
achieve their inhibitory activity by blocking catalytic/cofactor-binding sites of DNMTs or by targeting their regulatory messenger RNA sequences; however, their weak inhibitory potential indicates a need for the development of more potent inhibitory compounds in future.

Non-nucleoside analogues are another group of epigenetic drugs that could block DNMTs. They can effectively inhibit DNA methylation without being incorporated into DNA. Procainamide (also known as Pronestyl or Procanbid) is an antiarrhythmic agent, as well as an inhibitor of DNA methylation and thus it has been used to hypomethylate T cells in experimental systems and, consequently, to produce overexpression of lymphocyte function-associated antigen 1, making T cells autoreactive\^477. It has been under investigation in experimental and clinical settings\^478. Procaine hydrochloride (also called Novocain) and epigallocatechin-3-gallate are currently being investigated in the treatment of several diseases, rather than tumors\^479.

1.2.6.2 HDACs inhibitors. Inhibitors of histone deacetylase are an emerging class of targeted anticancer agents that mediate the regulation of gene expression and induce growth arrest, cell differentiation and apoptosis of tumor cells. They induce hyperacetylation in chromatin usually resulting in activation of certain genes and terminal cell differentiation and/or apoptosis in cancer cells\^383. Then, re-establishing normal histone acetylation patterns through treatment with HDAC inhibitors have been shown to have anti-tumorigenic effects. HDACi selectively alter the expression of a relatively small proportion of expressed genes (2-10%) in transformed cells\^480, despite what it may be anticipated by the wide distribution of HDACs in chromatin. The gene the most frequently involved is p21\textsuperscript{WAF1,CIP1} that resulted to be upregulated. Concerning the apoptosis of tumor cells, many mechanisms have been proposed such as alterations in BCL2 family protein expression, increase in Caspase activity, increase in sensitivity to FAS/FASL interactions and change in the expression of also genes like c-Myc and c-Ras\^481. HDAC inhibitors regulate gene expression by enhancing the acetylation of histones, and thus inducing chromatin relaxation and reactivation of silenced tumor suppressor genes\^322. Therefore, the effect on gene transcription may be a consequence of acetylation of a particular complex of histones and other proteins. In addition to negatively controlling the catalytic sites of HDACs, HDAC inhibitors cause selective changes in expression of Class II HDAC proteins\^482.

Due to the promising in vivo results of these agents, numerous clinical trials have been
An innovative epigenetic strategy for retinoblastoma treatment

initiated\textsuperscript{[483]}, with Vorinostat and Romidepsin already FDA approved. They are currently applied for treatment of certain types of T-cell lymphoma with cutaneous manifestations\textsuperscript{[361]}. Their cytostatic effect may be in part explained by inhibition of abnormal HDAC expression in tumor cells and by interfering with aberrant HDAC recruitment to promoter regions\textsuperscript{[484]}. HDACs may also affect other targets other than histones, that are acetylated and possibly substrates of HDACs, including transcription factors, DNA repair proteins, and cell cycle regulators\textsuperscript{[485]}.

HDAC inhibitors are heterogeneous, some deriving from natural molecules, whereas some developed by synthesis. Their classification is based on their chemical nature and mechanism of inhibition, such as their affinity for the HDACs of Classes I, II, and IV. They have high specificity, although presenting extreme side effects. They are categorized into several groups, including hydroxamic acids, cyclic tetrapeptides and analogues, short-chain fatty acids (aliphatic acids), benzamides and epoxides\textsuperscript{[483]}. The numerous clinical trials designed for these drugs may elucidate some critical questions remained to address\textsuperscript{[486]}.

\textit{Hydroxamic acids} are the most important class of HDAC inhibitors; they are very effective, targeting the catalytic site of Class I and II HDACs and they have emerged as promising and potent treatments for cancers\textsuperscript{[481]}.

\textbf{Trichostatin A} (TSA) was the first natural hydroxamate discovered to reversely inhibit HDACs\textsuperscript{[487]}. In the '70s, trichostatin A (TSA) and trichostatin C were initially isolated as fungistatic antibiotics from \textit{Streptomyces hygroscopicus}\textsuperscript{[488]}. Later, it has been demonstrated the very potent activity of trichostatic acid in inducing Friend cell differentiation and specific inhibition of the cell cycle of normal rat fibroblasts in the G1 and G2 phases at the very low concentrations. TSA also causes an accumulation of acetylated histone species in a variety of mammalian cell lines and its \textit{in vivo} effect can be attributed to the inhibition of the histone deacetylase enzyme of Classes I and II\textsuperscript{[487]}. More recently, it has been reported that TSA can regulate the expression of the gene \textit{Drg-1}, identified as a candidate suppressor of metastases in colon cancer\textsuperscript{[489]}.

TSA-induced histone acetylation causes decondensation of interphase chromatin and it exerts its activity by complex linking of a zinc ion that is supposed to mediate the acetamide cleavage at the catalytic site\textsuperscript{[490]} (\textit{Fig. 1.6}).
Suberanilohydroxamic acid (SAHA also known as Vorinostat), is similar to TSA and it has been shown to inhibit cell proliferation in very low concentrations. It was previously selected among a library of 600 second-generation synthesized hybrid polar compounds as an active inducer of differentiation of murine erythroleukemia cells\textsuperscript{[491]}. Later on, it has been discovered that SAHA inhibits different classes of HDACs. In 2006, FDA approved it as a treatment for progressive, persistent, or recurring cutaneous T-cell lymphoma, or for patients following two systemic chemotherapies\textsuperscript{[492-493]}. It leads to hyperacetylation of histones as well as non-histone proteins, inducing apoptosis and sensitizing tumors to cell death processes and other drugs. The ability of SAHA to sensitize cancerous cells to other drugs makes it an interesting candidate for combination therapies, including epigenetic and non-epigenetic drugs. However, as SAHA has multiple targets, it also frequently induces many side effects such as anemia, diarrhea, fatigue, nausea, hyperglycemia, thrombocytopenia and anorexia\textsuperscript{[494]}.

M-Carboxycinnamic acid bishydroxamate (CBHA) is another potent HDACi\textsuperscript{[495]} and the structural basis for several derivatives, including Dacinostat\textsuperscript{[496]} and Belinostat\textsuperscript{[497]}, that was FDA approved in 2015 for the treatment of patients with peripheral T-cell lymphoma (PTCL)\textsuperscript{[498]}.

Amino-suberoyl hydroxamic acids (ASHAs) have recently been discovered to inhibit HDACs and transform cell proliferation at nanomolar concentrations\textsuperscript{[499]}. Other hydroxamates inhibitors, such as Oxamflatin\textsuperscript{[500]} and Givinostat (also known as ITF2357) are under investigation for several diseases, including cancer\textsuperscript{[501]}.

LAQ824 is a cinnamic hydroxamic acid analogue that significantly enhances the anti-tumor activity of adoptively transferred antigen-specific T-cells in preclinical models\textsuperscript{[502]}. LAQ824 inhibits \textit{in vitro} enzymatic activities and transcriptionally activates the \textit{p21} promoter in reporter gene assays. It also selectively suppress growth of cancer cell lines at sub-micromolar levels after 48-72 hrs. of exposure, whereas higher concentrations and
longer exposure times are required to retard the growth of normal dermal human fibroblasts\textsuperscript{[503]}.

Panobinostat (also known as LBH-589) is a cinnamic hydroxamic acid analogue and it has been recently tested in combination of radiotherapy for the treatment of lung cancer\textsuperscript{[504]}. In 2015, FDA approved Panobinostat in combination with Bortezomib and Dexamethasone for the treatment of patients with multiple myeloma\textsuperscript{[505]}.

A series of aryloxyalkanoic acid hydroxamides have also been synthesized and they are HDACi at nanomolar concentrations\textsuperscript{[506]}.

**Cyclic peptides and analogues** are a structurally complex group of HDAC inhibitors, naturally occurring and synthetic that all contain an unusual amino acid with a hydroxamic acid, epoxyketone, or ketone function in the side chain. Cyclic tetrapeptides contain a 2-amino-8-oxo-9, 10-epoxy-decanoyl as a half of the molecule, whereas cyclic peptides do not.

**Trapoxins A and B** belong to the family of hydrophobic cyclotetrapeptides, with the epoxyketone attached to the cyclic peptide core and they were isolated from *Helicoma ambies*. They can revert the altered morphology of cells induced by oncogene expression, due to an irreversibly and potent inhibition of HDAC activity. Despite this efficacy, both compounds have not yet been introduced in clinical trials owing to their instability and toxicity\textsuperscript{[254]}.

**Cyclic depsipeptides** are polypeptides in which one or more amino acid is replaced by a hydroxy acid, resulting in the formation of at least one ester bond in the core ring structure. Many natural cyclic depsipeptides possessing intriguing structural and biological properties, including antitumor, antifungal, antiviral, antibacterial, anthelmintic, and anti-inflammatory activities, have been identified from fungi, plants, and marine organisms. In particular, the potent effects of cyclic depsipeptides on tumor cells have led to a number of clinical trials evaluating their potential as chemotherapeutic agents. Although many of the trials have not achieved the desired results, Depsipeptide (also known as Romidepsin or FK228), is a natural bicyclic depsipeptide isolated from *Chromobacterium violaceum*\textsuperscript{[507]}. It is a stable prodrug that is activated by reduction with glutathione, after uptake into cells. Its reduced form, namely redFK, is the active
FK228 is more effective than other HDACIs in vivo models because of its fair stability in medium or serum and its hydrophobic nature facilitating its penetration through the cell membrane to afford the active species. It was then synthesized and has been shown to have clinical efficacy in patients with progressive, persistent or recurrent cutaneous T-cell lymphoma and, in 2009, it has received FDA approval for use in treatment, although anemia, anorexia, hyperglycemia, thrombocytopenia, fatigue and nausea have frequently been reported.

Cyclic hydroxamic-acid containing peptide (CHAPs), which are hybrids of trichostatin and trapoxin, have been further designed and synthesized. In such hybrids, a hydroxamic acid side-chain is attached to the cyclic peptide core. Several products have thus been prepared which differ by changing, the number of the amino acids constituting the ring structure, the chirality of amino acids and the side-chain structure. Among them, CHAP1 and CHAP31 are the most stable and most potent HDAC inhibitors.

Apicidin was initially isolated from two Fusarium species as a novel antifungal metabolite. Its potent HDAC-inhibitory activity led to evaluate it as an anti-proliferative agent and it is still under preclinical investigation. Chlamydocin-analogues are other cyclic peptides so selective and active, working at millimolar or even nanomolar concentrations.

Short-chain fatty acids, such as sodium butyrate (also known as Buphenyl) and its prodrug AN-9, and phenylacetate are relatively weak inhibitors of the HDACs, with activity at millimolar concentrations.

Valproic acid (also called VPA) is a relatively weak inhibitor of the HDACs, with activity at millimolar concentrations. It was previously used for many years as an antiepileptic drug: it is extremely well tolerated by patients, and its long-term effects are well known. The most commonly reported complaints are neurological symptoms (such as dizziness) that are generally transitory and reversible. Neurological symptoms may become excessive when VPA is combined with other agents; as well, its use during the first trimester of pregnancy is associated with an increased risk of major congenital malformations.
Phenylbutyrate is another relatively weak inhibitor that has been in the market for non-oncological uses and it has been shown to have activity as HDACi. In addition, it is currently under clinical trials for cancer treatment\[^{476}\].

**Benzamides** and their derivatives do not present structural similarity with other HDAC inhibitors. MS-275 (also known Entinostat or Benzamidine) is the most active benzamide derivative and induces hyperacetylation of nuclear histone in various tumor cell lines. MS-275 is a synthetic compound, showing relative selectivity and it has been under clinical investigation\[^{518}\].

CI-994 (also called as Tacedinaline or Acetyldinaline) is a synthetic benzamide derivative that shows relative selectivity in the HDACs inhibited. Its potential was confirmed as an oral cytostatic drug and preclinical studies have been conducted for the treatment of human acute myelocytic leukemia\[^{519}\]. CI-994 has been introduced in clinical trials for a number of tumor diseases. The clinical potential of this drug has been also established against colorectal cancer\[^{520}\].

MGCD0103 (also known as Mocetinostat) is dihydrobromide salt of a substituted 2-aminophenyl benzamide and it is given orally\[^{521}\]. Two novel synthetic compounds SK7041 and SK7068 cause accumulation of acetylated tubulin, but affect neither acetylation of histones nor inhibition of cell cycle progression\[^{522}\].

**Epoxides** are inhibitors from natural sources. Depudecin is a fungal metabolite isolated from *Alternaria brassicicola*\[^{523}\] and it can revert the cell morphology of v-Ras-transformed cells. It also inhibits HDACs, inducing hyperacetylation of histones in a dose-dependent manner\[^{524}\].

1.2.6.3 **Bromodomain inhibitors.** Bromodomains are conserved structural motifs associated with chromatin modifying proteins\[^{525}\], they are considered epigenetic reader domains and able to recognize acetylated lysine residues for chromatin modification\[^{526}\]. Bromodomain and extra-terminal (BET) proteins are known to mediate transcriptional elongation of acetylated chromatin, binding to the acetyl groups and their clinical importance has been investigated in many studies\[^{527}\]. These proteins present unique function and specificity and they are promising targets for therapeutic treatments. Bromodomain inhibitors are selective small molecules that competitively bind acetylated
lysine residues, causing the disruption of the interaction between BET proteins and acetylated histones. Displacing BET proteins from the histone, the inhibitors may lead to widespread downregulation of *c-Myc* gene and its target proteins\(^{[528-529]}\). They also can directly regulate expression of *BCL2*, an anti-apoptotic gene that is highly expressed in a number to tumor types\(^{[530]}\).

### 1.2.6.4 HATs inhibitors

These compounds have been identified to inhibit the catalytic activity of HATs in many cancers and diseases. Although they are not very selective and bind to multiple classes of proteins, HAT inhibitors seem promising for treatment of various diseases\(^{[314]}\).

*Bisubstrate inhibitors* were the first discovered to selectively inhibit HATs and re-express tumor suppressor genes in cancers\(^{[531]}\). *Lys-CoA* is high selective, however it is generally inactive in mammalian cell systems, exhibiting non-drug-like properties unless modified with the co-administration of moderately cytotoxic detergents or administered via microinjection. C646 may be the only potent and selective HAT inhibitor discovered so far. The compound binds at the specific enzyme and acts as a cofactor competitor\(^{[532]}\). It can mimic the pro-apoptotic effect of RNA-mediated HAT knockdown, which involves both extrinsic and intrinsic cell death pathways\(^{[533]}\).

*Natural inhibitors* suppress histone H3 and histone H4 acetylation by specific HATs. *Curcumin* seems to inhibit cell proliferation and inducing apoptosis in breast cancer *in vivo*\(^{[534]}\). *Garcinol* and *anacardiac acid* inhibits acetylation, as well as they induce apoptosis and downregulate global gene expression. These two natural compounds however exhibit relatively low potency, which limits the authenticity of their activity\(^{[535]}\).

### 1.2.6.5 Protein methyltransferase inhibitors

Methylation of lysine and arginine residues is catalyzed by protein methyltransferases (PMTs). The enzymatic activities have suggested pathological roles in cancer, neurodegenerative diseases, and inflammatory diseases. Inhibition of PMTs has been shown to stop these enzymatic alterations\(^{[536]}\).

*Selective inhibitor* BIX-01294 was the first inhibitor of a protein lysine methyltransferase. Although the drug has good potency in terms of blocking protein–protein interactions, it is toxic in cellular assays at high concentrations\(^{[314]}\).
Second-generation inhibitors include E72 and UNC321 incorporate 7-alkoxyamine tethered to the quinazoline core as a structural modification[537-538]. UNC0646 has a remarkable toxicity/function ratio in certain cell lines and it is also potent, selective, and has low cell toxicity making it an excellent inhibitor[539].

1.2.6.6 Histone methylation inhibitors. Newly synthesized molecules that inhibit trimethylation have been shown to re-activate developmentally regulated genes[314].

3-deazaneplanocin A (DZNep) selectively inhibits trimethylation of lysine 27 on histone H3 and lysine 20 on histone H4, and reactivates silenced genes in cancer cells. DZNep was shown to successfully induce apoptosis in cancer cells by selectively targeting polycomb repressive complex 2 proteins, which are generally overexpressed in cancer[540]. DZNep specificity was however challenged in a subsequent study, in which it has been observed to reactivate developmental genes, not silenced by DNA methylation[541].

1.2.6.7 Combined therapy. The interaction between different components of the epigenetic machinery has led to the exploration of effective combinatorial cancer treatment strategies, which involve use of both DNMT inhibitors and HDAC inhibitors together. Such combination treatment strategies have been found to be more effective than individual treatment approaches[250]. For instance, the de-repression of certain putative tumor-suppressor genes was only seen when DAC and TSA were combined[542]. Anti-tumorigenic effects of Depsipeptide were enhanced when leukemic cells were simultaneously treated with DAC[543]. Synergistic activities of DNA methylation and HDAC inhibitors were also demonstrated in a study showing greater reduction of lung tumor formation in mice when treated with Phenylbutyrate and DAC together[544].

Another promising strategy for cancer therapy is performed by a combination of epigenetic drugs with conventional chemotherapy that has been hypothesized to be more effective in treating drug resistant forms of cancer[431,545]. One study has shown that HDACi treatment demethylases and re-expresses tumor suppressor genes[546], resulting in the sensitization of cancer cells to other cytotoxic drugs. Additionally, HDACi in combination with the calpain protease inhibitor calpeptin has been shown to enhance growth inhibition of breast and ovarian cancer cells[547]. Furthermore, the combination of HDACi and TRAIL in mouse models was found to reduce tumor size by inducing apoptosis[548], and the combination of HDACi and GT-oligo increases ovarian cancer cell
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death[549]. Drug resistant cancer cells may be similarly sensitized by demethylation to other cytotoxic agents as well. Recent clinical studies suggest that pretreatment with epigenetic drugs can reduce cancer relapse and be more effective for treating drug resistant cancers. For example, one study determined that lung cancer patients who were treated with the epigenetic drugs DAC and HDACi prior to conventional chemotherapy had lower incidences of relapse[550]. Two other studies demonstrated that MAPK pathway inhibitors in combination with HDACi suppressed cAMP mediated resistance in melanoma cells[551] and that pre-treatment of platinum drug resistant ovarian cancer cells with HDACi and methylation inhibitors sensitized these cells to cisplatin-mediated cell death[552]. In this last study, epigenetic drug treatment resulted in the re-expression of RGS10, an important regulator of cell survival and chemoresistance in ovarian cancer. Hypermethylation and histone deacetylation silence this gene in drug resistant ovarian cancer cells, and re-expression of this gene made these cells susceptible to platinum drugs. Overall, these results indicate that pretreatment using epigenetic drugs in combination with conventional therapies may be beneficial for reducing cancer relapse and improving drug resistant cancer treatment. Similarly, to cancer, the role of epigenetic drugs in combination with other agent could be beneficial in treating numerous diseases[314] and becomes a key area of research for further development.
1.3 DRUG DELIVERY SYSTEMS

1.3.1 Economics of drug delivery

Development of a new drug molecule is expensive and time consuming. Since last decades, the number of newly developed molecules introduced by pharmaceutical industry on the market has been reduced due to the rising of research and process costs, alternative investment opportunities and erosion of effective patent life. To avoid these drawbacks, interest of investors and researchers has turned towards investing the role of drug delivery system (DDS). Drug delivery is entering the forefront of product strategy for pharmaceutical companies: using a carrier to deliver an already tested agent represents a highly profitable strategy to overcome inherent risks associated with the use of new chemical entities. Drug delivery could be described as a formulation or a device that enables the introduction of a therapeutic substance in the body to improve drug efficacy and safety by controlling its rate, time and place of release in the body. Delivering an agent to a specific site will include the administration of the therapeutic product (carrier and active compound), the release of the active compound by the product, and its subsequent transport across the biological membranes to the site of action.

Moreover, the use of novel drug delivery systems including an "old" chemical compound appears a cheaper and more rapid alternative for patients to receive an effective treatment. In this case, in fact, pharmaceutical companies may partially skip the extremely costly preclinical and clinical investigations, counting on previously proved and validated pharmacokinetic and -dynamic features of the drug. Most importantly, DDS can also be considered as an interface between the patient and the drug. In this, DDS can represent both a safety barrier against host microenvironment metabolic attack as well as drug induced undesired side effect.

The chemical and biological distinction between the drug and the device is important, as it is the criterion for regulatory control on delivery system formulations by the drug or medicine control agency.

At present, there are 30 main drug delivery products approved for clinical use that represent a worthy part of the pharmaceutical market. In the United States for example, the total annual income for this sector is approximately US$33 billion with an annual
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The main problems associated with systemic drug administration are essentially related to the biodistribution and bioavailability of pharmaceuticals throughout the body. To achieve the effective concentration the drug must be administered in large quantities, the majority of which will follow a different fate from the therapeutic one: in fact, once in contact with the blood stream the drug will undergo enzymatic changes, chemical degradation and immune system attack. Consequently, adverse toxic reactions and sensitization are common and contribute to reduce drug efficacy.

A "perfect" drug should exert its pharmacological activity only at the target site, using the lowest concentration possible and without negative effects on non-target compartments. Drug delivery either by means of soluble or particulate components can improve drug pharmacokinetic, drug bioavailability, sometime safety and efficacy and it is used in the view of having the drug targeted only to the site where its action should be exerted. Thus, recently, a new field of biotechnology is focused on improving carriers (molecules, polymers and vectors) functionalization, exploring the possibility of having selected targeting properties to cell targets. Unfortunately, very often the body recognizes...
the drug targeting system as non-self and unexpected toxicities could hamper the use of the same. It has been envisaged that ideal drug delivery systems should be made of self-powered, computer-controlled medical nanorobot system, named pharmacyte, capable of precise transportation, timing and targeted delivery of pharmaceutical agents to specific targets in the body.

1.3.2 Drug-delivery formulations in cancer therapy

Chemotherapy is the most common conventional treatment option for several cancers and it is used either alone or in combination with other therapeutic approaches that kills cancer cells by drug toxicity or by preventing cell division, either by stopping the nutrient uptake or by inhibiting the mechanism responsible for cell division. However, this approach is blunt and rarely successful for advanced stages of cancer, as pharmacologically active cancer drugs reach the tumor site with poor specificity and dose-limiting toxicity. A large number of pharmacological compounds are in fact administered in the blood stream; however, this method of treatment is doomed to result in some limitations. Some of the compounds used in therapy can be unstable in physiological fluids due to their short half-life or may become unstable because of modification by endogenous enzymes. This would in turn result in a reduction of effective dosage and in the appearance of metabolites with potential toxic side effects. Moreover, blood streamed free drugs may diffuse into any tissues and can exert their activity on healthy cells. Currently, available free chemotherapeutic agents are time-tested (half shelf-life controls) and confer good disease-free survival only for a limited period. For all these reasons, many efforts are made to develop new drug delivery systems.

