

A RETROSPECTIVE OBSERVATIONAL STUDY ON CASES OF SARCOMA TREATED WITH THE DI BELLA METHOD: RATIONALE AND EFFECTIVENESS

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ABSTRACT

Despite all the new developments in cancer therapy, the life expectancy of patients with sarcoma remains short. Since it was established as a cancer therapy, the Di Bella Method (DBM) has been able to increase survival rate and life quality, without overt toxicity, in comparison to what is described in the literature for the treatment of sarcoma with the same immunohistochemical, histologic and clinical features. We therefore treated 37 patients with sarcoma using the DBM protocol. The DBM therapy consists of somatostatin and analogues (octreotide), all trans-retinoic acid (ATRA), β -Carotene, axerophthol

dissolved in vitamin E, vitamin D, vitamin C, melatonin (MLT), proteoglycans, glycosaminoglycans, hydroxyurea, Sodium butyrate (Na-Bu). These molecules have antiproliferative, antiangiogenic, cytostatic, antioxidant, antimetastatic (differentiative) and immunomodulating properties. Moreover, the inclusion of ATRA, MLT and Na-Bu has increased the antitumoral properties of the therapy by extending them to cancer stem cells. Furthermore, the non-cytolytic and non-cytotoxic metronomic dosage of hydroxyurea has improved the outcome of DBM therapy by increasing anti-tumour capability. The results of this treatment revealed the effectiveness of the DBM. In conclusion, the multi-strategic objectives of the DBM are to inhibit proliferation-invasiveness and neoplastic angiogenesis, silence the survival system of cancer stem cells, enhance immunomodulatory and antioxidant activities, improve the vitality and efficiency of normal cells, and depress the efficiency and vitality of neoplastic ones.

KEYWORDS: Sarcoma; Growth Factor; Somatostatin; Melatonin; Retinoic Acid; Vitamin D; Vitamin E; Prolactin; Di Bella Method; D2 R agonists.

FOREWORD-INTRODUCTION

Sarcomas are a rare heterogeneous group of malignant tumours of mesenchymal origin, which comprise 1% of all malignant neoplasms in adults and 12% of malignant paediatric cancers. The histopathological spectrum of sarcomas is broad, presumably because the mesenchymal embryonic cells from which they emerge have the ability to mature into striated skeletal muscle, adipose tissue, fibrous tissue, bone and cartilage.^[1]

They are mainly divided into bone sarcomas and soft tissue sarcomas. The first are less common, with significant morphological heterogeneity and broad-spectrum biology^[2], while the more common second type includes at least 100 different histologic and molecular subtypes, with variable clinical behaviour.^[3] While part of this intratumoural heterogeneity could be explained in terms of clonal genetic evolution, an essential part includes a hierarchical relationship between sarcoma cells, governed by both genetic and epigenetic influences. The notion of this functional hierarchy operating within each tumour implies the existence of sarcoma stem cells. With progressive evidence, the literature is documenting, in osteosarcomas, the narrow causal relationship between the significant increase in serum GH rate during development and in prepubertal and pubertal ages, and its site of action, clearly prevalent in the osteocartilaginous growth areas.^[4]

Both soft tissue sarcomas and bone sarcomas are mainly treated with neoadjuvant chemotherapy, surgical resection and adjuvant chemotherapy. Radiotherapy is used less often and is generally applied when other treatments cannot achieve significant results.^[1,2,3] There is no unique treatment for individual subtypes of soft tissue sarcoma because of the broad biological spectrum. Treatment varies depending on tumour immunohistochemistry; chemotherapy is rarely a reliable treatment for the effective resolution of the disease.^[1]

In bone sarcomas, where surgery is not followed by disease relapse, five-year survival for localised disease is just over 70%, while in metastatic osteosarcomas and Ewing sarcomas it is just over 20%.^[3] In soft tissue sarcomas, 5-year post-diagnosis survival depends on the aggressiveness of the disease and on the precocity of diagnosis, with survival rates of just over 50%. In half of soft tissue sarcomas, the disease presents as or becomes metastatic. The main site of metastasis is the lung. In metastatic disease, survival is generally approximately 12 months.^[5]

Our retrospective observational study showed that progress in sarcoma therapy can be achieved with the DBM in terms of objective response, quality of life and survival. Patients with sarcoma were treated with the following DBM treatment protocol (Tab.1):

Medications	Chemical Composition	Dosage	Method of Administration	Frequency
Somatostatin	14-aa polypeptide	4 mg	Subcutaneous or preferably intravenous	Daily (12 night hours with infuser)
Octreotide LAR	Octreotide Acetate 8 aa	10 mg	Intramuscular	Weekly
Retinoid solution	All-Trans-Retinoic Acid Axerophthol Palmitate Beta-carotene Alpha Tocopheryl Acetate	0.5 g 0.5 g 2 g 1000 g	Oral	Daily (3 administrations)
Vitamin C	L-Ascorbic Acid	4 g	Oral	Daily (lunch and dinner)
Vitamin D3	1,25-diOH-Tachysterol	30 drops = 1 ml approximately = 1 mg	Oral	Daily (3 administrations)
Tetracosactide Acetate	Tetracosactide Acetate	0.25 mg	Subcutaneous	3 administrations per week, every other day, with infusion
Bromocriptine	Bromocriptine	2.5 mg	Oral	½ tablet twice a day
Cabergoline	Cabergoline	0.5 mg	Oral	½ tablet twice a week
Chondroitin Sulfate	D-glucuronic acid (GlcA) N-Acetyl-D-galactosamine (GalNAc)	500 mg	Oral	3 times a day
Glucosamine	D-Glucosamine	500 mg	Oral	3 times a day
Ursodeoxycholic Acid	Ursodeoxycholic acid	300-450 mg	Oral	Daily
Melatonin	Melatonin 12% Adenosine 51% Glycine 37%	100 mg	Oral	Daily
Sodium Butyrate	C ₄ H ₇ NaO ₂	500 mg	Oral	2 times a day
Hydroxyurea	Hydroxyurea	500 mg	Oral	2 times a day
Calcium Carbonate	CaCO ₃	500 mg	Oral	2 times a day
Calcium Levofolinate	Calcium Levofolinate Pentahydrate	22 mg	Oral	Once a day, every other day
Sucrosomial Iron	Sucrosomial Iron	14 mg	Oral	Once a day, every other day
Acetazolamide	Acetazolamide	250 mg	Oral	½ tablet twice a day
Diethyldithiocarbamate	Diethyldithiocarbamate	200 mg	Oral	Daily

CASES

Patients with Sarcoma treated with the DBM and evaluable for this study all have a medical history of diagnosis, tumour stage and grade, along with treatments that were performed prior to treatment with DBM. (Tab. 2)

MEDICAL HISTORY									
File no.	Date of birth	DIAGNOSIS	O/M	diagnosis date	Stage	Grade	Surgical interventions	Chemotherapy	Group
438	14/02/1950	Endometrial Stromal Sarcoma	M	Mar-1997	III	G 3	surgery in 1997	no	C
N1	25/11/1968	Fibrosarcoma	M	Jun-1999	II	G 2	1999 - 2004 surgical removal of lung and pelvic metastases	yes	E
N2	28/06/1990	Osteosarcoma	O	25/09/1995	II B	G 4	1996 - bone resection	yes	E
779	11/12/1972	Liposarcoma	M	25/09/2007	I	G 1	radical resection (347-1525046)	no	C
2653	06/10/1938	Synovial sarcoma	M	01/12/2009	III	G 3	left thigh amputation 12/2009	no	C
783	10/08/1968	Reticulosarcoma	M	01/01/2007	IV	undefined	no	no	A
NO	04/04/1966	Osteosarcoma	O	09/09/1997	II B	undefined	Jan-98	yes	E
480	19/03/1999	Osteosarcoma	O	01/09/2003	II B	G 3	thigh (2003) lung (2005)	yes	E
33	05/02/1940	Osteosarcoma	O	05/09/1997	III	G 4	thigh 1997	yes	E
42	06/07/1967	Mesenchymal chondrosarcoma	O	08/05/1997	III B	G 3	surgery in 1997/99 - relapsed in 2007 - relapsed in 2009	yes	E
1716	24/02/1952	HISTIOCYTOMA	O	1996	II A	G 3	1996 - 2001 (relapse)	no	D
1132	29/12/1950	Epithelioid fibrosarcoma	M	Aug-2007	IV	G 2	primary tum. 08/07 - pulm. lobectomy 03/08	no	D
1718	16/08/1930	Malignant fibrous histiocytoma	O	Oct-2000	I	G 2	radical resection	no	C
368	03/09/1988	Bilateral	O	Mar-2002	III	G 3	2000-2001-2002-2005	no	C