To protect drugs from rapid degradation and increase their bioavailability, several authors tried to formulate those using DDS. These systems in fact will convey numerous beneficial features: a good DDS should be design to improve drug half-life in the blood stream and to shelter the molecule from the host enzymatic attack; moreover, DDSs also form a barrier to avoid unwanted interaction of drug with host tissues.

Furthermore, DDS can be also engineered to time-deliver the active molecule, delaying and prolonging the biological interaction at site, to control the rate at which a drug is released. Improving safety-efficacy ratio of classical drugs has been attempted using different methods, such as individualizing drug therapy, dose-titration and therapeutic
drug monitoring and it would allow opening the drug therapeutic window. DDSs are also flexible to be design for different delivery routes of administration: the choice depends on the disease, the effect desired and the product available. Drugs may be administered directly to the organ affected by disease or given systematically and targeted to the diseased organ. Various methods of systemic DDS are classified depending on the anatomical routes, including gastrointestinal, parenteral (such as subcutaneous or intravenous), transmucosal, transnasal, transdermal\cite{561}.

Controlled-release technology can be applied to virtually all routes; for example, transdermal delivery, aerosol sprays, encapsulated cells, soft gels and a variety of programmable and implanted drug-delivery devices. The delivery systems currently available enlist carriers that are either simple, soluble macromolecules (such as monoclonal antibodies, soluble synthetic polymers, polysaccharides and particulate biodegradable polymers) or more complex multicomponent structures (microcapsules, micelles, liposomes, and cell-based-carriers). Here below an overview of the most common nano-evices in use and implemented in research and common practice, some of them even used in clinics for diagnostics and treatments.

1.3.2.1 Beads. Beaded system consists of multiple, small beads that are composed of inert (such as polystyrene) or magnetic substances. These elements are normally designed for oral delivery; in fact, they are often pH sensitive so that the release can be selectively achieved in different part of the gastric system depending on the environmental acidity. The active drug is overlaid on the beads surface and encased in a delivery capsule. This process is used to achieve long-acting drug levels associated with the convenience of once-a-day dosing and it produces a pharmacokinetic pattern roughly similar to a zero-order pattern, with $C_{\text{max}}$ obtained approximately 4 to 6 hours after ingestion and sustained levels observed for 24 hours after initial dosing. Normally particles may range from 50nm to 20µm, with surface functional groups such as carboxyl- or amino- groups for covalent coupling of proteins or other ligands\cite{555}.

1.3.2.2 Carbon nanotubes. Carbonanotubes (CNTs) have received a great deal of attention in biomedical fields: in fact, they are characterized by unique structures and properties. Nanodimensional sheets of carbon polymer (single wall SWCNT) or several layers wrapped around coaxially (multi walls, MWCNT) provide large hole axial space
to accommodate molecules and vast surface areas for rich surface functionalities. Indeed, the majority of applications are not performed using pristine CNTs but using oxidized CNTs that expose reactive carboxyl- groups to favor biointeraction. In this fashion, they show increased solubility in biological fluids and reduced toxicity together with increased reactivity so that can be used as transporters for the delivery of biomolecules and drugs in vitro and in vivo. Functionalized CNTs have been employed to deliver treatment in several sites of the body, given their ability to be transported through the blood stream and to enter the cytoplasmic space as non-viral vector for gene therapy thanks to needle-like endocytosis processes that pierce the cell membrane. Most relevantly, functionalized MWCNTs have been used to deliver therapies to the central nervous system, like siRNA-based anti-stroke therapy injected to animal brain, resulting in motor functional amelioration of injured animal[562]. More specifically, CNTs have been used as nanocarriers to transport anticancer drugs, genes, and proteins for chemotherapy[563], including topoisomerase inhibitors, platinum (Pt)-based drugs, and antimicrotubules. Given the negative charge concentrated on functionalized CNTs, these carriers are excellent vectors for gene, protein and nucleotides in cancer treatment especially achieved with the delivery of siRNA to tumor cells resulting in a significant inhibition of tumor growth[564]. Moreover, using combinations of light energy, CNTs have also been applied as mediators for photo-thermal therapy (PTT) and photo-dynamic therapy (PDT) to directly destroy cancer cells without severely damaging normal tissue using infrared energy[565].

1.3.2.3 C-60 fullerene. Similar to CNTs, fullerenes (C60) are nanoscale carbon materials with unique photo-, electro-chemical, and physical properties; however, their inherent hydrophobicity limits their use in biology and thus leads to the research in developing water-soluble fullerene derivatives[566]. In recent years, various fullerene derivatives and surfactant-coated C60 had been reported, such as C60-(OH)n[567], C60-PEI[568], malonic acid derivatives[569] and sugar derivatives[570]. Nevertheless, the employment of fullerenes for drug delivery is still at an early stage of development[571], and up to now, there are only a few reports about fullerene derivatives being used for the delivery of anticancer drugs[572].

1.3.2.4 Nanodiamonds. Another carbon-based delivery and diagnostic system is the so-called nanodiamonds (NDs), attractive agents for use in medico-biological applications, largely due to their great biocompatibility, stable photoluminescence, commercial availability, minimal cytotoxicity, and ease of purification[573]. NDs could be
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functionalized and conjugated to a variety of molecules for the purpose of cell labeling and drug delivery that can improve their solubility, direct them to specific binding sites on target cells and tissues, and reduce their effects on normal tissues\textsuperscript{[574]}. The diversity of ND functionalization broadens the scope of their potential diagnostic and therapeutic applications. They are suitable for controlled drug-delivery applications because of their capability to release drugs slowly and consistently and have abundant capacity for drug loading due to their large surface area:volume ratio\textsuperscript{[575]}. Moreover, NDs could also be used to solubilize and efficiently deliver water-insoluble chemotherapeutic agents to several tumor cells\textsuperscript{[576]}. They can be used as biomarkers or for cell labeling and tracing because they do not interrupt cell division or differentiation and have less cytotoxicity\textsuperscript{[577]}.  

1.3.2.5 Virus-mediated nanocarriers. Several virus-based nanocarriers have been reported as an emerging drug delivery system\textsuperscript{[578]}. The versatile hierarchical assembly of viral coat protein subunits provides a natural and easy way of drug packaging. Virus-like particles (VLPs) can easily meet the requirements needed for a drug nanocarrier system, such as biocompatibility, water solubility, and high uptake efficiency. Moreover, VLPs can be modified with polymers such as PEG to improve their half-life in the host by moderating their immunogenicity\textsuperscript{[579]}.  

1.3.2.6 Dendrimers. Dendrimers (also called star polymers) are a unique class of repeatedly branched polymeric macromolecules with numerous arms radially extending from the center, resulting in a nearly perfect 3D geometric pattern\textsuperscript{[580]}. They comprise a series of branches around an inner core, the size and shape of which can be modulated as per the requirement. In particular, their surfaces are designed with functional groups to increase or resist biopermeability and to optimize biodistribution, receptor-mediated targeting, therapy dosage, or controlled release of drug, as well their interior void space may be used to encapsulate small-molecules metals or labeled imaging probes. Production of dendrimers can be customized accordingly to need for shape, dimension and nature of trapped molecules. Smart materials are increasing their versatilities, as they can be selectively sensitive to different specific environments, such those existing within different intracellular compartments. Those dendrimers used for cancer therapy, such as oligonucleotide-linked dendrimers, are designed to improve the therapeutic index of cytotoxic drugs: these polymers have been endowed with specific chemical moieties to direct delivery to cancerous cells and in
some cases are able to overcome multidrug resistance in tumors via bypassing efflux pumps\[581]\.

**1.3.2.7 Micelles.** Micelles formed by self-assembly of amphiphilic block copolymers (5-50nm) in aqueous solutions are of great interest for drug delivery applications. The drugs can be physically entrapped in the core of block copolymer micelles and transported at concentrations that can exceed their intrinsic water-solubility. Moreover, the hydrophilic blocks can form hydrogen bonds with the aqueous surroundings and form a tight shell around the micellar core. As a result, the contents of the hydrophobic core are effectively protected against hydrolysis and enzymatic degradation. In addition, the corona may prevent recognition by the reticulo-endothelial system (RES) and therefore preliminary elimination of the micelles from the bloodstream. The fact that their chemical composition, total molecular weight and block length ratios can be easily changed, allows control of the size and morphology of the micelles. Functionalization of block copolymers with cross linkable groups can increase the stability of the corresponding micelles and improve their temporal control\[582]\.

1.3.2.8 Liposomes. Alec Bangham first produced liposomes in England in 1961. They are colloidal carriers, formed spontaneously when certain lipids are hydrated in aqueous media and consist of an aqueous volume entrapped by one or concentric bilayers of natural and/or synthetic lipids. They are made of amphipathic phospholipids where one end of each molecule is water soluble, while the opposite end is water insoluble. Accordingly, with their different lipid nature drugs can be encapsulated in liposomes, either in the phospholipid bilayer, in the entrapped aqueous volume, or at the bilayer interface. Liposomes are stable, biocompatible, biodegradable, self-assembled phospholipid membranes. Accordingly, with their structure and production design, they are classified as multilamellar, small unilamellar, or large unilamellar, and they range in size from 25nm to 10μm in diameter. The size and morphology of liposomes can be customized using different method of preparation and composition and serve delivery of drugs, vaccines, and genes for a variety of disorders, including cancer\[583]\.

Liposomes have been clinically tested as carriers of antineoplastic and antimicrobial drugs, chelating agents, steroids, vaccines, and genetic materials\[584-585]\.

Their surface characteristics allow liposomes to targeted delivery hydrophobic drugs without eliciting an immune response\[586]\ and undergo an enhanced permeability and retention effect for preferential extravasation from tumor\[587]\.

In some cases, liposomes attach to cellular membranes and
appear to fuse with them, releasing their drugs into the cell. Liposomes eventually become subject to enzymatic degradation and/or phagocytic attack, leading to release of drug for subsequent diffusion to tumor cells: in this case liposomes are taken up, the phospholipid walls are acted upon by organelles called lysosomes, and the medication is released.

Liposomal nanomedicines represent one of the most advanced classes of drug-delivery systems, with several currently in the market and many more in clinical trials\(^\text{[588]}\). There are several examples of liposomal formulations for conventional small-molecule drugs\(^\text{[589]}\) and some of those have been FDA approved, including liposomal doxorubicin, PEGylated liposomal doxorubicin, PEGylated liposomal daunorubicin\(^\text{[590]}\). Accordingly to their nature, they show different mechanisms of action for achieving drug release at target, for example cationic liposomes are reported to bind by electrostatic interactions to negatively charged phospholipid head groups, preferentially expressed on tumor endothelial cells\(^\text{[591]}\).

The surface features of the different liposomes also drive the choice for the route of administration and the more suitable pharmaceutical preparation. Mesophasic proliposomal system may be used for transdermal delivery\(^\text{[555]}\) where the liposomal suspension might be incorporated into an ointment and gel base. The proliposomes system was found to be superior to PEG-based ointment system and it was developed for controlled, prolonged and localized delivery via topical route. The prolonged pharmacodynamic effect of novel proliposomal ropivacaine oil, together with its delayed elimination and prolonged redistribution to plasma, is compatible to depot-related slow-release and similar to the performance of other liposomal local anesthetics. The advantage of the proliposomal oil lies in its ease of preparation and its extended shelf-stability (>2 years) at room temperature\(^\text{[592-593]}\).

The next generation of DDS represents a strategy for molecular targeting the drug to cancer cells and via antibody-mediated or other ligand-mediated interactions and it includes immuno-liposomes, in which mAb fragments are conjugated to liposomes. They are an integration of biological components capable of tumor-cell recognition with delivery technologies\(^\text{[594]}\). The immuno-liposomes offer a number of theoretical advantages as compared with other antibody-based anticancer strategies and appear to be non-immunogenic and capable of long circulation in the blood stream even with repeated administration \(^\text{[595]}\). Similarly, ligand-based targeting using growth factors, hormones, vitamins (e.g., folate), peptides or other specific ligands is being pursued in conjunction
with liposomes. As well, artificially engineered antibodies have been used as a conjugate to thermostensitive liposomes (affisomes) for the delivery of paclitaxel\textsuperscript{[596]}. Furthermore, liposomes have also been investigated for the delivery of gene therapy, transferring nucleic acid-based gene-silencing molecules to tumor cells, in order to ensure a targeted molecular intervention and achieve a higher level of specific action than conventional cytotoxic chemotherapy.

1.3.2.9 Nanoparticles. Nanoparticles (NPs) may vary in size and nature. They are typically metal based or polymer-based and span from few to several hundred nanometers. They can be engineered as nanoplatforms for effective and targeted delivery of drugs, and imaging labels, as they are able to overcome many biological, biophysical, and biomedical barriers. Targeting strategy can vary according to their nature, for example, iron-based system can be directed by magnetic field exposure. However, the most common targeting strategy is ligand-mediated where a specific interaction between NPs and cancer cell surface is desired. In cancer therapy, the ligand-targeted NPs are expected to deliver cytotoxic agents selectively and specifically to tumor cells via receptor-mediated endocytosis, thereby enhancing intracellular drug accumulation. A variety of tumor-targeting ligands, such as antibodies, folate or growth factors and cytokines has been used to facilitate the uptake of carriers into target cells\textsuperscript{[597]}. Their properties could be attributed to their small sizes; lower toxicity, maximized bioavailability and they could be used to deliver small-molecule drugs, peptides, proteins and nucleic acids, either alone or in combinations.

Despite extensive research on NP systems for cancer therapeutics, there are only a few tumor-targeted nanotechnology-based drug-delivery systems approved by FDA and European Medicines Agency\textsuperscript{[590,598]}. Recent advances in diagnostic technology led to development of bioaffinity NP probes for molecular and cellular imaging, targeted NP drugs for cancer therapy, and integrated nanodevices for early screening and detection of cancer. These developments raise exciting opportunities for personalized oncology in which genetic and protein biomarkers are used to diagnose and treat cancer, based on the molecular profiles of individual patients\textsuperscript{[586]}. NP-based DDS present several advantages, including the possibilities to reduce the lack of selectivity of anticancer drugs, targeting the chemotherapeutics to specific sites, either actively or passively\textsuperscript{[558]}; to overcome multidrug primary or acquired resistance\textsuperscript{[576]}. An innovative epigenetic strategy for retinoblastoma treatment
which is mostly due to increased efflux pumps in the tumor cell membrane; to defeat low aqueous solubility of anticancer drugs, increasing their bioavailability\textsuperscript{[599]}. Moreover, NP systems have not only the ability to carry loaded active drugs to cancer cells presenting high efficacy and less side effects, but also, if combined with existing optical imaging technologies, to increase the resolution of tumor imaging\textsuperscript{[600]}. A wide variety of nanoparticles, including magnetic or gold-based, are used for diagnosis-cum-therapy of different cancer types, by visualizing tumors and carrying out targeted delivery of drugs with reduced toxic side effects\textsuperscript{[601]}. Hence, they allow a theranostic approach towards cancer treatments, combining therapy and diagnosis into a single procedure or molecule\textsuperscript{[602]}. They also can deliver genetic materials to both primary and metastatic cells for genetic therapy. NP formulations appear quite stable with high carrier capacity, have longer shelf life and improvement in biodistribution of cancer drugs, as well they are suitable for administration of both hydrophilic and hydrophobic substances through oral, nasal, parenteral and intraocular routes\textsuperscript{[603]}. Magnetic NPs are very commonly used and based on the ferromagnetic elements Co, Ni, and Fe\textsuperscript{[604]}. They can be also exploited to mediate a hyperthermic adjuvant effect in conjunction to deliver drugs in order to achieve high intra-tumoral concentration. Magnetic NPs are indeed in use during thermal ablation for cancer treatment, and can be considered as a minimally invasive option to exploit together with conventional therapies\textsuperscript{[605]}. During thermoablation, cells heated to temperatures ranging 41-47°C begin to show signs of apoptosis\textsuperscript{[606]}, while temperatures above 50°C are associated with less apoptosis and more necrosis\textsuperscript{[607]}. Thus, iron oxide NPs have been used as both diagnostic and therapeutic nanoscale materials to treat deep tissue tumors. With minimal drawbacks, they continue to be actively investigated because of their minimal toxicity and potential for rapid heating\textsuperscript{[608]}. Similarly, nickel-based NPs can be used for tumor targeting by serving either as inducers of hyperthermia in response to an externally applied magnetic field (as drug-delivery platforms)\textsuperscript{[609]} or directly as pro-apoptotic oxidant agents\textsuperscript{[610]}. Gold-based nanoparticles (GNPs) are classically used as DDS for cancer therapy\textsuperscript{[611]}. They are highly stable nanometer-sized colloidal suspensions, presenting versatile scaffolds for drug delivery\textsuperscript{[612]}, and they can be functionalized obtaining multiple receptor targeting, multimodality imaging and multiple therapeutic actions against
cancer. Different subtypes of GNPs such as gold nanospheres, nanorods and nanocages have been reported based on shape and physical properties\textsuperscript{[613]}. Colloidal GNPs have great potential to overcome delivery limitations because of their biocompatibility and low toxicity. In cancer cell lines, surface properties of GNP have been shown to regulate their cellular uptake, intracellular release, and distribution in subcellular compartments\textsuperscript{[614]}.

In addition, GNPs could be heated with shortwave radio-frequency fields. By labeling GNPs with antibodies against particular cancer cells, higher concentrations of GNPs could be achieved within the target. Once the particles are internalized, radio-frequency fields applied to cells result in localized heat leading to cell death\textsuperscript{[607]}.

Polymeric NPs may deliver chemotherapeutic drugs with less interaction with healthy cells, resulting in enhanced efficacy, reduced toxicity, controlled and long-term release rates, prolonged bioactivity, increased patient compliance due to less administration frequency, and the ability to co-deliver multiple drugs with synergistic effects at the same site\textsuperscript{[615]}. PNPs are available in different types, such as nanospheres and nanocapsules, depending on their particular application\textsuperscript{[616]} and their versatility has been increased with the use of smart materials for their production. Smart materials in fact react to environmental stimuli such as temperature, pH to suit delivery purposes. Moreover, through chemical surface functionalization, NPs have been designed to enhance drug targeting specificity, lowering systemic drug toxicity, improving treatment absorption rates, and providing protection for pharmaceuticals against biochemical degradation. For instance, PNPs coupled with ligands and aptamers have been reported as a way to actively target cancerous cells that further induce receptor-mediated endocytosis for intracellular delivery.

Quantum-dots (QDs). Among the different NPs, quantum may be considered as the most suitable for diagnostic purposes. They are spherical light-emitting NPs composed of a semiconductor material, mostly containing selenium metal compounds, with dimensions that range from few nanometers to microns. Different sized quantum dots colloid solutions once excited with electricity or light emit a constant wavelength fluorescent signal that can be exploited for medical purposes. It is easy to imagine the wide application that this might have in cancer screening, for marker detection in biological fluids, classification of tumor from biopsies, and for high-resolution biomolecular and cellular imaging\textsuperscript{[617]}. Moreover, novel functionalized and fluorescent QDs were developed with high drug-loading efficiency, low cytotoxicity, and favorable cell
compatibility and they are promising candidates for drug delivery and cellular imaging\cite{ref618}. These multifunctional QDs are promising vehicles for the co-delivery of nucleic acids and chemotherapeutics, and for real-time tracking during cancer treatment\cite{ref619}.

QDs also have great potential in photodynamic therapy, where they act either as photosensitizers themselves or as a carrier\cite{ref620}.

\textbf{1.3.2.10 Cell-based carriers.} The delivery systems that we have briefly described might, in different ways hold some features that limit their application, including the reduced amount and/or dimensions of molecules that can be delivered, the restricted carrying capacity and the possibility of altering the drugs release\cite{ref621-622}. Alternatively, the different cell-based carriers might be in theory considered as a very effective and reliable DDS, almost ideal, for the attractive possibilities to deliver therapeutic compounds, especially those that have reduced half-lives and are rapidly inactivated \textit{in vivo} following administration\cite{ref623}. Cell based carriers have been introduced since the seventies, however in the last few years, some have been widely developed and optimized, as possible vehicles for therapeutic compounds. They could be identified in two main categories:

\textit{Transduced cells} that are capable of expressing pharmaceutically relevant agents. These cells have been transduced with selected genes and with different vectors. Usually gene transfer is performed to endow the cell with a specific feature, for example to express a fluorescent protein to track the behavior of the cell \textit{in vivo} or to correct a genetic defect as well as to make the target cell susceptible to the action of a selected drug\cite{ref624}. They include myeloid precursors, fibroblasts, mesenchymal stem cells, neural progenitor cells, epidermal stem cells, hematopoietic stem cells, platelets, muscle progenitor and muscle stem cells. In addition, cell carriers have been reported as capable of delivering immunomodulatory molecules at sites of interest\cite{ref625}. The main problems associated with the use of this technology is to regulate the duration of the effect of the transduced cells, the safety of the construct and their distribution in the body as it is difficult to control. However, the possibility of engineering stem cells for the \textit{in vivo} production and release of selected therapeutics appear promising and this area will certainly enjoy new applications hopefully up to the clinic\cite{ref626}.

\textit{Cell carriers}. In general, carrier cells can be loaded with drugs or therapeutics. They can be transplanted in tissue compartment or enter the blood stream, therefore they be
designed to release their content either systemically or at site of action, where the target might be other cells or a tissue following a controlled release rate. They can be used to provide delivery for example of missing enzymes and hormones that might in turn affect the metabolism and function of neighboring cells and tissues\[627\].

Cell carriers investigated so far include both bacterial cells and animal cells. Bacterial cells have been used as non-living cell envelope preparations from gram-negative cells, devoid of cytoplasmic content, while preserving morphology and surface antigenic structures\[628\]. These ghosts have been successfully investigated mainly as adjuvant particles to improve an immune response against the ghost-derived target antigens.

Animal cells can include macrophages, erythrocytes, platelets, islets, hepatocytes and fibroblasts and they have been suggested as possible vehicles for therapeutic compounds\[629\]. In particular, macrophages could be loaded with drugs by way of ingested nanoparticles. This approach requires that macrophages are adoptively transferred to the recipient after ex vivo loading with nanoparticles conjugated with drugs and/or contrasting agents\[630\]. The majority of them are immediately sequestered in the liver and in the spleen\[631\].

In the majority of cases the carriers are actual endogenous cells, they produce little or no antigenic response, and upon aging or being damaged can be removed from the circulation following a physiological route.

### 1.3.3 Erythrocyte-based carriers

Since the mid-seventies, the use of erythrocytes (also called Red Blood Cells RBCs) as drug carriers has been originally proposed to improve drug delivery. Here below I listed only few of the biological features that erythrocytes hold and that are pivotal for their use as carriers.

Erythrocytes are naturally designed to constitute potential biocompatible cellular carriers for the delivery of molecules through the blood stream. They are the most common cells of blood, responsible for oxygen transport and normal human RBCs are discocytes, with a typical biconcave shape and an ellipsoidal disc with depressions located in the center on both sides. The process of erythrocyte formation within the body is known as erythropoiesis. In a mature human being, erythrocytes are produced in red bone marrow under the regulation of a hemopoietic hormone called erythropoietin\[632\]. The average human erythrocyte is 8.6μm in diameter and 1.9μm in thickness (2.5μm in periphery and
In mammals, RBCs are anucleated and lose their organelles during maturation. They also appear yellowish with a central pallor. The elastic, biconcave disk provides a large surface-to-volume ratio for oxygen delivery and better flexibility to squeeze through narrow capillaries, of 2-3μm in diameter. The blood volume of a normal adult human male is about 7% of body weight and about 6.5% in a female and therefore, the average content of erythrocytes in healthy men and women is 5.4x10^6 and 4.8x10^6 per μl, respectively. A human body is commonly endowed with 2.5x10^{13} RBCs continuously produced by stem cells in the bone marrow, at a rate of 2 million per second. As they mature, the erythrocytes appear quite simple in structure, they lose their nuclei, and organelles, become disk-shaped, and begin to produce hemoglobin. This molecule is a tetramer composed of four monomers held together by weak bonds. It consists of two pairs of polypeptide chains, the globins, each having an attached heme-molecule composed of iron and a protoporphyrin molecule. The iron atom has a free valence and can bind one molecule of oxygen. Thus, each heme-containing protein can reversible hold one molecule of oxygen and thus, it is responsible for O₂-CO₂ binding inside the erythrocytes. The main role of erythrocytes is in fact the transport of O₂ from the lungs to tissues and the CO₂ produced in tissues back to lungs. Thus, erythrocytes are a highly specialized O₂ carrier system in the body. Because a nucleus is absent, all the intracellular space is available for O₂ transport that is guaranteed by about 270 million hemoglobin molecules per RBC. In addition, because mitochondria are absent and because energy is generated anaerobically in erythrocytes, these cells do not consume any of the oxygen they are carrying. Erythrocytes have a life span of around 100-120 days travelling the circulatory system before degenerate and being selectively removed and destroyed by macrophages in the reticulo-endothelial system (RES), also known as the monocyte-macrophage system, especially in the spleen and liver. The breakdown products are recycled; hemoglobin is break down into globin and heme. Globin degraded to amino-acids, while iron reused in hemoglobin synthesis.

Their membrane is dynamic, semi-permeable components of the cell, associated with energy metabolism in the maintenance of the permeability characteristic of the cell of various cations and anions. The surface area of mature RBCs is about 136μm² but can swell to a sphere of approx. 150μm³. Their membrane is strictly connected with the membrane skeletal proteins that are organized in a uniform shell and their shape can undergo a number of reversible transformations. An important determinant of RBC survival is its deformability, which is affected by the internal viscosity, the
surface/volume of the cell and the intrinsic deformability of the membrane. The RBCs act as an osmometer since they shrink when placed into a hypertonic solution or swell when placed into a hypotonic solution. Thus, they can reach a critical hemolytic volume, giving rise to holes on the membrane ranging from 10nm up to 500nm. These processes are usually reversible and following hemolysis the holes close and the cell resumes its biconcave shape\textsuperscript{[626]}.

The presence or absence of antibodies in plasma and of certain inherited antigens (A, B and Rh factor) on the surface of RBCs determines in part the different blood types. These antigens are proteins, carbohydrates, glycoproteins or glycolipids that can trigger an immune response if they are foreign to the body. The two most important human blood group systems are ABO and the RhD antigen and a safe blood transfusion or re-injection depends on careful blood typing and cross matching. For instance, blood group AB individuals have both A and B antigens on the surface of their RBCs, and their blood does not contain any antibodies against either A or B antigen. Therefore, an individual with type AB blood can receive blood from any group (with AB being preferable), but cannot donate blood to any group other than AB. They are known as universal recipients. At the contrary, blood group O (also called blood group zero in some countries) individuals do not have either A or B antigens on the surface of their RBCs, and their serum contains IgM anti-A and anti-B antibodies. Therefore, a group O individual can receive blood only from a group O individual, but can donate blood to individuals of any ABO blood group (i.e., A, B, O or AB). Because it is compatible with anyone, O negative blood is often overused and consequently is always in short supply.

Indeed one of the most important features is the possibility of increasing the membrane permeability. This allows external content to enter the RBCs during favorable conditions and then to be trapped inside when condition regress to normal. Resealed red blood cells have been exploited extensively for both temporally and spatially controlled delivery of a wide variety of drugs, bioactive and therapeutic. Once are loaded with active agents, erythrocytes still retain morphological, immunological and biochemical properties similar to those of native cells. Moreover, they are completely biodegradable without generation of toxic products and show high biocompatibility especially when autologous erythrocytes are employed. In comparison to other carriers, RBCs have a prolonged lifetime in circulation and can be safely eliminated through fixation of complement and uptaken by macrophages\textsuperscript{[636]}.

Potentially, a wide variety of chemicals can be encapsulated or coupled to the membrane, including biopharmaceuticals, therapeutically active peptides and proteins, nucleic acids-
based biologics, antigens and vaccines, that all are widely exploited candidates for target therapy. Erythrocytes are also suitable for the delivery of therapeutic agents that have reduced half-lives and limited tissue penetration\cite{629,637-640}. Moreover, carrier erythrocytes can be employed as circulating bioreactors due to the presence of several enzymatic activities that can directly affect the loaded molecules and, in the case of loaded prodrugs, give rise to the active drug itself\cite{463}.

Upon infusion into the compatible organism, drug-loaded RBCs can serve as the intravenous slow-release carriers and/or targeted drug delivery systems especially to target the drug to the RES\cite{629}, until they become aged due to the gradual inactivation of their metabolic pathways. After their natural life span in systemic blood stream, the cell membrane loses its natural integrity, flexibility and chemical composition. These changes will in turn result in the destruction of the senescent RBCs upon passage through the spleen trabecules\cite{641}. In fact, when erythrocytes become old, certain catabolic changes occur leading a loss of both plasticity and resistance to osmotic and mechanical damage. These changes hinder the passage of the red cells through the microvasculature, which may convey to either lysis of the cell in circulation or phagocytosis in RES. In the first scenario, erythrocyte opsonization (coating by compounds appetizing phagocytes) by immunoglobulins and complement promoting phagocytosis give rise to direct destruction of carrier erythrocytes, lysis, aggregation, immune reactions, cellular uptake, adhesion to vascular endothelium and rapid elimination via phagocytosis and entrapment in the microvasculature\cite{642}. Once in the reticulo-endothelial system, the erythrocyte is attacked by lysosomal enzymes that cause the breakage of the cellular membrane and the degradation of the hemoglobin by the heme-oxygenase enzyme. Aged or abnormal erythrocytes could be then destroyed by the phagocytizing macrophages of the RES, including peritoneal macrophages, hepatic Kupffer cells, alveolar macrophages of the lung, peripheral blood monocytes\cite{643} and vascular endothelial cells\cite{644} that recognize the cells and remove them from circulation.

It is noteworthy that the possibility exists to engineering modifying the RBC surface and loading capacity to benefit delivery efficacy and improve their application as drug release or site-targeted delivery systems for a variety of bioactive agents for different therapeutic scopes\cite{639,645}.  

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1.3.3.1 Drug-erythrocyte associations. There are two major approaches for establishing a physical association between the therapeutic compound and erythrocyte carriers. The most widely used formulation is drug encapsulation in erythrocytes using several loading procedures, while the second approach occurs through the attachment of the ligand to RBC membrane. During all these procedures, blood group antigens on RBCs surface are not destroyed and blood typing is retained after drug loading or membrane binding\[646-647\].

Encapsulation of drug or other bioactive agents in resealed erythrocyte carriers can be performed by several procedures. Some of these methods have a physical nature whereas the others are chemically based and they all provide encapsulation of diverse agents including antibiotics, steroids, antimicrobial agents, proteins and genetic materials into RBCs. The optimal characteristics for a compound to be successfully encapsulated in erythrocytes include a considerable degree of water solubility, resistance against inactivation within the erythrocytes, the lack of physical and/or chemical interaction with erythrocyte membrane or the other cell constituents, and well-defined pharmacokinetic and -dynamic properties\[648\]. At the end of procedures, they reserve most of their volume available for the encapsulated substance and once in the stream, the drug-loaded erythrocytes serve as slow circulating depots\[649\] and target the drugs to the RES\[650\]. Moreover, erythrocytes provide a two-ways shelter action preventing drug premature inactivation and degradation by endogenous factors and, at the same time, protecting the organism against the toxic effects of the drugs thus avoiding immunological reactions.

Drug loading in erythrocytes include several osmotic-based methods, chemical perturbation of the membrane, electroporation, entrapment by endocytosis, loading by electric cell fusion and by lipid fusion.

Osmotic-based methods constitute the benchmark methods for the encapsulation of substances in erythrocytes, developed during the 1970s. Erythrocytes have the ability to undergo reversible swelling and shape changes in a hypotonic solution or under stress, accompanied by an increase in the permeability of the membrane. RBCs can increase in volume by 25-50% leading to an initial change in the shape from biconcave to spherical adapting additional volume while keeping the surface area constant\[651\]. Therefore, the cells can maintain their integrity up to a tonicity of 150 mosM/kg, above which the membrane ruptures, releasing the cellular contents. Exposure of erythrocytes to a
hypotonic solution creates transient pores of 200-500 Å in the erythrocyte membrane, and allows drugs to enter and become permanently entrapped after the cells have been resealed with a specific isotonic buffer solution. Osmotic stress can alter erythrocyte morphology thereby accelerating their removal from the circulation by the RES\textsuperscript{652}. Erythrocyte ghosts are what remains after cell lysis and depletion of cellular contents and can be resealed by restoring isotonic conditions, having the drug inside. Upon incubation, the cells are able to resume their original biconcave shape and recover original impermeability\textsuperscript{653}. This methodology has been widely used and customized, applying some variations including hypotonic dilution, or dialysis and they have been applied in the encapsulation of various compounds, such as lipids, enzymes and other drugs.

Hypotonic dilution was the first method studied and developed for the encapsulation of chemicals into erythrocytes and is the simplest and fastest way to include\textsuperscript{654}, low molecular weight drugs. According to this method, a volume of packed erythrocytes is diluted 2-20 times with aqueous solution containing the selected drug obtaining a hypo-osmotic environment. The solution tonicity\textsuperscript{7} is then restored adding a hypertonic buffer and the loaded cells collected by centrifuge separation\textsuperscript{655}. The major drawbacks of this method include low entrapment efficiency\textsuperscript{656} and a considerable loss of hemoglobin and other cell components. This reduces the circulation half-life of the loaded cells that are readily phagocytosed by RES macrophages\textsuperscript{654}. This method is used for loading several chemical compounds, including enzymes, bronchodilators and anticancer drugs\textsuperscript{651}.

A more severe version of hypotonic dilution is the hypotonic hemolysis. The cells can maintain their integrity in an isotonic environment. Reduction of solute concentration in the external fluids leads to membrane ruptures. At peri-lysis point, some transient pores are generated on the membrane that lead to cell lysis and loss of cellular contents. The rational for using these ruptured erythrocytes to reconstruct drug carriers is because ruptured membranes can be resealed by restoring isotonic conditions. Upon incubation, the cells resume their original biconcave shape and recover original impermeability\textsuperscript{654}. This method also hold an historical value as it was used in 1986 by Leslie McEvoy and colleagues to evaluate the role of trans bilayer phospholipid distribution in the recognition and phagocytosis of erythrocytes by macrophages, finding that the asymmetrical distribution of phospholipid is the physiological feature that prevents RBC interaction with RES\textsuperscript{658}. 

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As a optimization of this process, hypotonic hemolysis was later adapted for the standardization of production of a novel carrier by the Italian National Council of Research (CNR) which filed a patent, identified as WO2010/070620 for a engineered erythrocyte-based drug delivery system, referred to Erythrocyte-Magneto-FHA-Virosomes (EMHV) developed to improved ability of erythrocytes to release therapeutic compounds to specific target cells\(^{[639]}\). This system is currently in use in our lab and constitutes the base of the majority of our experimental activities, as we focus on the development and implementation of new anticancer therapeutics for personalized medicine\(^{[463,639-340,645]}\). Various modified version of similar process can be exploited to develop automatized systems: "Red cell loader" is a new procedure for the encapsulation of non-diffusible drugs into human erythrocytes, developed by Magnani and colleagues\(^{[636]}\).

To optimize loading, several procedures were coupled: for example, hypotonic stress was associated with dialysis, another widely used method for concentration/size separation\(^{[659]}\). Semi-permeable dialysis membrane maximizes the intracellular/extracellular volume ratio for macromolecules during lysis and resealing. In the process, an isotonic and buffered suspension of erythrocytes with a suitable hematocrit is placed in a conventional dialysis tube immersed in 10-20 volumes of a hypotonic buffer at 4°C. The tonicity of the dialysis tube and, as a consequence, the resealing of loaded erythrocytes, are then restored at 37°C by directly adding a calculated amount of a hypertonic buffer (usually containing adenosine, glucose and magnesium chloride) to the surrounding medium or by replacing the surrounding medium by isotonic buffer. The drug to be loaded can be added by either dissolving the drug in isotonic cell suspending buffer inside a dialysis bag at the beginning of the experiment or by adding the drug to a dialysis bag after the stirring is complete.

Hypotonic dialysis has high entrapment efficiency, in vivo survival and integrity of RBCs and loading capacity. Conversely, the drawbacks include a long processing time, heterogeneous size and the need for special equipment.

Chemical perturbation of the membrane is based on the increase in membrane permeability of erythrocytes when the cells are exposed to certain chemicals. In 1973, Deuticke and colleagues showed that the permeability of erythrocyte membrane increases upon exposure to polyeneantibiotic such as amphotericin B\(^{[660]}\). Later, this method was successfully used to entrap the antineoplastic drugs in human and mouse
Electroporation, also known as electro-insertion or electro-encapsulation, is performed using an electrical pulse to encapsulate bioactive molecules\textsuperscript{662-663}. A strong external electrical field brings about irreversible changes in an erythrocyte membrane and thus, transient electrolysis has been used to generate desirable membrane permeability for drug loading\textsuperscript{664}. The erythrocyte membrane is opened by a dielectric breakdown and subsequently, the pores are able to admit molecules of different size. The procedure involves suspending erythrocytes in an isotonic buffer in an electrical discharge chamber. This method of encapsulation is a good alternative to other commonly employed techniques and its entrapment efficiency is about 35%, as well the life span of the resealed cells in circulation is comparable with that of normal cells. Various compounds such as enzymes, drugs and latex particles of diameter 0.2μm\textsuperscript{665} can be entrapped within erythrocytes by this method.

**Entrapment by endocytosis** involves the dilution of 1:10 of packed erythrocytes with a particular buffer, followed by incubation. The various candidates entrapped by endocytosis include primaquine, vinblastine, hydrocortisone and chlorpromazine, which are known to induce uniconcave shapes called stomatocytes in the cell membrane. The pores created by this method are then resealed and the vesicle membrane separates endocytosed material from cytoplasm, thus protecting it from the erythrocytes and vice-versa\textsuperscript{666-667}.

**Loading by electric cell fusion** involves the initial encapsulation of drug molecules into erythrocyte ghosts followed by adhesion of these cells to target cells. The fusion is accentuated by the application of an electric pulse, which causes the release of an entrapped molecule. An example of this method is loading a cell-specific monoclonal antibody into an erythrocyte ghost\textsuperscript{668}. An antibody against a specific surface protein of target cells can be chemically cross-linked to drug-loaded cells that would direct these cells to desired cells\textsuperscript{669}.

**Loading by lipid fusion** is another method to encapsulate drug into erythrocytes. Lipid vesicles containing a drug can be directly fused to human erythrocytes, which lead to an exchange with a lipid-entrapped drug. This technique was used for entrapping inositol
monophosphate to improve the oxygen carrying capacity of cells\cite{670}, even though its entrapment efficiency is very low.

*Membrane binding* of ligand to RBC membrane represents an alternative to encapsulation strategy to achieve RBC loading of active molecules. As for encapsulation, it could be reversible or irreversible. Drugs can be coupled to erythrocyte surface using variety of covalent and non-covalent cross-linkers, as well as anchored onto circulating naïve RBCs using recombinant fusion proteins with specific affinity to them. Erythrocyte membrane has the potential to provide an extended surface area that may be used for the attachment of multiple copies of protein or other therapeutic molecules. Lack of isolation of a drug from blood en route to the therapeutic site would represent an obvious downside of surface coupling vs encapsulation. However, the problem of premature inactivation and side effects can be partially avoided using pro-drug formulations resistant to plasma inhibitors\cite{671}. On the other hand, surface coupling strategies avoid damaging encapsulation procedures and therefore offer theoretical advantages of drug loading without compromising RBC biocompatibility. In addition, binding therapeutic drugs to erythrocyte surface circumvents issues related to drug release\cite{672}. Notably, coupling to RBC surface resolves diffusional limitations: even enzymes that react with small, membrane permeable substrate are more active when bound to the RBC surface than when incorporated within the cell\cite{673}.

Techniques for coupling different molecules to RBC membranes have been designed during past decades, in the process of development of reagents for immunological reactions of agglutination. Numerous cross-linking agents and procedures have been applied to conjugate proteins and other antigens and biological molecules to RBCs of different animal species. However, subsequent studies revealed that these conjugation methods grossly damage erythrocyte membrane, reducing their plasticity, resistance to lytic agents and biocompatibility.

Several practical strategies for coupling therapeutics to carrier RBC surface have evolved and have been tested *in vitro* and *in vivo* in last two decades. These strategies can involve: 1) chemical coupling of agents directly to RBC surface 2) coupling of a receptor that binds a therapeutic agent to erythrocyte membrane 3) conjugation of therapeutics with affinity ligands that bind to erythrocytes thereby anchoring cargoes on them. Binding can be achieved with antibodies, antigens, enzymes, cytokines and other biologically active compounds explored for systemic delivery.

Animal studies showed that surface coupling to RBC could be used for improvement of
antigen delivery, masking of RBC antigens, clearance of pathogens from blood and intravascular delivery of therapeutics that supposed to act within the vascular lumen[671]. The most widely used strategy for membrane binding of ligands to erythrocyte carriers is the "avidin-biotin" approach with several applications in drug delivery during the last two decades[674]. Biotinylation of intact mammalian erythrocytes could be performed either by attachment to the amino groups by means of biotin N-hydrosuccinimide ester (NHS-biotin) or by oxidation of the induced aldehyde groups of the erythrocyte membrane by biotin hydrazide. Avidin-biotin bridges have been used for reversible membrane binding of several bioactive agents, such as bovine serum albumin (BSA)[675] and HIV-1 tat protein[676].

1.3.3.2 Delivery strategies. According to the preferred therapeutic approach, circulating erythrocytes are used as either carriers for the sustained release of the drugs or to target the drugs to specific organs[677], using two major strategies in the delivery of the chemical compounds.

**Drug release strategy** involved the release of the therapeutics and/or imaging agents encapsulated into carrier erythrocytes. Releasing can occur either slowly, for example via diffusion through RBC plasma membrane[678] and/or its eventual degradation[679], or rapidly, e.g. via lysis of carrier RBC by plasma complement[672]. The maintenance of the normal oxidant/antioxidant balance in erythrocytes during drug encapsulation may help to produce loaded cells with characteristics similar to those of normal erythrocytes[680], without RES involvement and, in this case, the drug-loaded cells can be used as slow-release carriers for the entrapped drugs. Dosage is designed to obtain a prolonged therapeutic effect by continuously releasing medication over an extended period after administration of a single bolus[681]. Several mechanisms can contribute to this purpose including passive diffusion out of the loaded cells into circulation, specialized membrane-associated carriers[682], accumulation of the loaded erythrocytes into the lymphatic nodes, and drug release upon hemolysis in this sites and hemolysis in the injection sites[683].

**Targeted drug delivery**, involving RES or not, is an important physiological strategy using erythrocytes as carriers in drug delivery and it is among their mostly attractive applications in the recent decades. Accelerated removal and RES targeting occur when a considerable fraction of carrier is be trapped by the RES organs within a short time.
period after re-injection. Once RES macrophages and other phagocytes naturally recognize loaded RBCs their fate is to be processed by these sites, which are the intended site of action of the drug\textsuperscript{[684-685]}. Therefore, targeting outside these sites is minimal with a consequent reduction of side effects and effective dose\textsuperscript{[686]}. The delivery strategy results appropriate for the treatment of specific disease states\textsuperscript{[687]}. Biotechnological modification has been prompted to increase RES sequestration of loaded RBC and to increase selectivity towards certain organs such as the liver and the spleen. For instance, exposure of loaded carriers to membrane stabilizing agents increases the targeting index of the erythrocytes to RES via decreasing the deformability of these cells\textsuperscript{[688]}. As well coating the carrier cells by anti-Rh or other types of antibodies may be another method that makes the erythrocytes more recognizable by RES macrophages. Pre-exposing the carrier erythrocytes to thermal shock, oxidant compounds, the agents reactive to the sulfidryl group-containing portions of the cell membrane, the enzyme neuraminidase and the proteolytic enzymes also have been exploited to improve RES targeting of carrier erythrocytes with some degree of success\textsuperscript{[554]}. On the other hand, treatment of loaded RBCs with glutaraldehyde enhances their stability and, consequently, the release of the encapsulated substance is reduced\textsuperscript{[689-690]}. It is possible to direct loaded erythrocytes to target organs other than those belonging to RES system. The various approaches may include the co-encapsulation of elements that help specific localization. For example, localized disruption and release can be achieved with application of ultrasound waves and consequently microvessel ruptures due to targeted microbubble destruction\textsuperscript{[691]}. As well, wheat lectin extracted pre-treating the erythrocytes loaded with anticancer drugs improves their targeting index to neoplastic cells\textsuperscript{[692]}.  

1.3.3.3 Advantages and disadvantages. At this point, and for the purpose of this thesis, a clear summary of the most important features of erythrocyte-based DDS is necessary to emphasize their beneficial aspect encouraging their use in drug delivery, as well as to stress some drawbacks that might jeopardize our research. These in fact must be the focus of future effort for therapeutic optimization\textsuperscript{[693]}. Here is the list of some of the beneficial feature that enable their use as system of choice in certain situations as drug delivery system preferred to other widely used carrier systems (\textit{Table 3}).
<table>
<thead>
<tr>
<th>Physical/Chemical rheology</th>
<th>Beneficial aspects</th>
<th>Risk evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>High degree of uniform size and shape of the carrier (monodispersion)</td>
<td>[484-485]</td>
<td>For human erythrocytes, only autologous, compatible or O negative blood can be used [699].</td>
</tr>
<tr>
<td>Complete biodegradability</td>
<td>[682,696-697]</td>
<td>Possible erythrocyte agglutination [699].</td>
</tr>
<tr>
<td>Ability to circulate throughout the body (physiological role) reaching all compartments</td>
<td>[638]</td>
<td></td>
</tr>
<tr>
<td>Safe elimination mechanisms, including fixation of complement and uptake by macrophages</td>
<td>[636]</td>
<td></td>
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<table>
<thead>
<tr>
<th>Carrier loading optimization</th>
<th>Beneficial aspects</th>
<th>Risk evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>High performance of encapsulation</td>
<td>[700]</td>
<td>Possible leakage of certain encapsulated substances from the loaded erythrocytes [699,703].</td>
</tr>
<tr>
<td>High amount of loaded drug within a small cell volume (quantum delivery)</td>
<td>[696]</td>
<td>Altered erythrocyte physiology due to the loaded molecules [699].</td>
</tr>
<tr>
<td>Long life span in circulation</td>
<td>[682,701]</td>
<td>Difficulty to storage loaded viable erythrocytes prior to re-entry the host body [699].</td>
</tr>
<tr>
<td>Versatile carriers used to deliver a wide variety of compounds</td>
<td>[640,702]</td>
<td>The need of using of conditioning agents (isotonic buffers with essential nutrients, nucleosides or chelators, lyophilization) as well as low temperature to stabilize products [699].</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Risks of variability for poor standardization during preparation [699].</td>
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<tr>
<th>Therapy localization</th>
<th>Beneficial aspects</th>
<th>Risk evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Possibility of localized concentration of the therapeutic agent to the target tissue, magnetic driven concentration</td>
<td>[643,639,645,704-706]</td>
<td>Limited carrier activity in non-RES organs [708].</td>
</tr>
<tr>
<td>Targeting the RES organs towards the physiological route</td>
<td>[682,698,707]</td>
<td></td>
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</tbody>
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<tr>
<th>Therapy release</th>
<th>Beneficial aspects</th>
<th>Risk evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Improving of drug pharmacokinetic and -dynamic parameters (increasing therapeutic window)</td>
<td>[463,645,699,704]</td>
<td>Limited drug half-life due to easy uptake and accelerated <em>in vivo</em> removal by the RES (as result of modifications occurred during loading procedures) [708].</td>
</tr>
<tr>
<td>Sustained release into the circulatory system acting as a reservoir for the loaded drug</td>
<td>[696]</td>
<td></td>
</tr>
<tr>
<td>Ideal zero-order drug-released kinetics</td>
<td>[709]</td>
<td></td>
</tr>
<tr>
<td>Systemic drug clearance and activity in blood are prolonged</td>
<td>[698,699,709-710]</td>
<td></td>
</tr>
<tr>
<td>Prevention of drug degradation and inactivation by host agents</td>
<td>[463,682,701,709]</td>
<td></td>
</tr>
<tr>
<td>Prevention of any undesired immune response against the loaded drug</td>
<td>[711]</td>
<td></td>
</tr>
<tr>
<td>Considerable increase in drug dosing interval in chronic therapy</td>
<td>[710,712]</td>
<td></td>
</tr>
<tr>
<td>Bioreactor activity that converts loaded prodrugs by RBC endogenous enzymes</td>
<td>[463,713]</td>
<td></td>
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<tr>
<th>Toxicity</th>
<th>Beneficial aspects</th>
<th>Risk evaluation</th>
</tr>
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<tbody>
<tr>
<td>Targeting the RES organs (physiological route)</td>
<td>[682,698,707]</td>
<td>Possible contamination due to the blood source, the equipment used and the loading environment [714-715].</td>
</tr>
<tr>
<td>Protection of the organism against toxic effects of drugs</td>
<td>[704]</td>
<td>Intravascular hemolysis, including acute vascular, renal and immune reactions to free hemoglobin and hypoxia [708].</td>
</tr>
<tr>
<td>High biocompatibility as the use of autologous cells, minimize the possibility of triggered immune response</td>
<td>[682,698,699]</td>
<td>Off target drug leakage [699,703].</td>
</tr>
<tr>
<td>Decrease drug side effects</td>
<td>[712,716]</td>
<td></td>
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</tbody>
</table>

The success of blood derivatives technology highly depends on the reliability of the products. In the case of erythrocyte carrier production, good laboratory practices are applied and guidelines are set for the standardization of important product features. Indeed, the success of resealed erythrocytes products as a drug delivery system depends equally on final chemo-physical conditions as well as on their shelf-life duration. The most common storage media include oxygenated Hank's balanced salt solution (HBSS)
containing 1% soft gelatin, and acid-citrate-dextrose at 4°C. The cells are well recovered after liquefying the gel by placing the tube in water bath at 37°C followed by centrifugation. In this environment, erythrocytes remain viable in terms of their physiologic and carrier characteristics for at least 2 weeks at this temperature. The addition of calcium-chelating agents or the purine nucleosides improve circulation survival time of cells upon re-injection. Exposure of resealed erythrocytes to membrane stabilizing agents, such as dimethyl sulfoxide (DMSO), followed by lyophilization or glass filtration has been reported to enhance their stability upon storage. The resultant powder was stable for at least one month without any detectable changes. The major disadvantage of this method is the presence of appreciable amount of membrane stabilizers in bound form that remarkably reduces circulation survival time. Alternatively, another method utilized for storage has been cryo-preservation of RBCs in liquid nitrogen. Another strategy to increase storage stability includes encapsulation of a prodrug that undergoes conversion to the active drug only at body temperature in a way that therapeutic activity is maintained up to administration.

Once administered into the blood stream, efficacy of resealed erythrocytes is related to their life-span that depends upon their size, shape, and surface electrical charge as well as the extent of hemoglobin and other cell constituents lost during the loading process. There are standard methods used to determine in vivo survival time, for example labeling of cells by fluorescent markers such as fluorescein isothiocyanate or entrapment of 14C sucrose or gentamicin. These methods are necessary also to describe the circulation survival kinetics of resealed erythrocytes that usually shows a typical bimodal behavior with a rapid loss of cells during the first 24 hrs. after injection, followed by a slow decline phase with a half-life about days or weeks.

1.3.3.4 Characterization. After loading of therapeutic agent on RBCs, the carrier cells may be exposed to physical, cellular as well as biological evaluations. Physical characterizations include shape and surface morphology, cell size and volume, energy metabolism, deformability, surface pH, density gradient separation, stability, drug release and drug content. Cellular investigations involve hemoglobin content, cell volume and cell recovery, osmotic shock and fragility, turbulence shock and erythrocyte sedimentation rate. Biological evaluations include sterility, pyrogenicity and animal toxicity.
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**Shape and surface morphology** decide their life span after administration. The morphological characterization of erythrocytes is undertaken by comparison with untreated erythrocytes using microscopy imaging\cite{718}. In the majority of cases, light microscopy reveals no change in resealed cells apart from the presence of few spherical erythrocytes (spherocytosis). Electron and phase contrast microscopy are normally used to check for morphological changes induced by osmosis-based encapsulation methods\cite{719}. This analysis may reveal few stomatocytes, a form of spherocytosis with an invagination in one point of the disk and some cells of smaller size (microcyte)\cite{693} while the majority of the cells maintain their biconcave discoid does not change shape after the loading procedure.

**Deformability** affects the life span of the cells and the ease of passage through narrow capillaries and RES. This parameter determines the rheological behavior of the cells and depends on the visco-elasticity of the cell membrane, viscosity of the cell contents, and the cellular surface-to-volume ratio. The deformability is measured by passage time of definite volume of cells through capillary or polycarbonate filters\cite{693}.

**Stability** is assessed by means of the incubation of the cells in the autologous plasma or in an iso-osmotic buffer, setting hematocrit between 0.5% and 5% at temperatures of 4°C and 37°C\cite{720}. To improve stability and prolong shelf life of carrier, erythrocytes are stored as lyophilized powder cells in an amber color vial to minimize light interaction\cite{668}.

**Drug release rate** is the most important parameter for evaluation of the state of resealed erythrocytes. In fact, this parameter regulates their pharmacokinetic behavior *in vivo*. Normally hemoglobin is released at the same time of because drug release as it involves the loss of cell membrane integrity (hemolysis). The drug release is controlled by molecular weight and liposolubility of the drug. The ratio between the rate of hemoglobin and the rate of drug release depend on the mechanisms involved in the release of the substance encapsulated within the erythrocytes\cite{638}. There are mainly three ways for a drug to efflux out from the erythrocyte carriers: phagocytosis, diffusion through the membrane of the cells and activation of a specific transport system. RBCs are normally removed from circulation by the process of phagocytosis. The membrane surface state (rigidity or presence of antibodies) determines degree of cross-linking determines whether liver or spleen will preferentially remove the cells. In this
case, a quick delivery of drug to RES compartment occurs. The rate of diffusion of the drugs out of RBC depends upon the rate at which a particular molecule penetrates through a lipid bilayer and it is great for a molecule with high lipid solubility. Therefore, when the release rate of lipophilic drug is considerably higher than that of hemoglobin as the drug may diffuse readily thanks to passive diffusion. When the rate of drug release is instead comparable to that of hemoglobin, drug release occurs thanks to cell lysis, as the drug cannot be released by mere diffusion mechanism. Hence, hydrophilic drugs may become attached to cell structures requiring the lysis of the cell for drug release. Moreover, most of the drug molecules enter cells by a specific membrane transport system that also ensures the drug release.

Measurement of the internalized drug is performed to verify efficacy of the chosen encapsulating method, at the end of the process on isolated loaded erythrocytes. The process involves the deproteinization of packed, loaded cells with acetonitrile, followed by complete lysis and centrifugation. The clear supernatant is analyzed for the drug content using spectrophotometer or HPLC.

**Hemoglobin content** may be impaired by the alterations in the permeability of the membrane of RBCs during the encapsulation procedure. The process involves the deproteinization of cell membrane, followed by hemoglobin assay using a cell suspension by recording the absorbance of supernatant at 540nm on a spectrophotometer.

**Percent cell recovery** involves counting the number of intact cells per unit volume of packed erythrocyte before and after loading the drug. The goal is to minimize the loss during the encapsulation procedure to maximize cell recovery.

**Osmotic fragility** detects the effect of loading process on the RBCs to check the possible changes in cell membrane integrity and the resistance of these cells to osmotic pressure of the suspension medium. The test is carried out by suspending cells in media of varying sodium chloride concentration and determining the hemoglobin released. In most cases, osmotic fragility of resealed cells is higher than that of the normal cells because of increased intracellular osmotic pressure.

**Turbulence fragility** depends upon changes in the integrity of cellular membrane and reflects resistance of loaded cells against hemolysis resulting from turbulent flow within
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It is determined by repetitive passages of cell suspension through 30-gauge hypodermic needle at 10 ml/min flow or vigorously shaking the cell suspension using a multiple test tubes orbital shaker. In both cases, hemoglobin and drug released after the procedure are determined. The turbulent fragility of resealed cells is found to be higher. Moreover, the turbulence fragility index is defined as the shaking time producing 20% hemoglobin release from erythrocytes.

**Erythrocyte sedimentation rate** (ESR) is an estimate of the suspension stability of RBCs in plasma and is related to the number and size of the red cells and to relative concentration of plasma protein, especially fibrinogen and α, β globulins. This test is performed by determining the rate of sedimentation of blood cells in a standard tube.

The potential of erythrocytes based carriers are better exploited in *in vivo* applications as this drug delivery system can reach a wide spectrum of pharmacological and therapeutic targets: they can be used for liver-deficiency/therapy, delivery of antiviral agents, enzymes and NPs, treatment of hepatic tumor and for parasitic disease, removal of RES iron overloads and toxic agents. They also may function as circulating bioreactors, improving oxygen delivery to tissue.

**1.3.3.5 Erythrocyte carriers in cancer.** Over the last decades, erythrocytes have been studied as carriers of chemotherapeutic agents for targeting the RES. However, to date the majority of the drug delivery studies based on drug-loaded erythrocytes that are in the preclinical phase are focused on the delivery of anticancer molecules.

Loading anti-cancer drugs into carrier erythrocytes reduced drug toxicity towards the body and improves their delivery to tumors via several mechanisms: for example, some study exploit antibody specific recognition of tumor cells, while other are relying on an unspecific mechanisms called Enhanced Permeation and Retention (EPR) proper of the solid tumor microenvironment.

Liposomes, linear polymers and polymer micelles represent the most popular carriers for anti-cancer drugs. However, RBC carriers may find a niche in tumor treatment, providing a wide range of anti-cancer encapsulated drugs and formulations with prolonged circulation. Carrier erythrocytes are also expected to improve drug and detection probe delivery, to have less adverse effects, and thus result in improved detection and treatment.
of tumors[732]. For instance, loading the hydrophobic anti-tumor agent dequalinium into mouse RBCs supplied much longer half-life in circulation than PEG-liposomal formulation[733].

The treatment of carrier erythrocytes with certain substances gives rise to alterations in the properties of the loaded RBCs and, especially, to a greater receptiveness of the RES in the macrophages. In particular, their cross-linking treatment with glutaraldehyde enhances the properties as a carrier system, and the carrier erythrocytes appear more stable and resistant to turbulences. The output of the encapsulated substance from these erythrocytes into the circulatory flow is reduced[696]. However, the treatment with glutaraldehyde increases the selectivity of the erythrocytes towards the RES[734], increasing their uptake by macrophages and other cells exerting active phagocytosis[735].

Few encouraging examples of using loaded erythrocyte based therapy support the effort to optimize and advance erythrocyte carrier technology. For example, doxorubicin-loaded RBC delivered the cargo into macrophages[736] and accumulated in the liver after intravenous injection in animal models[737]. This treatment was used against lymphoid tumors in dogs. In this case, doxorubicin inflicted unexpected substantial chronic suppression of myeloid cells however did not result in marked cardiac toxicity, a hallmark adverse effect[738]. Notably, a formulation of human autologous or blood typing and cross-matching compatible erythrocytes encapsulated with a related anthracycline antibiotic daunorubicin has been tested in patients with acute leukemia and showed a more prolonged drug level in plasma and lesser side effects than after injection of free drug[739]. Similarly, doxorubicin-loaded autologous erythrocytes re-infused in patients with lymphomas, provided reduction of peak level and extension of drug level in plasma of patients resulting in significant elevation of the area under the curve and reduction of side effects comparing with free drug[740].

Delivery of drug carriers to solid tumors relies in major part on the EPR effect mediated by abnormally high permeability of tumor vasculature and lack of effective lymphatic drainage. In the context of vascular permeability and tumor extravasation via EPR effect, large RBCs represent less effective delivery platform than sub-micron carriers such as liposomes.

**Erythro-Magneto-FHA-Virosomes**® (**EMHV**). Delivery of drug carriers to solid tumors relies in major part on the EPR effect mediated by abnormally high permeability of tumor vasculature and lack of effective lymphatic drainage. In the context of vascular
permeability and tumor extravasation via EPR effect, RBCs represent an effective delivery carrier platform.

Recently, in the laboratory the Institute of Clinical Physiology (IFC) of Consiglio Nazionale delle Ricerche (CNR) in Siena, an engineered modified erythrocyte-based delivery system was developed and patented. The Erythro-Magneto-FHA-Virosomes® (EMHV) represents a versatile carrier suitable for the delivery of active molecules of different chemical nature. For the production of EMHVs, erythrocytes are enriched with Fe$_2$O$_3$ paramagnetic nanoparticles, normally used in diagnostics. Once in the blood stream, the application of a biocompatible external magnetic field concentrates the carrier in selected areas of the body. Moreover, the erythrocyte membrane is decorated with the fusogenic glycoprotein filamentous hemagglutinin (FHA). EMHVs are therefore endowed with "virosome" properties that enhance their ability to attach and fuse with the host cell membrane. Once the EMHV is anchored to the target cell, a complete EMHV-target membrane fusion occurs within thirty minutes, with the complete release of the EMHV contents inside the host cells$^{[639]}$.

EMHVs have been extensively characterized$^{[463]}$ and have been used as an effective DDS, which reliably conveys drugs to site of action sparing the normal tissue from toxic effects. EMHVs were used to deliver gene therapy, based on the use of synthetic DNA Elk-1 decoy in a model of syngenic porcine vascular smooth muscle cells in vitro, preventing cell migration and proliferation$^{[640]}$. More relevantly, a preclinical investigation on the use of epigenetic therapy with DAC was successfully carried out, demonstrating the feasibility of drug delivery to a selected cancer site in vivo. Moreover, these experiments also indicated that the use of EMHV to deliver DAC increases its effectiveness as an anticancer age in a xenograft model of responsive human prostate cancer. Interestingly, EMHVs conveyed DAC therapy resulted in an anti-proliferative effect also in human non-responsive human prostate cancer model$^{[645]}$. Currently, our effort continues to investigate the possibility of using EMHVs for combinatory therapy with different chemotherapeutics or molecule of different chemical nature.

Following in the footsteps of these early successes, optimization of encapsulation of different pharmaceutical agents is pivotal for the development of novel therapeutic strategies. Erythrocytes as carrier may be suitable to convey different types of molecules in high concentration, however the choice of the active agent might require adaptation of the encapsulation procedures. Here I have reported a briefly list of the possible drugs that could be delivered by these carrier. The list is based mostly on the need encountered in our research for innovative anticancer therapy, under ongoing development in our lab.
Inhibitors of epigenetic enzymes have demonstrated promising results for the treatment of several tumors, although some reports emphasized the need to improve drug stability in solution as well as maximize delivery efficacy, reducing toxic side effects and prolong epigenetic outcomes. For instance, the remarkable therapeutic potential of free DAC is dramatically hampered by its systemic instability once free circulating into the blood stream. Many formulations have been designed to protect it from rapid degradation, using different carriers such as liposomes, polymer based system and NPs linking in the attempt of improving the pharmacokinetic and -dynamic principles that govern the action and disposition of demethylating agents. The main body of the successful results from this thesis will relate to the characterization and the use of DAC included into our EMHV delivery system to treat solid tumors in animal models.

Biodrugs include peptides, RNA- and DNA-based therapeutics and can target cancer cells, interfering with the expression of specific protein and promoters of gene involved in tumor growth and progression. In general, biodrugs are inherently unstable, potentially immunogenic and typically require a delivery vehicle for efficient transport to the targeted cells.

Molecular beacons (MB) may be peptide-, RNA- or DNA-based molecules. Peptide-based MBs are Förster resonance energy transfer (FRET)-based target-responsive probes, which offer control of fluorescence emission in response to specific cancer targets and thus are emerging as promising tools for in vivo cancer diagnostics, image guidance and therapy. They show great potential due to their theranostic proprieties, although present selective delivery, specific activation and detection sensitivity and thus, the need to be targeted by DDS. RNA-based molecular beacons (MB) against cancer protein have been designed and DNA-based MBs could be also custom synthesized, against an intracellular target homologues nucleic acid sequence that is recognized and reported.

MBs are hairpin-shaped and present an internally quenched fluorophore, whose light emission is restored when they bind to their targets nucleic-acid sequence and undergo a spontaneous conformational change. Therefore, if the target nucleic acid is present, the event of MB-target hybridization can occur, causing the activation of the fluorophore with fluorescent light emission. The probes are particularly suited for tracing specific sequences of nucleic acids and they can be also used as novel theranostic tools as the protein sequence hybridization will impede transcription.
Among cancer regulatory targets, \textit{BIRC5(Survivin)} is a member of the inhibitor of apoptosis family (IAPs)\textsuperscript{[744-745]}. Besides its inhibitory action on apoptosis, \textit{Survivin} is also involved in the regulation of cell division. Its protein expression is up-regulated in several cancers as it regulates tumor progression and evasion of apoptosis. In line with its physiological roles, it is expressed also in several healthy tissues. The high expression of Survivin in cancer cells correlates to poor prognosis and resistance to chemotherapeutic treatment, thus making this protein an attractive target in anticancer therapy. The dual role of Survivin in cells, regulation of cell division and inhibition of apoptosis, combined with controversial data concerning the expression in normal tissues, emphasize the need to have an appropriate control release for selected inhibitors to test both \textit{in vitro} and \textit{in vivo} studies. Recently, various biological drugs have been developed, targeting Survivin expression. Among them, MB against mRNA-Survivin (SURV-MB) has become one of the agents used for the development of our EMHV-mediated anticancer therapy. To overcome limitation of the instability and to allow better permeability, we successfully used SURV-MB-loaded EMHVs to investigate Survivin role in retinoblastoma.

\textbf{RNA-based therapeutics} can be classified by the mechanism of activity and include inhibitors of mRNA translation (antisense), agents of RNA interference (RNAi), catalytically active RNA molecules (ribozymes), and RNAs that bind and block targeted proteins and other molecular ligands (aptamers).

RNA interference, also known as post-transcriptional gene silencing (PTGS) pathway, is a biological process in which endogenous microRNAs (miRNAs) and double-stranded exogenous small interfering RNAs (siRNAs) molecules inhibit gene expression, typically causing degradation of specific target mRNAs, translational repression and sequence-specific cleavage of complementary mRNA. At present, many RNA-based therapeutics have reached clinical testing\textsuperscript{[746]}. Challenges with their delivery, specificity, stability, and immune activation have spawned improvements in nucleic acid carriers and the development of chemically modified oligonucleotides\textsuperscript{[747]}.

\textbf{Ribozymes} are catalytic RNAs that function as enzymes and do not require proteins for catalysis. They are self-processing RNAs that cleave mRNA transcripts\textsuperscript{[746]}. However, the substrate recognition domain of ribozymes can be artificially engineered to stimulate site-specific cleavage and to be allosterically activated by effector molecules, which has led to the development of artificial "riboswitches" as biosensors and synthetic biological tools\textsuperscript{[748]}. 

\textit{An innovative epigenetic strategy for retinoblastoma treatment}
Ribozymes require chemical stabilization before the delivery to the target cells and some of them have shown encouraging results\textsuperscript{[749-750]}, although presenting some severe side effects. For example, Survivin (BIRC5)-ribozyme was able to catalyze Survivin(BIRC5)-mRNA degradation.

**Aptamers** are single-stranded nucleic acids that bind and block targeted proteins with high affinity and specificity due to their stable-three dimensional shapes\textsuperscript{[751]}. Many RNA aptamers exist as hairpin-like monomers that bind targets via unpaired nucleotides, but some others function as multiplexes. They are often modified during chemical synthesis to increase their resistance to nucleases and improve pharmacological properties\textsuperscript{[746]}.

**DNA-based biodrugs** are also available: Decoys (hijacker molecules) are short synthetic double-stranded (DNA) oligodeoxynucleotides that compete with promoters and can sequester and block related transcription factors, preventing their binding at target promoters. There are few encouraging reports of anticancer activity due to decoys used against the regulation pathways for HSF1 and NFkB, two key role proteins in cancer development. Although they are intended for potential therapeutic applications however, they might be better suitable as combinatory strategy for multi-transcription factor-mediated processes that may require multiple regulatory elements to be inhibited in varying combinations\textsuperscript{[752]}.

### 1.3.4 Ocular DDS

In the attempt to develop a therapeutic strategy for retinoblastoma, one of the most important features to consider is how to deliver the treatment to the cancer lesion. In this effort, one must consider that the eye is a close system, however highly supplied by major blood vessels. Moreover, the eye, for anatomic and functional classification, belongs to the central nervous system, and similarly to the brain is protected by biophysical barriers. For this, topical and systemic administration of drugs to the eye is highly inefficient and, consequently, ocular drug delivery is an extremely challenging endeavor\textsuperscript{[753]}. The main drawbacks are bioavailability and uncontrollable targeting of conventional ophthalmic preparations. Different drug delivery systems have been investigated for these purposes, including a controlled-release of the drug, drug targeting and penetration enhancement of the drug\textsuperscript{[754]}.
**Topical eye drop** is the most convenient and suits patient compliance\(^ {[755]}\) as it is a non-invasive route of administration, even though delivery of therapeutic drug levels to the targeted ocular tissues is partially achieved and maintained for short time.

In the treatment of ocular diseases, topical application has been therefore the main route of drug administration, especially to the anterior segment of the eye. Advances are established by modulation of conventional topical solutions with permeation and viscosity enhancers. However, rapid clearance from the eye often results in short intraocular residence times and therapeutic effect can only be achieved with frequent administrations. On the other hand, for posterior segment of the eye, drug can be delivered by using different routes, although the presence of blood retinal barriers hinders the systemic disposal. In these cases, repeated intravitreal injections represent the most common and widely recommended route of drug administration however, they cause several side effects and poor patient tolerance\(^ {[756]}\). Periocular injections are less invasive and better accepted, however they lead to compromised drug permeation, due to the ocular barriers. To overcome these limitations there is the need for controlled and sustained release of the drug in these compartments and research has been focused towards development of drug releasing devices and nanoformulations for treating chronic vitreoretinal diseases. These novel devices and/or formulations must hold some requirement such as an easy formulation, no/negligibly irritating action, high precorneal residence time, sustained drug release rate, and enhanced ocular bioavailability of therapeutics\(^ {[1]}\). To date, several ocular drug delivery systems, such as emulsion, ointments, suspensions, aqueous gels as well novel ocular DDS, like nanotechnology-based drug delivery, implants, contact lenses, microneedles and *in situ* thermosensitive gels have been developed.

*Hydrogels* have been investigated since 1965 as ocular drug delivery systems and increase of their loading capacity, optimization of drug residence time on the ocular surface and biocompatibility with the eye tissue have been the main focus of previous studies\(^ {[757]}\). Then, the development of new therapies for treating various eye conditions has led to a demand for extended release delivery systems, which would reduce frequent ocular instillation while still achieving therapeutic drug levels in the target tissues for a specific length of time.

Investigation led to the use of conventional drug delivery systems as potential intraocular carriers. NP-based systems designed for retinoblastoma therapy have improved rates of
drug delivery to the posterior segment of the eye and have increased the intravitreal half-life of chemotherapy agents, thus highlighting their potential in treatment of this cancer\(^7\). The intravitreal delivery of biodegradable drug-loaded polyester-based microspheres in the ocular tissue exhibited reduced rates of toxicity to surrounding normal structures\(^7\), as well dendrimers or gold-tethered liposomes containing chemotherapeutic agents significantly decreased tumor volume in retinoblastoma preclinical models\(^6-7\). Even in the case of ocular delivery, gold-based NPs conjugated with chemotherapeutics can strongly absorb near infrared light, which enables the subsequent drug release and the photo-thermal destruction of cancer cells\(^7\). The light-responsiveness of gold and other photosensitive nanocarriers infers considerable potential for Rb because of the regular use of lasers in the treatment of retinal disease.

As bioengineering advances, various non-implantable and implantable drug delivery devices have been also developed. Colloidal carriers may allow targeted drug delivery and provide protection to substances that are sensitive to degradation. To improve bioavailability and delivery rate, several biodegradable polymers have been used as ocular drug carrier for controlled-release systems. They release the drug at time of self-degradation and are finally absorbed by the body\(^4\). Furthermore, novel natural polymers, like arabinogalactan, xyloglucan, gum cordia, locust bean gum and carrageenan have demonstrated the possibility to safely deliver drugs at a controlled rate in different ophthalmic formulations\(^3\).

Numerous novel treatment options have been as well explored and include molecularly targeted or gene therapies and systemic or local drug delivery systems. In particular, molecularly imprinted soft contact lenses (SCLs) hold high potentials as novel drug delivery systems for the treatment of eye disorders. This technique is used for the preparation of polymers with specific binding sites for a template molecule; it is a versatile and effective method in optimizing the drug release behavior and enhancing the loading capacity of SCLs.

It is undoubtable that the applicability of these novel different ophthalmic formulations is increasing, however, to date tremendous effort is still required to establish them on a commercial scale. Consequently, there is a need for designing new delivery strategy and optimize eye treatment. In this project, we focused on the necessity to develop the engineered erythrocyte-bases DDS that could recapitulate all the beneficial characteristics of the erythrocyte carriers and that could be locally and safely concentrate
in the eye. More specifically, we investigated the potential of the magnetically driven EMHVs to deliver novel therapies against Rb. The application of the external biocompatible magnetic field ensured carrier localization. EMHV would favor drug release and concentration at site of cancer lesion, sparing adjacent healthy ocular tissues in an orthotopic mouse model, generated by using Weri-Rb1 human retinoblastoma cells, where both epigenetic therapies and newly synthesized biodrug against deferentially expressed gene markers of Rb were tested. Therefore, carrier erythrocytes could be applied in many experimental settings, encouraging the "bench to bedside" translation to clinical approaches.
2 AIMS

Retinoblastoma is a devastating solid tumor that affects children and youngsters, for which treatment is still mostly confined to surgery and chemotherapy. The main aim of this work was to characterize and develop an innovative strategy for the local delivery of new therapies to treat retinoblastoma. Moreover, our activity has been focused on standardizing a preclinical investigation set developing an orthotopic model that could be versatile for the study of a novel DDS for the delivery of newly synthesized biodrugs of different chemical nature.

As retinoblastoma is known to arise from epigenetic alterations, besides RB1 inactivation and/or silencing, the work aimed at targeting reversible epigenetic gene silencing that sustain cancer development. To identify key genetic actors that mediate cancer phenotype in retinoblastoma cells a step-wise experimental design was set, that started by proving the anti-proliferative effect of DAC on Weri-Rb1 in vitro and in vivo. We aim at studying, by microarray analysis, the time course of differential gene expression to identifying potential key genes and pathways mostly affected by the epigenetic treatment. Methylation analysis of the selected gene panorama will give indication on the mechanism of action of DAC that could have either a direct effect on methylation of genes, or a more blunt effect on cell survival. We will use computational analysis of predictive gene-gene co-expression network of interaction and we will perform validation of the predictive investigation by means of qPCR.

While confirming DAC demethylating activity and anti-proliferative effect on Weri-Rb1, a comparison between single DAC treatment and combination of treatment with other epigenetic drug, namely trichostatin A, a HDAC inhibitor will be also performed as part of the optimization of the new therapeutic approach to identify the most effective and less toxic epigenetic treatment in Rb. This set of experiment will shed light on the epigenetic regulation of Weri-Rb1 and also indicate which one, between DAC alone and the combination of the two drugs, could be the best treatment worthy of further experimental investigation.

The development of a localized delivery strategy is in fact a major goal of my research, therefore I implemented and optimize the inclusion of only DAC into a cell-based carrier. The erythrocyte based engineered DDS named the Erythro-Magneto-FHA-Virosome®
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(EMHV), was patented by the CNR laboratory in which I carried out the whole experimental work. EMHV-s provide a magnetically driven mean to concentrate drug delivery at site of cancer lesion. My project aims at describing the localized activity of EMHV-loaded DAC both in xenograft and in orthotopic models. It is expected that the use of the carrier will highly improve the efficacy of DAC, opening the therapeutic window of this FDA approved anticancer agent. These experiments are mandatory for the further development of the therapy in clinical set.

Furthermore, in accordance with our analysis of microarray and gene expression in vitro and ex vivo indicating Survivin as a major role-play gene in Rb, I decided to investigate for the first time a newly synthesized biodrug, a molecular beacon (MB) against Survivin-mRNA (SURV-MB) to prevent expression of this widely expressed pro-survival effector Rb in cancer cells. Moreover, the use of a MB to impede gene/protein expression could result in both therapeutic and diagnostic effect. I aimed at describing the internalization of MB within Weri-Rb1 and to preliminary verify their potential as diagnostic tool. To test their potential anti-proliferative effect, MB will be used both as free agent as well as loaded into the EMHV. For this a characterization of MB inside EMHV is also due.

Overall, this could be considered as a forerunner project to identify new genetic biomarkers for retinoblastoma and to describe relevant genetic networks sensitive to epigenetic regulation to exploit as therapeutic targets. This project is also set to develop new therapeutic strategy based on the use of an innovative carrier for minimally invasive localized treatment of Rb that holds potential for clinical application. The combination of our results from computational prediction, network interaction and carrier optimization could provide the basis for innovative tools in personalized medicine.
3 MATERIALS & METHODS

Reagents

Superparamagnetic NPs were purchased from nanoscreenMAG, Chemicell, Berlin, Germany. Filamentous Hemagglutinin from Bordetella pertussis (FHA), 5-aza-2’-deoxycytididine (DAC) and Trichostatin A (TSA) were purchased from Sigma-Aldrich, Milan, Italy. TurboFect Transfection Reagent was purchased from Thermo Scientific, Waltham, Massachusetts, USA. Molecular Beacon (MB) for Survivin-mRNA has been modified by the laboratory of IFAC CNR in Florence, immobilizing the fluorophore/quencher pair at the extremities: 5’(ATTO647N)-CGACGGAGAAAGGGCTGCCACGXCG(BBQ)-3’ X=C6-dT Thio, and then kindly donated for the scope of this project.

Cell culture

For all the experiment, the human derived Weri-Rb1 retinoblastoma cell line was used, as it represent a widely accepted model for the study of retinoblastoma. Weri-Rb1 retinoblastoma cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in culture medium RPMI 1640 supplemented with 10% Fetal Bovine Serum, 2mM L-glutamine, in presence of 100U/ml penicillin-streptomycin, at split ratio of 1:2 twice a week. Weri-Rb1 cells were used both for in vitro study and for xenograft and orthotopic implantation.

Confocal Laser Scanning Microscopy (CLSM) analysis

After 24 hours of incubation, Weri-Rb1 cells at a density of 5x10^5 were seeded in 6-well microtiter plates. This cell line grows in suspension and recapitulates all the feature of human cancer. The culture medium could contain MB against Survivin-mRNA, either administered in free form or loaded inside the erythrocyte-based delivery system EMHVs. Free biodrug was internalized inside cells using the transfection reagent, following manufacturer's instructions, meanwhile the loading inside EMHVs was performed following the CNR patented method, described below. In a sample, fresh medium was replaced with no adding of Survivin-MB-EMHVs to visualize naïve cell structure (control). For microscopy investigation of drug inclusion,
cells were let adhere on the coverslip using a mix of ice-frozen acetic acid and ethanol 100%. Cells were washed in 1X PBS buffer, fixed with cold ethanol and then transferred on a slide. Nuclei were counter stained with DAPI. Cells were washed twice with 1X PBS and a coverslip is mounted on the slide, using an anti-fade medium. Fluorescence and bright-field images were captured by CSLM, Leica TCS SP5 inverted microscope system, equipped with sources emitting from the UV to the visible. DAPI fluorescence was detected using excitation at 405nm and recording emission at 454nm while the fluorescence of Survivin-MB was detected using excitation at 635nm and recording emission at 670nm.

**Cytofluorimetric (FACS) analysis**

Werri-Rb1 cells at a density of $5 \times 10^5$ were seeded in 6-well microtiter plates for cell cycle assays. The culture medium in which cells were re-suspended might be the same [CTRL] or contain free DAC at the concentration of $2.5 \mu M$ (corresponding to the therapeutic dose of $1.7 \mu g$), in order to confirm the previous data [DAC 1.7μg]. This treatment was also delivered towards the CNR patented erythrocyte-based delivery system named EMHV and so $1 \mu g$ DAC loaded-EMHVs could be tested on Werri-Rb1 [DAC 1μg - EMHV]. Cells could be also treated with free TSA at increasing doses of 75, 150 and 300ng [TSA 75ng, TSA 150ng, TSA 300ng] to detect the most effective anti-proliferative condition, and then a combined therapy involving free therapeutic DAC and free TSA at different doses after 24 hours incubation might be detected [DAC 1.7μg - TSA 75ng (24h); DAC 1.7μg - TSA 150ng (24h); DAC 1.7μg - TSA 300ng (24h)]. Moreover, Survivin-MB loaded-EMHVs were used on Werri-Rb1 cells, to detect its activity for cell cycle arrest.

After 24, 48 and 72 hours of incubation, control and epigenetic treated cells were harvested and analyzed by FACS. Werri-Rb1 cells treated with loaded Survivin-MB were instead analyzed after 6 and 24 hours incubation. Nuclei were stained with $10 \mu g/ml$ propidium iodide (PI) in hypotonic solution (1X PBS containing 0.1% sodium citrate and 0.1% Triton X-100) for 30 minutes at 4°C in the dark for assessment of cell cycle phases. Apoptotic cells were detected by Annexin V test (BioVision). The control and treated cells, except whose treated with DAC-EMHV, were suspended in 1X binding buffer and incubated at room temperature for 15 minutes with Annexin V-FITC, as well Propidium Iodide (PI) was added for nuclei staining following manufacturer's instructions. Conversely, EMHVs cannot be analyzed using apoptosis kit due to their physical and
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morphological features.
Flow-cytometry was carried using Becton-Dickinson FACScan CentroII and data were analyzed by FlowJo software.

**Quantitative qPCR experiments**

QPCR was performed to quantify mRNA levels in some of the relationships evidenced in particular by regulatory paths governed by master regulators. Total RNA was extracted from Weri-Rb1 cells using NucleoSpin RNA isolation kit (Macherey-Nagel) according to the manufacturer's instructions. RNA concentration and purity was determined by Picodrop spectrophotometer. For each sample, 1μg of total RNA was reversely transcribed using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). Gene expression was determined by DyNAmo Flash SYBR Green qPCR Kit (Thermo Scientific), using the PikoReal Real-Time PCR System (Thermo Scientific). The relative expression of target genes will be evaluated using the comparative cycle threshold method and all samples were analyzed in triplicate.

Amplification conditions were: 7 minutes at 95°C followed by 40 cycles of 10 seconds at 95°C, 20 seconds at 60°C and 20 seconds at 72°C. The relative expression of target genes was evaluated using the comparative cycle threshold method, with b-actin used for normalization.

**Primers used:**

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<td>5'-AGATTTCAACAGGCACCTGCCA-3'</td>
<td></td>
</tr>
</tbody>
</table>
An innovative epigenetic strategy for retinoblastoma treatment

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRAD</td>
<td>5'-CAATGTCCAGGCGCTGTTTGAA-3'</td>
<td>5'-AAAGGCCCACTCTTGGCGCTGTTA-3'</td>
<td>60</td>
</tr>
<tr>
<td>TRAF2</td>
<td>5'-TGTGCCTGCGTATCTACCTGAA-3'</td>
<td>5'-TCAATACGTGCTCCCGGTTAT-3'</td>
<td>60</td>
</tr>
<tr>
<td>TOLLIP</td>
<td>5'-TGGCCAAGAATTACGGGCATGAC-3'</td>
<td>5'-ACCGTGCACTGGATGACCTTAT-3'</td>
<td>60</td>
</tr>
<tr>
<td>P73</td>
<td>5'-TACTGCCAGATCGCAAGACAT-3'</td>
<td>5'-CGTGCTGCTCTTCTTGTAAC-3'</td>
<td>60</td>
</tr>
<tr>
<td>DAP3</td>
<td>5'-AGTGCCGGTGAGGTAATCAAT-3'</td>
<td>5'-GCGCCCTCATGCAATCATTT-3'</td>
<td>60</td>
</tr>
<tr>
<td>CASP6</td>
<td>5'-AGGTGCAGCCTGCTGGTGGA-3'</td>
<td>5'-AGGGCTGCATCCACCTACCT-3'</td>
<td>60</td>
</tr>
<tr>
<td>BCL_XL</td>
<td>5'-TGCAAGTGTTGAGTGCCTGAT-3'</td>
<td>5'-TGAAAGCGTTCTGCGCTTTT-3'</td>
<td>60</td>
</tr>
<tr>
<td>FAS</td>
<td>5'-AAAGCTAGGGACTGACATCA-3'</td>
<td>5'-GTGGCGGTGAGTITTTATATCCA-3'</td>
<td>60</td>
</tr>
<tr>
<td>BIK</td>
<td>5'-TGGACTGGTTTCTGCGCATGACTGA-3'</td>
<td>5'-ACTGCCCTCCATGCTATTCCA-3'</td>
<td>60</td>
</tr>
<tr>
<td>b-actin</td>
<td>5'-TGCGTGACATTAAGGAGAAG-3'</td>
<td>5'-GCTCGTAGCTCTTCTTCCA-3'</td>
<td>60</td>
</tr>
</tbody>
</table>

**Methylation specific PCR (MSP)**

DNA methylation patterns in the CpG islands of *CASP8*, *FAS* and *BIK* were assessed by methylation specific PCR, based on the sequence differences between methylated and unmethylated DNA after sodium bisulfite modification. Weri-Rb1 cells were treated for 48 hrs. with DAC 2.5 uM and successively genomic DNA was extracted from treated and untreated cells using QIAamp DNA mini kit. Following the manufacturer's instructions, genomic DNA was subjected to bisulfite modification by the Thermo Scientific EpiJET Bisulfite Conversion Kit. Successively, modified DNA from control and treated cells was used for MSP reactions. Amplification conditions were: 3 minutes at 95°C, 40 cycles of 30 seconds at 95°C, 60 seconds at primers Tm and 60 seconds at 72°C, followed by a final extension of 2 minutes at 72°C. PCR products were separated on a 2.2% agarose gel containing ethidium bromide and visualized under ultraviolet illumination.
The primer pairs specific for methylated (M) and un-methylated (U) sequences were the following:

**Primers used:**

<table>
<thead>
<tr>
<th>MSP</th>
<th>Primer</th>
<th>Sequence</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CASP</strong>&lt;sup&gt;765&lt;/sup&gt;</td>
<td>M-Forward</td>
<td>5'-GTT GGT TTT ATT TAG TTC GGC-3'</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>M-Reverse</td>
<td>5'-CCC TAT CGA TAA CAA ATA ATA TAC-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>U-Forward</td>
<td>5'-GTT GGT TTT ATT TAG TTT GGT-3'</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>U-Reverse</td>
<td>5'-CCC TAT CAA TAA CAA ATA ATA TAC-3'</td>
<td></td>
</tr>
<tr>
<td><strong>FAS</strong>&lt;sup&gt;766&lt;/sup&gt;</td>
<td>M-Forward</td>
<td>5'-AGTTTCGGCGTTTTTCGGAGATTATTGC-3'</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>M-Reverse</td>
<td>5'-CACCCCGCCGAAACGAACC-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>U-Forward</td>
<td>5'GGTAGTTTTGGTGTTTTTTGGAGATTATTGT-3'</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>U-Reverse</td>
<td>5'-CACCCACACAAAAACAAAACTTTAAC-3'</td>
<td></td>
</tr>
<tr>
<td><strong>BIK</strong>&lt;sup&gt;767&lt;/sup&gt;</td>
<td>M-Forward</td>
<td>5'-GGGAGTCGTGTTAGGTTTTATC-3'</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>M-Reverse</td>
<td>5'-GAACAAAAAAAATACGTTTCGAA-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>U-Forward</td>
<td>5'-GGGGAGTTGTGTTTAGGTTTTATC-3'</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>U-Reverse</td>
<td>5'-CAAACAAAAAAAATACCATTTCAAA-3'</td>
<td></td>
</tr>
</tbody>
</table>

**Preparation of loaded EMHVs**

Human erythrocytes have been prepared accordingly to the previously described protocol<sup>463</sup>. Briefly, whole blood was prepared by gradient centrifugation at 400g for 30 minutes and then washed twice in 1X PBS.

2x10⁹ erythrocytes were lysed in 250μl lysis buffer for 60 minutes at 0°C and the isotonicity was then restored by adding 130μl of resealing buffer, supplemented with 2μg of FHA, 0.1mg of 50-70nm super-paramagnetic NPs and the required amount of drug, like 0.1mg of DAC or 2μg of Survivin-MB.

After 45 minutes incubation at 8000g for 15 minutes at 4°C, loaded EMHVs were washed twice with 1X PBS, resuspended in 1X PBS and conserved at 4°C until used.

Using previous HPLC investigation, the drug amount inside the DDS was standardized and 2x10⁹ DAC-EMHVs could accommodate about 10μg of total drug. Moreover, the active phosphorylated form of DAC represented the 50% of total loaded drug into the EMHVs.
**Animals**

According to ministerial guidelines, animals used in preclinical models in "Toscana Life Sciences" animal facility were housed in microisolators in autoclaved cages with polyester fiber filter covers, under germfree conditions. All food, water, and bedding were sterilized and the animals were maintained in an ambient temperature of 23±2°C in rooms having a 12 hours light/dark cycle.

Mice will be anesthetized by 2.5% isoflurane during manipulation.

**Xenograft**

Xenograft tumors were established monolaterally in Nude-Foxn1\textsuperscript{nu/nu} female mice (Harlan Laboratories, Udine, Italy) by means of a subcutaneous injection into the left flank consisting of 5x10\textsuperscript{6} Weri-Rb1 cells suspended in 0.2ml of a 1:1 mixture of ice-cold Matrigel\textsuperscript{TM} basement membrane matrix (BD Bioscience, MA, USA) and 1X PBS, using a 20-gauge needle syringe. Accordingly to ministerial animal welfare body, the experimental protocol used was classified as minimally invasive.

Once xenografts started growing, their sizes (mm\textsuperscript{3}) were measured twice a week with digital caliper and the volume was calculated according to the modified ellipsoid formula \(1/2(\text{Length x Width}^2)\). Tumor xenografts were allowed to grow approximately up to 100mm\textsuperscript{3} and this volume was selected as the initial stage for beginning the treatment.

Mice were randomized, anesthetized and prepared for tail vein injection with the selected treatment. Before starting the experimental setting, the measurement procedures need to be standardized: tumor mass could be in fact evaluated by measuring sizes using either digital caliper, or with ultrasound scan system Visualsonic (VEVO2100). Tumor volumes have been then compared to the corresponding initial volumes, in order to normalize the data (\(X = 100 \times \text{volume}_1/\text{volume}_0\)).

Two independent experiments were performed with at least six mice assigned to each group. Animals were assigned to five different groups in each experimental setting, receiving biweekly intravein (I.V.) injections of 300\(\mu\)l treatment, over 3 weeks. They might be treated as follow: 1X PBS [CTRL]; 75\(\mu\)g free DAC (corresponding to the therapeutic dose of 2.5 mg/kg) [DAC 75\(\mu\)g]; 45\(\mu\)g free TSA [TSA 45\(\mu\)g]; 75\(\mu\)g free DAC followed by 45\(\mu\)g free TSA (after 24h) [DAC 75\(\mu\)g - TSA 45\(\mu\)g (24h)]; 2x10\textsuperscript{9} DAC loaded-EMHV (containing 10\(\mu\)g) followed by a static magnetic field, in which two cylindrical (0.7 cm diameter, 0.5 cm high) earth magnets (52N) were externally applied to the xenograft mass not impeding movements for 30 minutes immediately after I.V.
injection, to achieve intra-tumor localization and accumulation [DAC 10μg - EMHV - MF]. The magnetic field application was repeated at time of each injection. Mice were then allowed recovering and monitored for signs of distress. At the end of the treatment course or when the tumor volume reached approximately 1300mm³, mice were sacrificed by CO₂ asphyxiation and the retinoblastoma mass, liver and kidneys were harvested. Half of these samples were stored in liquid nitrogen using OCT, whereas the remaining parts have been processed using TRIAZOL and store at 4°C until additional analysis.

Orthotopic tumors
Orthotopic tumors were established monolaterally in the right eye of Nude-Foxn1<sup>nu/nu</sup> female mice (Harlan Laboratories, Udine, Italy) by means of an sub-retinal injection consisting of 1x10⁴ Weri-Rb1 cells suspended in 10μl 1X PBS, using a 32-gauge needle syringe, modifying the experimental method of Bond and co-workers[769]. In particular, the procedure has been carried out using an eco-guided micromanipulator (VEVO2100 ultrasound system) and the globe was pierced laterally through the conjunctiva and the sclera to reach vitreous cavity. The needle will be held steadily in this position, depressing the plunger slowly. Accordingly, to ministerial animal welfare body, the experimental protocol used was classified as moderate invasive. Once orthotopic tumors started growing 2-3 weeks post-injection, they present the classical leukocoria sign of retinoblastoma and their sizes (mm³) have been evaluated once a week after 3D imaging reconstruction. Tumors were allowed to grow approximately up to 30mm³ and this volume was selected as the initial stage for beginning the treatment. Mice were randomized, anesthetized and prepared for tail vein injection with the selected treatment.

Before each injection, tumors were measured using the ultrasound scan system and compared to the corresponding initial volumes, in order to normalize the data (X=100x volume<sub>1</sub>/volume<sub>0</sub>). Animals were assigned to two different groups in each experimental setting, receiving biweekly I.V. injections of 300μl treatment, over 3 weeks. They might be treated as follow: 1X PBS [CTRL] or 75μg free DAC (corresponding to the therapeutic dose of 2.5 mg/kg) [DAC]. Mice were then allowed recovering and monitored for signs of distress. At the end of the treatment course or when the tumor volume reached approximately 90mm³, mice were sacrificed by CO₂ asphyxiation and both eyes (naïve and within the orthotopic tumor), liver, kidneys were harvested. Half of these samples were stored in liquid nitrogen using OCT, whereas the remaining parts have been processed using TRIAZOL and store at 4°C until additional analysis.
Morphological analysis of orthotopic tumor

To carry out morphological investigation on the implanted tumor, eye bulbs from experimental animals were collected, and put in sucrose 30% overnight. The washed bulbs were cryo-protected in OTC, snap-frozen in liquid nitrogen and then store at -80°C until required. Each animal donated both eye, as the non-treated eye would serve as an internal control. A morphological descriptive investigation was performed on whole eye slice (50 um thick) observed under a light microscope.

Statistical analysis

The cell cycle phases are expressed as Mean±SD of at least n=3. Three-Ways ANOVA were applied to compare the effect of different treatments on cell cycle phases (sub G1, G0-G1, S, G2-M) and One-Way ANOVA was used to compare the effect of DAC-EMHV's treatment at selected time points (24, 48 and 72 hours).

The apoptotic cells were expressed as Mean±SD of at least n=3. Statistical analysis was performed with One-Way ANOVA independently for early and late apoptosis on log-transformed data to improve normalization. Pairwise comparisons were tested using Tukey’s honestly significant difference criterion.

For the significance of gene expression values in qPCR validation, the statistical analysis of ΔCt values was based on One-sample T-Test and expressed as Mean±SEM.

The in vivo results are expressed as Mean±SEM of n=6. One-Way ANOVA and One-Way ANOVA repeated measures when required, were applied to compare the tumor mass reduction in the evaluation of the xenograft implant or orthotopic tumor after selected treatment using data collected at the last injection.

Ethics statements

Human red blood cells were obtained from transfusion bags collected from anonymous healthy voluntary donors, which have given their written informed consent carried out in accordance with Italian Government law. It was not necessary the approval from an institutional review board (ethics committee) since neither direct human participation nor involvement of human studies have been foreseen in this work. Samples have been provided by Azienda Ospedaliera Universitaria Senese.
The preclinical studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the International guidelines on handling of laboratory animals and applying the 4Rs to experiments (in accordance with NIH and European Commission recommendations). The protocols for Animal Experiments were approved by the Ethics Committees of the Toscana Life Sciences and the Istituto Superiore di Sanità (ISS) on behalf of Italian Minister of Health (Permit Number: # CNR-030314 and # CNR-101013).

Animal well-being was monitored accordingly to Langford and colleagues[770].
4 RESULTS

The retinoblastoma epigenetic panorama is a growing field of investigation. In this work, I focused on the possibility of developing and characterize innovative therapeutic approach to treat Rb and reverse gene silencing that induces cancer phenotype. In particular, I focused on the characterization of the role of timing the effect of a demethylating agent in reverting the silenced tumor-suppressor genes that are inactivated in retinoblastoma. This investigation revealed a time-dependent activity of DAC on Weri-Rb1 cell viability and proliferation due to a regulation of pivotal gene methylation.

**In vitro anti-proliferative effect of therapeutic DAC on Weri-Rb1**

The *in vitro* efficacy of therapeutic DAC was tested on Rb cell line Weri-Rb1. The cell cycle profile and apoptosis were both measured by FACS analysis at 24 up to 72 hrs., showing that at the latest time point, DAC at the concentration of 2.5μM (corresponding to the therapeutic dose of 1.7μg) induced a significant enrichment in sub G1 phase comparing to untreated cells, suggesting a possible activation of apoptotic response (**Fig. 4.1a**). This increased sub G1 phase reached statistical significance at 72 hours (CTRL 9.2±2.3 vs DAC 1.7μg 32.8±2.2) (ANOVA *P<0.05).

**Fig. 4.1a:** FACS analysis. Cell cycle in Weri-Rb1, using propidium iodide at 24, 48 and 72 hrs. after DAC treatment. Mean ± SD. *P<0.05.
To assess if the treatment could induce the activation of apoptotic response, Annexin V test was also performed. The analysis confirmed that therapeutic DAC exerted an apoptotic effect in retinoblastoma cells (Fig. 4.1b). In particular, the epigenetic treatment induced a reduction of alive cells and, as a consequence, an enrichment in late apoptosis at 72 hours after treatment, reaching statistical significance if compared to control (CTRL 60.6±0.9 vs DAC 1.7μg 14.4±1.3) (ANOVA *P<0.05).

![Fig. 4.1b: FACS analysis. Apoptosis in Weri-Rb1, using Annexin V-FITC at 24, 48 and 72 hrs. after DAC treatment. Mean ± SD. *P<0.05.](image)

With these analyses, we confirmed the previously encouraging results obtained in our laboratory at IFC CNR. Comparing this first set of data, still unpublished, with our new experiment, we confirmed that the time-dependent gene co-expression distribution in Weri-Rb1 after therapeutic DAC treatment induces an epigenetic reprogramming of retinoblastoma cells. Through a microarray analysis, the most prominently induced gene expression after DAC treatment was investigated, mapping the time-course dependent differential gene expression (DGE) profile of both pro- and anti- apoptotic genes. The authors also investigated the relevance of methylation regulation for these genes, indicating a possible mechanism of tumorigenesis.
Here, referring to that same microarray and after a thorough investigation with gene-pathway association mapping analysis carried out in the laboratory, I decided to expand the panel of genes of interest. These genes have been selected based on their molecular functions, which might be altered in numerous chronic disorders and especially related to cancer (Table 4.1, Fig. 4.2). For these fifteen genes, the differential expression varied with a threshold greater or equal to 2 at each of the selected time points. Using a gene-gene co-expression network, we described their connected representations: GeneMania was used to generate the networks, showing co-expression dynamics among the connected genes building the interactomes (Fig. 4.3) and the network configurations were built from the log-expression ratio values.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Molecular functions</th>
<th>Pathways Commons Network Visualizer</th>
</tr>
</thead>
<tbody>
<tr>
<td>RELA (NFkB)</td>
<td>protein binding; identical protein binding; kinase binding; enzyme binding; transcription activator binding; phosphate binding; transcription repressor binding; anion binding</td>
<td>It is a ubiquitous transcription factor involved in several biological processes. Upon degradation of the inhibitor, it moves to the nucleus and activates transcription of specific genes.</td>
</tr>
<tr>
<td>HSF1</td>
<td>protein binding</td>
<td>Its product is a transcription factor rapidly induced after temperature stress that is playing a role in the regulation of lifespan. Expression of this gene is repressed by phosphorylation, which promotes binding by heat shock protein.</td>
</tr>
<tr>
<td>CASP8</td>
<td>protein binding; identical protein binding; cysteine-type endopeptidase activity; cysteine-type peptidase activity</td>
<td>This gene encodes a member Caspase family, involved in the extrinsic apoptosis pathway. Sequential activation of Caspase plays a central role in the execution-phase of cell apoptosis. This protein is involved in the programmed cell death induced by FAS and various apoptotic stimuli. The N-terminal FADD-like death effector domain of this protein suggests that it may interact with FAS-interacting protein FADD.</td>
</tr>
<tr>
<td>BAX</td>
<td>protein binding; BH domain binding; identical protein binding; protein heterodimerization activity; protein domain specific binding; protein dimerization activity; BH3 domain binding; death domain binding</td>
<td>The encoded protein belongs to the BCL2 protein family, forming a heterodimer with BCL2, and functioning as an apoptotic activator. It interacts with, and increases the opening of VDAC, which leads to the loss in membrane potential and the release of cytochrome C. The expression of this gene is regulated by P53.</td>
</tr>
<tr>
<td>TRAP2 (PSMD2)</td>
<td>protein binding; enzyme regulator activity</td>
<td>It encodes one of the non-ATPase subunits of the 19S regulator lid of the proteasome, a multicatalytic proteinase complex that can cleave peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway. It may also participate in the TNF signaling pathway.</td>
</tr>
<tr>
<td>BIRC5 (Survivin)</td>
<td>protein binding; identical protein binding; protein heterodimerization activity; caspase inhibitor activity; caspase regulator activity; cysteine-type endopeptidase inhibitor activity; protein dimerization activity; enzyme binding; cobalt ion binding; enzyme regulator activity; endopeptidase inhibitor activity; endopeptidase regulator activity; peptidase inhibitor activity; Ran GTPase binding; peptidase regulator activity</td>
<td>It is a member of the inhibitor of apoptosis (IAP) gene family, which encodes negative regulatory proteins that prevent apoptotic cell death. Gene expression is high in most tumors.</td>
</tr>
<tr>
<td>RRAD</td>
<td>protein binding</td>
<td>Ras related glycolysis inhibitor and calcium channel regulator.</td>
</tr>
<tr>
<td>Gene</td>
<td>Protein Binding</td>
<td>Enzyme Activity/Other Functions</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------</td>
<td>------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>TRAF2</td>
<td>protein binding; sphingolipid binding</td>
<td>The encoded protein is a member of the TNF receptor associated factor (TRAF) family and it directly interacts with TNF receptors and forms a heterodimeric complex with TRAF1. The interaction of this protein with TRADD ensures the recruitment of IAPs for the direct inhibition of caspase activation.</td>
</tr>
<tr>
<td>TOLLIP</td>
<td>protein binding; kinase binding; Toll-like receptor binding; enzyme binding</td>
<td>This gene encodes a ubiquitin-binding protein that interacts with several Toll-like receptor (TLR) signaling cascade components, regulates inflammatory signaling and is involved in interleukin-1 receptor trafficking and in the turnover of IL1R-associated kinase.</td>
</tr>
<tr>
<td>P73</td>
<td>protein binding; enzyme regulator activity</td>
<td>This gene encodes a member of the p53 family of transcription factors involved in cellular responses to stress and development.</td>
</tr>
<tr>
<td>(ARHGAP24)</td>
<td>protein binding; protein domain specific binding</td>
<td></td>
</tr>
<tr>
<td>DAP3</td>
<td>protein binding; protein domain specific binding</td>
<td>This gene encodes a 28S subunit protein that also participates in apoptotic pathways which are initiated by tumor necrosis factor-alpha, FASL, gamma INF.</td>
</tr>
<tr>
<td>CASP6</td>
<td>cysteine-type endopeptidase activity; cysteine-type peptidase activity, protein binding</td>
<td>This gene encodes a protein member of Caspase family that is processed by Caspases 7, 8 and 10, and is thought to function as a downstream enzyme in the Caspase activation cascade.</td>
</tr>
<tr>
<td>BCL2L1</td>
<td>protein binding; identical protein binding; protein heterodimerization activity; caspase inhibitor activity; BH domain binding; caspase regulator activity; protein domain specific binding; cysteine-type endopeptidase inhibitor activity; protein dimerization activity; enzyme regulator activity; endopeptidase inhibitor activity; endopeptidase regulator activity; peptidase inhibitor activity; peptidase regulator activity</td>
<td>The proteins encoded by this gene belong to the BCL-2 protein family. These proteins are located at the outer mitochondrial membrane and have been shown to regulate outer mitochondrial membrane channel (VDAC) opening, controlling the production of reactive oxygen species and release of cytochrome C for the intrinsic apoptosis pathways.</td>
</tr>
<tr>
<td>BIK</td>
<td>protein binding; BH domain binding; protein heterodimerization activity; protein domain specific binding; protein dimerization activity</td>
<td>The protein encoded by this gene shares a critical BH3 domain with other death-promoting proteins, such BAX, that is required for its pro-apoptotic activity, and for interaction with anti-apoptotic members of the BCL2 family, and viral survival-promoting proteins. Since the activity of this protein is suppressed in the presence of survival-promoting proteins, it is suggested as a likely target for anti-apoptotic proteins.</td>
</tr>
</tbody>
</table>

**Table 4.1**: 15 selected pro-apoptotic or tumor-suppressor genes showing upregulation (log>2) in the microarray analysis, after therapeutic DAC treatment. Gene functions and their pathways are reported for each gene.
**Fig. 4.2:** Number of genes involved in molecular functions of interest for cell regulation

**Fig. 4.3:** Interaction network of selected 15 genes obtained from Biogrid database and visualized using Cytoscape.
In **Fig 4.3** the association map of the selected genes generated by Cytoscape is depicted: CASP8 interacts with other selected genes namely CASP6, FAS and TRAF2. BIK gene with high differential expression interacts with BCL2L1. Remaining eight genes did not show any direct interaction among selected genes. Many of these genes were involved in similar biological processes (**Table 4.1**) along with genes constituting connected component such as BIK, BIRC5, BAX and BCL2L1. Most relevantly, these genes are involved in process related to heterodimerization activity. Dimerization is crucial in retinoblastoma as RB protein binds and inhibits E2F family transcription factors constituted of E2F and DP protein, as dimerization partner.[771-772]

**Validation of DGE in Weri-Rb1 after DAC treatment**

After computational data reading, the expression profile of the fifteen selected genes was monitored after epigenetic treatment at different times and then validated using qPCR. Relative quantification of mRNA levels of these genes was evaluated at 48, 72 and 96 hours after treatment and compared to control. Data were shown as a ratio between treated (DAC) and untreated cells and normalized to b-actin (**Fig. 4.4**). Notably, a significant upregulation of pro-apoptotic genes CASP8, FAS and BIK and the tumor suppressor gene RRAD, was detected at each time point consolidating their early involvement in essential gene pathways to apoptosis in Rb. Most interestingly, the expression of other pro-apoptotic genes involved on the intracellular signaling at mitochondrial level, such as BAX and BCL_XL (also known as BCL2L1) expression was significantly up regulated only at the latest time point. On the other hand, BIRC5 (also known as Survivin) underwent an early down-regulation, favoring cell apoptosis. Similar, but opposite, gene expression fluctuations were detected for TRAP2 and CASP6 (T-test *P<0.05).

![Fig. 4.4: qPCR analysis. Fold change (log2 transformed) of gene expression in DAC-treated samples relative to the controls at 48, 72 and 96 hrs., reported as Mean ± SD (T-Test *P<0.05).](image)
**Methylation status of selected genes after DAC on Weri-Rb1**

A DNA methylation analysis was then performed on *CASP8*, *FAS* and *BIK* as they resulted to be the most up regulated genes in treated Weri-Rb1 after qPCR validation. Changing in methylation status of these genes provides a unique insight into the role of epigenetics in gene regulation, which is reflected by the proportion of methylated DNA. Therefore, any potential alteration of the methylation levels compared with control would indicate the drug effect on the epigenetic regulation of gene expression.

More specifically, DNA methylation patterns in the CpG islands of *FAS*, *CASP8* and *BIK* were assessed because of the sequence differences between the DNA status after sodium bisulfite modification. *CASP8* and *BIK* showed a distinct methylation profile when comparing treated and control cells. Methylation profile changes for both *CASP8* and *BIK* after DAC epigenetic treatment: control (CTRL) presented the methylated (silenced) form of both genes, whereas DAC-treated cells showed unmethylated (reactivated) form of *CASP8* and *BIK*. On the contrary, using primer for the methylated sequence, *FAS* either did not appear amplified in CTRL or treated samples, and this suggested an indirect activity of DAC on *FAS* gene regulation (Fig. 4.5).

![Methylation analysis](image)

*Fig. 4.5: Methylation analysis. Agarose gel of PCR products after methylation analysis of *CASP8*, *FAS* and *BIK* genes using sodium-bisulfite conversion kit.*

Through methylation study, direct and indirect regulatory mechanism of action of DAC treatment it has been described on the selected gene expression profiles at 48 hrs. We can assume that an epigenetic treatment directly affects *CASP8* and *BIK* genes thought DNMT inhibition of their promoter. *FAS*, encoding for FAS trans-membrane protein, instead results unmethylated and its regulation is dependent on the extracellular stress signal and on the binding of DAC on the FAS death receptor.
Preclinical evaluation of DAC anti-tumoral effect

We described both anti-proliferative and apoptotic features of DAC on retinoblastoma cells in vitro. For a complete characterization of the epigenetic therapy, an in vivo evaluation of the anticancer effect is necessary. A preclinical study was set using a xenograft animal model, developed in our laboratory to test the therapeutic DAC effect on tumor growth. A three-week treatment of therapeutic DAC was administered by I.V. injection and its effect was evaluated after the latest injection. Tumor mass volume was measured using both digital caliper and the ultrasound system. A representative image of the off-line 3D reconstruction (left) of the tumor mass acquired by ultrasound system VEVO2100 (right) is shown (Fig. 4.6). The mass volume measurements are coherent with those acquired by digital caliper (Fig. 4.7). For ethical reasons, only initial and final measurement sections were acquired by VEVO2100 as animals needed to be anesthetized during sections.

Fig. 4.6: Weri-Rb1 xenografts. 3D ultrasound imaging of CTRL and DAC-treated xenografts.

Our results indicated that a significant reduction was obtained with DAC compared to untreated control, demonstrating for the first time an anti-tumoral activity of this demethylating agent on retinoblastoma (Fig. 4.7). (CTRL 1434.19±193.44 vs DAC 75μg 150.58±32.40). One-way ANOVA RM (*P<0.05) was used to demonstrate that the two treatments induced significantly different growth rate.
Fig. 4.7: Weri-Rb1 xenografts. Normalized tumor mass (mm$^3$) after I.V. biweekly DAC treatments. Mean values ± SEM. *P<0.05.

**Ex vivo validation of gene expression after DAC treatment**

Pro-survival and apoptotic gene expressions were monitored in collected samples of Weri-Rb1 xenografts after treatment with DAC to evaluate the occurrence of gene pathway changes and to confirm the previous in vitro gene profile. In particular, the expression of the same relevant genes analyzed in vitro was evaluated using qPCR. This analysis confirmed the pivotal role of the pro-apoptotic genes *CASP8, FAS* and *BIK*, as they appeared over-expressed following the administration of the DNA demethylating agent. Most relevantly to our purposes and similarly to in vitro results, the oncogene *BIRC5*(Survivin) expression was found to be down regulated at the end of the treatment (Fig. 4.8).

![qPCR analysis](image)

**Fig. 4.8:** qPCR analysis. Fold change (log$_2$ transformed) of gene expression in DAC-treated xenografts relative to the controls after sample collection, reported as Mean ± SD.
Development and epigenetic treatment of a retinoblastoma orthotopic model

One of the purposes of our investigation was to study Rb in its natural site. This will allow investigating the influence of the environment and of the spatial/localization of the tumor lesion on the effect of the therapy. To strengthen our preclinical investigation, an orthotopic model of retinoblastoma was also developed. Weri-Rb1 cells were injected in retro-orbital space of the eye. For this model, the growth was detected by ultrasound system: in fact, orthotopic Rb is an internal cancer detectable only by 3D ultrasound imaging techniques. For this reasons, animals were anesthetized only once a week during measurement sections. To treat this orthotopic tumor, therapeutic dose of DAC was administered by I.V injection and compared to untreated control. The preliminary results indicated a trend of anti-proliferative effect induced by DAC treatment when compare with controls (Fig. 4.9) (CTRL 4.7±0.3 vs DAC 75μg 1.3±0.5). Moreover, we verified the correct localization of the orthotopic tumor through morphological investigation. The tumor mass can be traced as a dark mass inside the ocular globe that appeared clear in control samples (Fig. 4.10).

![Graph](image)

**Fig. 4.9:** Orthotopic tumor of Weri-Rb1. Normalized tumor mass (mm$^3$) after I.V. biweekly DAC treatments. Mean values ± SEM. *P<0.05.

![Contrast phase microscopic imaging](image)

**Fig. 4.10:** Contrast phase microscopic imaging of naive (left) and tumor (right) mouse eye. Magnification at 20x.
Effect of HDAC inhibitors on Weri-Rb1: a comparative study and combined treatment

Our results demonstrated that methylation of genes can contribute to Rb tumorigenesis. However, methylation is only one of the most prominent epigenetic mechanisms for gene regulation expression.

To investigate other potential epigenetic regulatory mechanisms on Rb cell survival, I treated Weri-Rb1 with scalar doses of free TSA (75 - 150 - 300ng) and compared to control. TSA is an inhibitor of HDAC enzymes that regulates histone deacetylation.

Both the cell cycle profile and apoptosis were measured by FACS analysis at 24, 48 and 72 hrs. and a dose-dependent effect of TSA was detected. In particular, TSA 300ng induced an enrichment of sub G1 cell phase comparing to untreated cells, reaching statistical significance at the latest time-point (Fig. 4.11a) (CTRL 4.1±0.8 vs TSA 300ng 60.2±5.9) (ANOVA *P<0.05).

**Fig. 4.11a**: FACS analysis. Cell cycle in Weri-Rb1, using propidium iodide at 24, 48 and 72 hrs. after different doses of TSA treatment. Mean ± SD. *P<0.05.
To assess if TSA treatment could induce apoptosis in Weri-Rb1 cells, Annexin V test was also performed. The analysis indicated that only the highest dose of HDAC inhibitor exerted a pro-apoptotic effect in retinoblastoma cells (Fig. 4.11b). In particular, the epigenetic treatment induced a reduction of alive cells and, as a consequence, an enrichment in late apoptosis at 72 hours after treatment, reaching statistical significance if compared to control (CTRL 59.4±1.6 vs TSA 300ng 20.5±7.5) (ANOVA *P<0.05).

![Fig. 4.11b: FACS analysis. Apoptosis in Weri-Rb1, using Annexin V-FITC at 24, 48 and 72 hrs. after different doses of TSA treatment. Mean ± SD. *P<0.05.](image)

Our results indicated that both the demethylating agent DAC and the inhibitor of HDAC TSA induce apoptosis in Weri-Rb1 cell. A synergistic effect of both epigenetic drugs administered together has never been tested in Rb before. Previous non-shown results of combined treatment with these two molecules would indicate that the best apoptotic effect could be obtained administering therapeutic DAC 24 hours in advance. These preliminary results were obtained in different cancer cell lines. Here I administered DAC to the cells 24 hrs. before than TSA treatment in Weri-Rb1. Moreover, scale-doses of TSA were used to describe TSA effect. I measured a significant variation of cell cycle: after this combined treatment results reached statistical significance at 48 only after using the highest dose of TSA (CTRL 4.2±1.3 vs DAC 1.7µg + TSA 300ng (24h) 64.1±1.6); this effect remained consistently significant up to 72 hours (CTRL 3.3±1.3 vs DAC 1.7µg + TSA 300ng (24h) 82.9±2.5) (ANOVA *P<0.05) (Fig. 4.12a).
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**Fig. 4.12a**: FACS analysis. Cell cycle in Weri-Rb1, using propidium iodide at 48 and 72 hrs. after combined treatment with therapeutic DAC and different doses of TSA after 24 hrs. Mean ± SD. *P<0.05.

In order to assess if the combined treatment could increase the apoptotic response in Weri-Rb1 cells, Annexin V test was also performed. Accordingly with the cell cycle analysis, combined epigenetic treatments with the highest dose of TSA (300ng) induced anti-tumoral effect at 72 hrs. after treatment. In particular, a reduction of alive cells and, as a consequence, enrichment in late apoptosis was shown after 24 hrs., reaching statistical significance if compared to control (CTRL 59.4±1.4 vs DAC 1.7µg + TSA 300ng (24h) 22.1±1.9) (ANOVA *P<0.05) (**Fig. 4.12b**).
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**Effect of deacetylation inhibition in vivo: epigenetic therapy failure**

Following the encouraging results obtaining using HDAC inhibitor *in vitro*, I studied the effect of TSA on xenograft animal model. I had previously performed preliminary studies using scale-doses of single TSA to set an effective dose-response (range 15 - 45μg per injection, data not shown).

For the main body of the experiment, animals were treated with high TSA (45μg) alone or in combination with therapeutic DAC (75μg) given 24 hrs. before. These experiments showed that at the end of the treatments, TSA alone did not exert any significant effect in term of reduction of tumor size, when compared with controls (CTRL 1434.19±193.44 vs TSA 45μg 698.1±173.09). On the contrary, I could detect a trend of anti-tumoral effect using the combined treatment DAC/TSA if compared to control. However, this combined treatment failed to reach significance thought-out all the length of the experiment (CTRL 1434.19±193.44 vs DAC 75μg - TSA 45μg (24h) 285.19±64.64) (ANOVA *P<0.05).

When compared with previously shown results (*Fig. 4.7*), significant reduction in term of tumor growth was only obtained after therapeutic DAC treatment compared to control (*Fig. 4.13*).

*Fig. 4.12b: FACS analysis.* Apoptosis in Weri-Rb1, using Annexin V-FITC at 24, 48 and 72 hrs. after different doses of TSA treatment. Mean ± SD. *P<0.05.
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**Fig. 4.13:** Weri-Rb1 xenografts. Normalized tumor mass (mm$^3$) after I.V. DAC, TSA and combined DAC/TSA biweekly treatments. Mean values ± SEM. *P<0.05.

**New formulation for DAC epigenetic treatment: the use of EMHV DDS in vitro**

My results indicated that DAC used free represents a very effective epigenetic treatment for both *in vitro* and *in vivo* experiment. Combination with TSA did not improve significance when administered in preclinical models. For my further experiment on the characterization of a newly formulated epigenetic therapy inside a drug delivery system, I therefore selected DAC. My project was in fact focused on the possibility of improving DAC anti-tumoral effect, in order to reduce its numerous toxic side effects. To achieve this aim, I used a modified erythrocyte-based drug delivery system that is CNR patented. EMHV are normally produced in the laboratory and their content of DAC has been standardized (2x10$^9$ erythrocytes contain 10μg DAC). DAC-loaded EMHV were then tested both *in vitro* and *in vivo* on Weri-Rb1 cells.

For our experiment, we aimed at comparing similar dose of DAC either administered free or loaded into the EMHV. However, technical impediments related to in the use of FACS analysis of the treated cells in the presence of EMHV allowed us to use the maximum dose of 2x10$^8$ loaded EMHV per sample containing 1μg of DAC. For the same reason, apoptosis investigation using Annexin V was not performed as EMHV were detected as false negative.
The *in vitro* efficacy of DAC-loaded EMHVs was tested and the cell cycle profile was measured by FACS analysis at 24 up to 72 hours. DAC contained inside the engineered DDS was used at the dose of 1μg and was found to induce induced a significant enrichment in sub G1 phase comparing to untreated cells (*Fig. 4.14*) at 72 hours (CTRL 9.2±2.3 vs DAC 1.7μg 32.8±2.2 or DAC 1μg - EMHV 52.1±7.1) (ANOVA *P<0.05).

*Fig. 4.14*: FACS analysis. Cell cycle in Weri-Rb1, using propidium iodide at 24, 48 and 72 hrs. after free therapeutic DAC or DAC-loaded EMHVs. Mean ± SD. *P<0.05.

These results indicated that a lower dose of DAC carried by EMHVs would induce a stronger effect than a high dosage of free DAC. This would highlight the importance of the use of a localizing carrier, that also act as a bioreactor, activating DAC into its phosphorylated active forms before reaching intracellular targets[^463].

**DAC-loaded EMHVs as an anti-tumoral therapy *in vivo***

Following our encouraging results *in vitro*, I decided to evaluate the anticancer effect of this new therapeutic formulation of DAC using the xenograft model of Weri-Rb1. In this experiment, free therapeutic drug (75μg) was compared to an EMHV loaded dose of 10μg. This loaded dose was selected considering the murine hemochrome: an optimal dose volume of 300μl, containing 2x10⁹ erythrocytes, was given by I.V injection so that animals did not show any signs of distress (AWB guidelines). To carry this experiment,
the group of EMHV-treated animals was also administered with two external magnetic fields (52N) positioned on the site of tumor for thirty minutes. This treatment allowed EMHV localization and fusion to target cells.

At the end of the treatment with EMHVs, a comparable to free DAC anti-tumoral effect was detected in the animals (Fig. 4.15) (DAC 75μg 150.58±32.40 vs DAC 10μg - EMHV - MF 175.99±38.12). Noticeably, the dose included into EMHVs was 7.5 times lower that the free dose. We demonstrated that the use of EMHVs localized by a magnetic field increased both anti-tumoral effect and bioavailability of DAC.

![Fig. 4.15: Weri-Rb1 xenografts. Normalized tumor mass (mm$^3$) after I.V. free DAC and DAC-loaded EMHVs biweekly treatments. Mean values ± SEM. *P<0.05.](image)

The results of my investigation indicated that it is possible to treat Rb with DAC to epigenetically reprogramming gene expression among which *BAX*, *CASP8*, *FAS* and *BIK*, to revert the cancer associated cell proliferation and induce apoptotic phenotype. Moreover, we confirmed that the use of EMHVs carrier as DDS could be exploited to convey anticancer molecules to tumor lesions, increasing bioavailability and reducing dosage. I therefore tested the anti-proliferative potential of a biodrug, namely molecular beacons (MB) against BIRC5(Survivin)-mRNA in Weri-Rb1 cells.
A biodrug-based treatment for retinoblastoma to target pro-survival gene

EMHV are a versatile carrier and can host molecules of different nature, such as antibodies and nucleotides. Our validation studies on DGE stressed the importance of the gene that encodes for Survivin, that largely varies its expression after DAC treatment and it is known to be involved in cell survival pathways. More specifically in Weri-Rb1, a reduction of BIRC5 gene expression is associated with the apoptotic response. I decided to use the molecular beacons against Survivin-mRNA (SURV-MB) to impede its translation and reduce protein level inside the cells. MB holds the property to emit fluorescence signal once it reaches specific target inside the cells. For this, MB can be considered theranostics, able to reveal target presence and to modify its activity by inhibiting protein's synthesis.

In the first instance, we verified the targeting of SURV-MB inside Weri-Rb1 cells. We demonstrated that free MB could internalize into the cell after lipofectamin treatment and could reached their specific intra-cellular target (Fig. 4.16a) causing fluorescence emission in the cytoplasm.

To test their therapeutic effect, I loaded SURV-MB into EMHV. This required the characterization of the new therapeutic formulation. For formulation characterization, a study to verify loading was carried out: to visualize the internalization of MB inside EMHV, I used modified SURV-MB without the quencher that constantly emits fluorescence light. Confocal microscopy view of a general population of EMHV loaded with de-quenched SURV-MB confirmed that the majority of the EMHV produced ad hoc, contained the nucleotides sequence (Fig. 4.16b). Similar loading is likely to be obtained with active therapeutic SURV-MB that does not constantly emit fluorescence but exerts it signal when reaching the target.
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Fig. 4.16: CLSM imaging. a: Weri-Rb1 cells and free fluorescent MB for Survivin; b: EMHV loaded with fluorescent MB for Survivin; c: Weri-Rb1 cells and EMHV loaded with fluorescent MB for Survivin; d: naive Weri-Rb1 cells. MB (red), nuclei (blue, DAPI staining).

When EMHV loaded with the active fluorescent SURV-MB are administered to Weri-Rb1 cells, they fuse to the cell membrane and release the contains inside the cells. As shown in Fig. 4.16c, the released active SURV-MB can reach its mRNA target, generating a red fluorescent signal. DAPI (blue) fluorescent signal indicated the presence of the cell nuclei, as in control cells (Fig. 4.16d).

The effect of EMHV loaded active SURV-MB on Weri-Rb1 cells was detected at 6 hours and 12 hours after treatment. In comparison with control value, the treatment induced a cell cycle variation in the cell population with an enrichment of cells in sub G1 phase (Fig. 4.17). This would indicate that SURV-MB induced an inhibition on Survivin-mRNA translation altering cell survival pathways.

These preliminary results in vitro suggest the possibility of using SURV-MB as a theranostic agent to selectively target a primary pathway of cell survival in retinoblastoma.
**Fig. 4.17**: FACS analysis. Cell cycle in Weri-Rb1, using propidium iodide (PI) at 6 and 24 hours after treatment with EMHVs loaded with Survivin-MB. Mean ± SD.
5 DISCUSSION

Searching for the magic bullet

The principal objective for pharmaceutical concerns the discovery and the development of medicines that satisfy requirement of patients for better therapeutics or diagnostics. This will lead not only to offer new therapies for currently untreated diseases, but also to improve options for safer, more efficacious and more cost-effective treatments, including, for example, generic drugs or more recently developed biosimilar antibodies. This field of research and development (R&D) more often includes the optimization of novel tailored nanodevices for medical purposes.

Meanwhile, the discovery and development of new therapeutic molecules for the treatment of cancer have undergone many changes. One of the most prominent is a conceptual change from one-size-fits-all approach that relies on the cytotoxicity action of well-known chemotherapeutics to a more personalized medicine approach: the latter often focuses on the optimization and implementation of target-tailored drugs that exploit a particular genetic dowry of the cells to cause vulnerability to cancer.

Drug repositioning is another aspect of utilizing clinical approved drugs for disorders other than the diseases that they are approved for. As the Nobelist James Black commented that "The most fruitful basis of the discovery of a new drug is to start with an old drug". Indeed, one of the challenges of the drug discovery market is to convey investments into the development of new strategies that would encourage the scientific community to test approved drugs for different diseases. For example, FDA (www.cancer.gov)\(^{773}\) and EMEA originally approved DAC for the treatment of myelodysplastic syndromes, however it has been proposed and trialed for human solid metastatic tumors such as colorectal and ovarian cancer (www.clinicaltrials.gov)\(^{584}\). Here, we demonstrated that it would be possible to implement its use outside these boundaries and to candidate DAC as a possible epigenetic treatment in Rb. Indeed, a careful evaluation of dosing using carrier-mediated delivery indicated the possibility of using DAC at effective concentration lower than those required for clinical use in other solid tumor.

Another strategy that we investigated in this thesis is to identify of new therapeutic tools for cancer thought a in-depth insights on its biological systems that can be obtained with
advancement in the field of high throughput genomics, using technologies such as cDNA microarrays and RNA Seq. Growing information on common molecular pathways identified in different malignancies focuses the screening of safe-drug libraries and shortens R&D processes, resulting in an economic cost-effective management. In the last ten years, DNA microarrays has emerged as a powerful approach to study the transcriptome of individual cancers and allows identifying characteristic patterns of gene expression and led to an explosion of studies of the molecular pathogenesis of tumors. NGS and other high-throughput genome-wide techniques have revealed in fact extraordinary genome complexities, conlfiming heterogeneity, not only between different tumors but also within an individual cancer histotype\(^{774-775}\). In this study we have taken a "high-resolution" view of specific epigenetic variation in Rb to open the door to understanding of cancer development and progression. We also provided an insight into molecular mechanisms of Rb to find novel cancer biomarkers (treatable biomarkers). Starting from microarray analysis and using public available data from different individuals and different experiments it was possible to identification of key driver genes that could be epigenetically regulated. This was fundamental for studying and targeting those mechanisms that regulate Rb cancer phenotype and allowed us matching drug therapies to reverse malignant cell fate.

Although others as well as our success has led to the identification of novel molecular targets that become markers for therapies (feasible), these need to be translated into druggable target, followed by therapeutic effect validation. The importance of understanding the difference between biologically active molecules and drugs is well established in the drug discovery industry. In general, after a potential novel therapeutic target has been identified, there can be significant scientific and technical hurdles to discovering a novel and effective drug. It is crucial to link the proposed target to the clinical disease but also to demonstrate quantitative consequences of target modulation that are sufficient to deliver a therapeutically meaningful biological effect. Finally, translating efficacy determined in drug discovery phase to clinical relevance could be a hard challenge: the drug target interaction must be then revealed in relevant \textit{in vitro} and preclinical models. This in turn would also impinge on the choice of physic-chemical formulation, route of administration and dose-dependent schedule of experimental design.
The foggy landscape of retinoblastoma

When we embarked in the study of retinoblastoma we realized how, notwithstanding the clinical, psycho-social and economic relevance of Rb, few studies have taken a comprehensive look at differential gene expression patterns in retinoblastomas\textsuperscript{776}. The information of gene methylation profile of retina cells is also seldom reported\textsuperscript{777}, often in comparison to the altered profile described for Weri-Rb1 and Y79 cells\textsuperscript{778}. Only recently a panel of differential methylated genes in Rb was compiled\textsuperscript{779} showing the vast majority of those to be hypermethylated, with only few genes that showed low methylation status. For example, hypermethylation was shown for \textit{RB1} that regulates cell cycle and acts as tumor suppressor as well as for \textit{TP53} that regulates cell division and \textit{RASSF1A} that concur to inhibit cyclin D1 accumulation. Proto-oncogene \textit{SYK}, for example belongs to the hypomethylated gene group. In this panorama, only Nalini and coworkers\textsuperscript{780} investigated a cDNA microarray of pre- and post-chemotherapy Rb tumor treated with etoposide, vincristine and carboplatin. Among the differential expressed genes, they identify twenty-one key gene categories, pathways, biomarkers and phenotype that undergo altered regulation after chemioterapeutic treatment including \textit{RB1}, \textit{SYK}, \textit{BIRC5}. Nalini's work indicated that a global regulation of gene expression is induced by a harsh treatment resulting into a wide broad spectrum activity.

In this scenario our an ongoing research investigates the fine regulation on differential gene expression of Rb after treatment with a well-known demethylating agent, such as DAC. A thorough microarray-based computational investigation on the effect of therapeutic dose of DAC on Weri-Rb1 cells identified a panel of epigenetically up or down regulated genes and their time course gene expression\textsuperscript{195}. Gene-gene co-
expression networks at different time points are used to identify context-driven marker association of transcription factor regulated proteins that belong to master regulatory paths. We applied computational analysis to describing DAC-driven regulatory connectivity patterns as network biology provides valuable prototype to understand complexity of cellular system and generate hypotheses for treatable biomarkers. On the same line of thought our group[419] identified signature genes that could be further exploited as epigenetic markers for retinoblastoma and are shared with the DGE landscape of non-ocular associated cancer osteosarcoma with inference on possible biological processes. The identified evidences were partially validated using experimental methods demonstrating a strong biological correlation.

The aim of this work has been to characterize and develop an innovative strategy for the local delivery of new therapies to treat retinoblastoma. The research initiated to consolidate previously acquired data, targeting the reversible epigenetic gene silencing that sustains cancer development using DAC in Weri-Rb1 cells in vitro and in vivo. We have shown how analysis of both cell cycle variation and apoptosis emphasized to role of therapeutic DAC as an anti-proliferative agent in Rb. Particularly, it was interesting to follow the time-dependent variations of the percentage of cells in sub G1 phase as well as the decrease of alive cells at the latest time point of treatment. It is likely that these effects were mediated by the modulation of specific gene expression already described by our in house computational studies. I investigated the role of a higher number of all those genes that have been shown to be differentially expressed in epigenetic-drug treated Weri-Rb1 in order to identify new feasible genetic markers. Among these hundreds of genes, we selected relevant genes that are showing high differential expression equal or greater than log2 across all time points and are involved in pathways related to different cancers, such as pro-apoptotic pathways. The selected genes were involved in various pathways, namely: extrinsic apoptosis pathway (CASP8, FAS, DAP3), intrinsic apoptotic pathway (BIK, BCL2L1, BAX), TFN signaling pathway (TRAP2, TRAF2). Moreover, genes encoding for ubiquitous transcription factors (RELA, HSF1, TOLLIP) were selected for their involvement in several biological processes. BIRC5 was selected as it is known to be a member of the inhibitor of apoptosis (IAP) gene family and it is high expressed in several tumors. P73, a p53 family member, encodes for transcription factors related to stress response. CASP6 product is a downstream effector of Caspase cascade, likely to be regulated by apoptotic treatment. RRAD was selected as it holds a role in regulating metabolic processes in cancer. It is
interesting that the interaction network analysis showed that several of these selected genes are involved in a common pathway centered on CASP8 and correlating extrinsic apoptosis pathway effectors (FAS) and downstream intrinsic (mitochondrial) apoptosis pathway genes (BIK, BCL2L1, BAX).

Once the cancer target genes were identified, validation of computational analysis data became inevitable. qPCR is considered as a reference method to measure the selected gene expression. In this work, I validated the expression of the selected genes. In vitro I was able to verify the trend of expression fold-change after treatment at different time points that correlate with the presence of early and late apoptosis expression. In fact, both FAS and CASP8 that mediate extracellular early apoptosis appeared over-expressed at 48 hours after treatment. On the other hand, after cell damage, BAX and BCL2L1 are over-expressed at latest time points. Their protein products are known to be involved in the cytochrome C release from mitochondrion to the cytosol and are indirectly responsible for the formation of "apoptosome" that activates the downstream Caspase cascade\textsuperscript{[781]}. BIRC5, also known as Survivin, is a small member of the IAPs family, predominantly expressed in tumor versus normal tissues. It is considered a prime cancer-specific drug target and its down-regulation has been reported to sensitize cancer cells to various chemotherapeutic agents\textsuperscript{[782]}. Tumor cell lines in vitro culture are frequently used as the first line of study the effect of any treatment on cell proliferation, apoptosis and other cellular endpoints, that can easily be measured using different high-throughput methods. There are, however, some caveats with regard to the use of cancer cells overriding the signals that tell them to self-destruct and that may result in substantial phenotypic, genetic, and epigenetic alterations induced by environment\textsuperscript{[783]}.

Therefore, we corroborated our in vitro results with ex vivo validation from tumor samples obtained by explants from mice treated with therapeutic DAC after three weeks treatment. The treatment induced a significant anti-tumoral effect using a therapeutic dose scaled from clinical reports and tested on human xenografted derived cells. To best of our knowledge, we have reported, for the first time, the systemic use of DAC effect tested on ocular tumor.

Our results showed a similar landscape of gene expression variation ex vivo with the most relevant genes, namely FAS, BIK and CASP8, showing upregulation at the end of treatment. BIRC5 instead appeared down regulated, confirming its role as a major effector in cell survival. The biological effect on tumor mass reduction and in differential gene expression regulation in Rb suggested the possibility of repurposing DAC for retinoblastoma therapy.
DAC is a DMNT inhibitor that irreversibly anneals the methylating activity of the enzyme, however it might exert a more general dose-dependent cytotoxic effect\textsuperscript{[784]}. For these reasons, we explored the methylation patterns of those selected genes that showed a more prominent differential expression variation in Rb and the possibility to deliver DAC at the site of action so that reducing its cytotoxic effect.

Several previous investigations described the methylation profile of \textit{CASP8} on retinoblastoma cells\textsuperscript{[191]}. Our result confirmed this finding, suggesting that \textit{CASP8} profile could be referred as a positive internal control of our experiment. However, beyond \textit{CASP8} methylation, we were able, for the first time, to demonstrate that also \textit{BIK}, implicated in the intrinsic apoptotic pathway, is methylated in Rb and that DAC treatment is able to demethylate both genes, so increasing their expression in treated cells.

Their upregulation exerts a down-stream regulatory effect in the FAS-dependent apoptotic signaling thus inducing the block tumor cells growth. On the other hand, methylation analysis of FAS demonstrates its demethylated status, suggesting that in Rb the lack of apoptotic response and the consequent tumor growth are not dependent from the down regulation of cell surface stress signaling receptors, but from the silencing of down-stream \textit{CASP8} and \textit{BIK} apoptotic effectors.

Interestingly, \textit{FAS} is a member of the TNF-receptor superfamily, and its product mediates the extrinsic apoptosis pathway and it has been implicated in pathogenesis of several diseases. It is known that FAS-dependent apoptosis pathway is activated by extracellular stress signals of different nature (chemical, physical, metabolic).

Experimental oncology research and drug development both substantially require specific, clinically relevant \textit{in vitro} and \textit{in vivo} tumor models. For these, biomarkers identified through basic research need to be validated using a preclinical discovery phase. Once a target candidate has been identified, sufficient preliminary data need to be generated to support clinical trials.

Preclinical models need to take into account both of the molecular nature of the target and behavior of the chemical compound. The use of different models will be required for a detailed investigation of compounds targeting epi/genetic markers that might be sensitive to environmental characteristics. In this work, we have intensively used tumor xenografts to exhibit the relevant molecular characteristics of the corresponding human cancers. However, human tumor xenografts might encompass some limitations, as they
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do not replicate human stromal cell interactions as are not implanted at their native site. Despite these limitations, xenografts will retain their role as workhorses in drug discovery, however a higher degree of accuracy should be used. For this, we developed an orthotopic model in order to investigate the influence of environment and spatial localization of tumor lesions on the effect of the therapy. The morphological appearance of the tumor lesion obtained inside the engrafted animal eyes confirmed that we achieved optimization of the model with the correct localization in correspondence of the retinal tissue. Similarly, to our previous preclinical investigation, in this orthotopic model it was possible to evaluate the activity of systemic therapeutic dose of DAC. In this case, free DAC reached ocular department exerting a tumorstatic activity. The preliminary results of our studies are encouraging and need a deeper investigation for confirmation and refinement. Immunohistochemistry investigation is likely to verify the reduction of DNMT activities, as this enzyme family represents the direct target of DAC. In depth studies are needed to find the expected reduction in Ki67 signal, as already shown in our previous work on DAC activity on other tumors\textsuperscript{[645]}. A thorough analysis of the microenvironment and extra-cellular surroundings will enrich the understanding of Rb treatment.

**Exploring combinatory therapies (might disappoint you)**

In therapeutic development, a major hurdle is translating efficacy determined in drug discovery phase to practical application in clinics, as there are often discrepancies between drug efficacy *in vitro* and *in vivo* experimental models and final efficacy, in patients.

In the scope of this thesis, we learnt that not all the agents that exert a significantly high anti-cancer effect *in vitro* could maintain their promises when translated *in vivo*. The prominent example that we found of anti-cancerous effects *in vitro* and have lesser or no effect *in vivo* is that of HDAC inhibitor TSA in the treatment of Rb. In fact, based on the epigenetic pathogenesis of Rb, an increasing dose (75 - 150 - 300ng) of TSA was tested on Weri-Rb1 cells, obtaining striking anti-proliferative time-dependent effect in vitro at the highest dose suggesting that also the removal of histone acetylation is relevant in inhibiting tumor cells survival. Unexpectedly, the same results have not been obtained in *in vivo* experiments where the I.V. administration of TSA alone did not exert any
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significant anti-tumoral effect.

We can also hypothesize that the fine-tuned dose-dependent regulation of gene expression by DAC in Rb could lead to overcoming potential anti-tumoral drug resistance as investigated in other solid tumors\cite{785-786}. DAC hypomethylation activity might for example, unblock silenced genes involved in drug-efflux inactivation pathways. Clinical trials are instructed to verify the possibility of increasing chemotherapics' effect used in combination with low doses of DAC (NCT00925132; NCT01876641). However, in Rb the effect of combination of DAC and chemotherapics has not been tested so far. Further experimental evidence is needed.

These evidences also demonstrated a time-regulation in activity of TSA with the highest effect that was measured at time point of 72 hours. These positive results led us to test the possibility of administering a combined epigenetic treatment to maximize the anti-proliferative effect on Weri-Rb1. DAC was then administered 24 hours before TSA (75 - 150 - 300ng) obtaining an earlier anti-proliferative response at 48 hours. This indicated towards the inhibition of histone deacetylation by TSA in Weri-Rb1 could be facilitated by the effect of previous administration of DAC. Unexpectedly, the administration of TSA alone did not exert any significant anti-tumoral effect \textit{in vivo}. The association of the two epigenetic treatments equaled the efficacy of the DAC alone, indicating that probably the effect ascribed to the combined treatment is only due to the DAC activity. Also as regard, the administration of the combined epigenetic treatments (demethylation and acetylation), in order to maximize the anti-proliferative effect on Weri-Rb1 cells, has given rice to discordant results \textit{in vitro} and \textit{in vivo}. In fact, the administration of DAC 24 hours before TSA (75 - 150 - 300ng) obtained an earlier anti-proliferative response in Rb cells \textit{in vitro} comparing DAC treatment alone, suggesting that the inhibition of both DNA methylation and histone deacetylation is more efficient than each single treatment in removing epigenetic gene silencing. On the other hand, the association of the two epigenetic treatments \textit{in vivo} equaled the efficacy of the DAC 5 2alone, indicating that probably the effect ascribed to the combined treatment was only due to the DAC activity or because it has been known that hydroxamate-based HDAC inhibitors, although their promising anticancer activities, lead to poor \textit{in vivo} results due to their metabolic instability and poor pharmacokinetics\cite{787}.

In literature it is reported that use of HDACi as anticancer drug in retinoblastoma\cite{788}. Authors were able to demonstrate TSA effect \textit{in vitro}, however there are no reports of \textit{in
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in vivo Rb anticancer activity. It is interesting however that also in clinical trials, TSA failed to produce the expected effect in patients. "There is much to be said for failure. It is much more interesting than success", as quoted the British humorist Max Beerbohm in 1946 in his "Mainly on the air"[789].

It would be interesting to find out if, in an orthotopic model, a significant anticancer effect can be obtained by using a drug delivery system that protect HADC inhibitor by degradation and improve its pharmacokinetics. The use of a more sophisticated model may also provide indication of the HDAC inhibitor effect on its specific target (histone deacetylases), when investigated a full screening of AMDE processing of TSA will also shed lights on this discrepancy.

Better, faster

One of the major pharmacological challenges is to achieve drug effectiveness using dose levels below thresholds associated with off-target effects and cytotoxicity. To reach this goal, several strategies can be implemented: to delivery drug in combination with other therapeutic agents for generating a synergistic effect and/or to modify the chemical nature of the molecule both for strengthen its resistance to metabolic agents and/or to phantom it from RES action. However, in the past few years, the most commonly used approach to this issue has been to associate drugs to suitable drug delivery systems.

Toxicity of DAC as a single agent has been evaluated extensively to support oncology clinical trials. Cytotoxicity, non-specific targeting, structural instability, catabolism and poor bioavailability restrict the DAC broader clinical application. As already mentioned in introduction, toxicological effects of DAC are mainly characterized as hematological (leukopenia, anemia and thrombocytopenia)[450], myeloid (bone marrow hypoplasia), lymphoid (thymic/testicular atrophy)[790]. These effects are very much consistent with its cytotoxic biologic action at high drug concentrations. It has been reported that low doses of DAC can deplete DNMTs causing less significant DNA damage or cytotoxicity (www.dacogen.com)[791]. Indeed, dosage is a critical determinant for this epigenetic agent, characterized by a narrow therapeutic window. To overcome the issues associated with inefficient bioavailability, whilst facilitating the administration to enhance efficacy, several DDS-based strategy have been developed. In time, poly (lactic-co-glycolic acid)
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(PLGA) and poly(ethylene glycol) (PEG) based nanoparticles,[792] gelatinases-stimuli responding smart material nanoparticles[793] and lipid-based nanocarrier systems were implemented for the potential delivery of decitabine improving formulation design and characterization for in vivo assessment.[794] Nanoparticle-based drug delivery systems still have some limitations. Once administered in the blood stream, they must overcome the biological barriers and obstacles such as mucosa and the cellular and humoral arms of the immune system. Moreover, they can be sequestered by serum proteins and undergo opsonization. These nanocomplexes generally undergo rapid clearance. Furthermore, limitations can arise from the intricate tumor microenvironment and higher interstitial fluid pressure found in solid tumor tissue that could prevent diffusion of nanoparticles. Considering these drawbacks, a cellular carrier might represent a better choice of delivery system. Among them, erythrocytes stand out to be the most appealing in order to improve the pharmacokinetics, biodistribution and pharmacodynamics of therapeutics or diagnostics. Erythrocyte technology to develop a drug delivery system has been long explored as blood and its components represent the natural biological carrier for drug delivery in the whole body.

Despite the apparent simplicity and obviousness of this strategy, RBC-based drug delivery is a highly challenging task. In general, many aspects of production, storage and regulatory affairs complicate industrial and clinical translation of natural biological carriers. Furthermore, the negative impact of transfusion-transmitted infectious diseases in the eighties almost decimated RBC drug delivery research. Nowadays, RBC carriers seem to be back of fashion, with growing recent literature available to witness new scientific interest.[795-797]. Indeed, there are some companies exploring preclinical studies of RBC drug delivery.[584,798] However, very few original works are detailed, where RBCs are being explored for delivery of therapeutics, including nanoparticles and other novel nanomaterials.

In this scenario, our research has a prominent place. In this work, we showed that DAC effect and bioavailability could be improved by a drug delivery system based on the use of engineered erythrocytes (EMHVs).

Ongoing research activity in our laboratory led to characterize the EMHVs delivery system both in vitro and in vivo. EMHVs accommodate paramagnetic nanoparticles that are instrumental for selective localization to specific tissue and site of actions. These FDA approved NPs are commercially available and used in low concentration to avoid
accumulation in target organs. Furthermore, to facilitate anchoring and subsequent membrane fusion of EMHVs to host cells, filamentous hemaglutinin was inserted during resealing procedure. These both are the ciphers that make EMHVs standing out against the field of drug delivery. In addition, we proved that EMHVs are a versatile carrier that could accommodate high quantity of molecule of different nature. For different studies, EMHVs were prepared with chemotherapics (doxorubicine, cisplatin), antibodies (patented antibody against colon cancer), oligonucleotides (decoys against NFkB, HFS1), radioactives for nuclear medicine and fluorescent nanoparticles and dyes for diagnostics. Recently, we developed an innovative formulation for DAC loaded inside EMHVs, and we have standardized the loading procedure to obtain constant yield of DAC inclusion\[639\]. This formulation was successfully used for the treatment of prostate cancer. Interestingly, in preclinical model, not only we were able to obtain a significant response in androgen-sensitive prostate cancer, but also we explored the possibility of treating also non-responsive, hormone-refractory tumor. Due to their remarkable long life span in circulation, they act as potential reservoirs for a slow, controlled and sustained release of their cargoes. Furthermore, EMHVs act as active bioreactors due to the enzymatic phosphorylases that they possess. Indeed, DAC is a pro-drug that will be modified inside the EMHVs and presented to its target in the active phosphorylated form at time of intra-cellular release\[463\]. Here, for the first time, it was demonstrated that DAC has therapeutic effect in Rb and that its bioavailability and pharmacokinetic is improved using EMHVs delivery system. EMHVs loaded with DAC were able to exert a significant anti-tumoral effect in Rb xenograft model similar to that obtained with free DAC treatment even if the dose administered by EMHVs is 7.5 times lower. Therapy localization by magnetic exposure and increased FHA-mediated membrane fusion, as well as carrier protection and bioreactor activity of EMHVs enhanced DAC bioavailability and its therapeutic efficacy.

More to the point

Briefly, we can conclude that EMHV delivery system has documented advantages: they can accommodate fragile biologics, which should circulate as long as possible preserving their properties and preventing loss of function; they can act as circulating reservoirs as
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slow-drug delivery systems where a prodrug is converted by red cell resident enzymes into its active form; EMHV s preventing opsonization and fast removal by the mononuclear phagocyte system, especially in the liver and spleen. Finally, one of the most interesting EMHV s feature, is the possibility of being localized in specific tissues by magnetic forces. For this, we will extend our research focusing on the possibility of using EMHV s in an orthotopic model of Rb reaching through the blood-stream the retro-ocular compartment, often invaded by resident cancer. To date, in fact, ocular drug delivery with conventional systems, such as drops, hydrogel and other disposals, suffer from lack of control in targeting of therapy and poor drug bioavailability. On the other hand, more localized ocular injections are hardly accepted by patients. To reduce these drawbacks, a systemic EMHV therapy could be enriched at the site of cancer by a biocompatible magnetic field exposure that would likely be tolerated by patients.

In our experiment with magnetic exposure, we achieved EMHV s and drug localization both in superficial district (flank xenograft tumor mass\cite{645} and in deep tissues (lung, unpublished). Ongoing experiment also indicated that EMHV s could enrich in the CNS (motor cortex) once injected I.V. overcoming the blood brain barrier. These acquired experiences would lead us to develop the optimal localization strategy to reach the ocular compartment. To accomplish this task, the magnetic source will have a pivotal role. A cylindrical heart magnet is normally used in our studies, however, a collaboration with the department of robotics of the Scuola Superiore Sant'Anna (Pontedera, Pisa), will provide us with a customized tunable source of magnetic field, able to focus magnetic forces toward selected tissue. This devise will be instrumental for the optimization of EMHV-loaded drug delivery to the Rb lesion in the eye.

**Shedding light on Beacons (MB)**

Development of sensitive, specific molecular probes is one of the central challenges in bioimaging. In fact, the last two decades have witnessed the implementation of MBs in a variety of fields, including target analysis and molecular and cellular imaging. MBs have become an effective molecular tool able to enhance gene expression or inhibit the production of deleterious proteins, thus serving as excellent candidates for gene therapy. With the use of MB, the synthesis, transport, and distribution of mRNA in living cells can be monitored with good spatial-temporal resolution to provide important information for functional genomics. In this work, we tested the possibility of using SURV-MB
against Survivin-mRNA, as a theranostic agent for Rb. Survivin is the product of \textit{BIRC5}, which is known to be over-expressed in several tumors including retinoblastoma and that we demonstrated to be down regulated in Rb after DAC treatment. Many evidences suggested that targeting Survivin expression with the use of drug delivery system can be considered an effective anticancer strategy and several clinical trials using inhibitors such as YM155 have been instructed, however with limited success\textsuperscript{[798]}. Inefficient \textit{in vivo} delivery and poor bioavailability are suspected to be two of the culprits responsible for the poor translation. This would leave ample room of maneuver for continuing innovation to realize the potential of targeting Survivin, ranging from better understanding of mechanistic the action of Survivin to more rational design of nanocarriers to deliver therapeutic cargoes in a targeted manner.

Few original research works have been carried out using MB targeting Survivin-mRNA in solid tumors and up to recently, only for diagnostic development\textsuperscript{[799-800]}. Moreover, there is only another report of the use on molecular beacon in Rb cells to induce photodynamic-mediated cell killing\textsuperscript{[801]}. We characterized SURV-MB as a diagnostic tool in Rb by detecting a significant burst of fluorescence enhancement upon target binding. The results demonstrated that delivery of MB targeting Survivin-mRNAs produced a strong signal, confirming the diagnostic potentials of SURV-MB. For this first step, we used lipofectamine as internalizing adjuvant, given the hydrophilic nature of MB. In fact, as for all the oligonucleotide therapeutics, such as siRNA, miRNA, aptamers and decoys, MBs would be more efficiently reaching their target if associated with drug delivery systems, such as fusogenic peptides, pH-sensitive lipoplex or liposomes. Most frequently, gold-NPs are used as an effective vector for MB intra-cellular bioimaging\textsuperscript{[802-804]}.

To develop an optimal therapeutic formulation, we used EMHV\textsuperscript{s} to deliver SURV-MB into Rb. Contextually, we verified that this carrier, with enhanced fusion and release properties, could efficiently deliver the biodrug into the cytoplasm of targeted cells. Upon reaching intracellular target, we were able to track SURV-MB internalized amount, as well as its distribution. Finally, SURV-MB loaded inside EMHV exerted an anti-proliferative effect in Rb suggesting the possibility of using this new biodrug-based EMHV\textsuperscript{s} formulation for theranostic purposes. Cell cycle analysis would suggest that with the investigated treatment protocol, the increased amount of sub G1 was a major contribution to the cell death. The data suggested that the designed molecular beacon...
might provide a potential alternative for Rb therapy and secure the ground for future investigation.

To refine and confirm these preliminary results, it would be necessary to deeply investigate the effect of SURV-MB-loaded EMHV's in vivo, especially in orthotopic model. From these experiments, we could verify the benefit of using EMHV's in reducing effective dosage in comparison to freely administered SURV-MB, and the ability of a novel tunable magnetic source to efficiently localize EMHV-loaded therapy at eye level. Furthermore, we could obtain strong indications about the theranostic nature of SURV-MB in Rb and move to design a translatable protocol for personalized medicine. The efficient design, versatility of features and functionalities, reproducible chemical synthesis and modification, target binding selectivity and affinity together with relatively rapid tissue penetration, low immunogenicity and rapid systemic clearance are often considered exploitable features that would make MBs the ideal recognition elements for use as therapeutics.

Testing the effect of SURV-MB-loaded EMHV new formulation, we suggested that SURV-MB could be a good theranostic tool9 for the targeting of BIRC5(Surviviv)-mRNA. After high-resolution investigation of epigenetically regulated gene networks, we selected BIRC5 among feasible targets for cancer treatment in Rb and we are confident that it could "run for" the role of druggable candidate.

Fig 5.2: epi/genomic biomarker diagram II (after investigation)
In conclusion, we provided preclinical evidence of a successful new formulation of a localized epigenetic treatment of retinoblastoma. Contextually the basis for investigating the potential of repositioning DAC as an epigenetic drug to treat the ocular cancer was established. After an integrative bioinformatics analysis, we validated selected gene candidates to become feasible targets for developing a high-precision medicine treatment and we proved the druggability of Survivin gene as a possible therapeutic target in retinoblastoma, characterizing the theranostic potential of a DNA-based biodrug, Molecular Beacon against Survivin-mRNA. Moreover, the use of the in house-developed EMHV's carrier allowed localized tissue and cell-specific drug delivery to cancer cells together with a lowering of dosage and reduction of off target effect.

This work has a place in the field of preclinical investigation for retinoblastoma cancer therapy. Indeed the study of retinoblastoma has revealed guideline principle for epigenetically regulated initiation and progression. We proposed an integrative bioinformatics approach that revealed expected gene network and interaction of relevant pathways involved in apoptosis, however most interesting also unveiled unexpected causal relationships emerged because of DGE investigation. We widen the concept of precision medicine opening two fronts of access: 1) we optimized the potential of the commonly re-known as "ideal" natural biocarrier erythrocytes creating novel EMHV formulations. Being aware of the many hurdles, intrinsic to R&D of erythrocyte technology, such as safety, stability, toxicity, as well as production, large scalability and regulatory issues, I am confident that researches like mine will lead the path toward the autologous use of erythrocyte in clinics envisaging customized production for each patient; 2) the introduction of a treatment based on the use of a biodrug such as SURV-MB not only suggests the need for further preclinical investigation of the EMHV loaded formulation in relevant orthotopic or humanized models, but also indicates the possibility of implementing a theranostic approach for specific gene targets in retinoblastoma. One of the future research challenge could be, via an integrate approach that merges wet-lab/NGS investigation and high-resolution genomic and proteomic technology, to identify early players in the genetic landscape of Rb. A therapy based on EMHV-loaded MB against those targets could result in highly reliable tool for early diagnosis and cure of Rb and MB might change its meaning from Molecular Beacons to Magic Bullets.
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