		chondrosarcoma							
L. DI BELLA	28/12/1998	Fibrosarcoma	M	05/01/1999	III	undefined	no	at onset	B
1981	23/10/1934	Polymorphous spindle cell sarcoma	M	Aug-2008	III	G 4	resection in 8/2008 - arm amputated 11/2008 – pulm. nodules 3/2009	no	C
2066	20/08/2007	Metastatic embryonal rhabdomyosarcoma	M	Apr-2009	IV	undefined	resection in 2009	yes	E
936	19/10/1936	Retroperitoneal leiomyosarcoma	M	1997	I	undefined	1997 - 2005 (relapse, colon)	no	C
6536	04/01/1975	Monophasic synovial sarcoma	M	06/02/2015	I	undefined	Surgery	no	C
6640	23/11/2003	Ewing sarcoma	O	25/01/2012	II	undefined	Surgery + RT + CT	yes	E
9846	04/09/1968	Retroperitoneal liposarcoma	M	13/08/2018	II B	G 1	Surgery	no	C
10413	02/04/2010	Monophasic synovial sarcoma	M	05/07/2019	I	G 2	Surgery		C
10420	26/03/1975	Leiomyosarcoma	M	18/06/2019	IV	undefined	Surgery + CT	yes	E
10457	30/10/1985	Ewing sarcoma/PNET	O	10/04/2019	IV	undefined	Surgery + CT	yes	E
10585	09/11/1964	Dermatofibrosarcoma	M	26/03/2019	I	undefined	Surgery	no	C
10853	02/06/1944	Angiosarcoma	M	13/09/2019	I	G 1	Surgery	no	C
11115	29/07/1977	Chondrosarcoma	O	13/09/2016	I	G 2	Surgery + RT	no	D
3674	03/10/1952	Myxosarcoma	M	09/04/2011	III	G 1	Surgery	no	C
3694	06/09/1976	Desmoid tumour	M	30/04/2010	IV	undefined	no	yes	B
4345	16/06/1954	Chondrosarcoma	O	01/10/2001	I	undefined	multiple	no	C
4530	07/12/1985	Leiomyosarcoma	M	17/02/2011	I	G 1	surgery in 2011 – 2012 (bladder relapse) - 2013 (pelvis relapse)	yes	E
4637	25/07/1980	Extraosseous	M	14/06/2012	IV	G 3	Surgery	no	C

		osteosarcoma							
4810	24/11/1978	Myxosarcoma	M	01/03/2012	IV	G 1	Surgery	yes	E
7603	30/05/1967	Sternal osteosarcoma	O	01/01/2013	IV	missing	Surgery	yes	E
9093	22/09/1975	Ewing sarcoma	O	11/04/2008	I	missing	surgery + CT in 2008	yes	E
9135	02/02/1992	Angiosarcoma	M	13/07/2017	I	G1	surgery	no	C
9671	13/08/1946	Leiomyosarcoma	M	27/06/2005	I	missing	surgery	no	C

The summary diagram below summarises the patient's conditions when first seeing the physician prescribing the DBM, the stage, and result of the DBM treatment in the patient. From this data, the increase in survival (quantified in years) can be seen compared to the percentages published in the AIOM guidelines. (Tab. 3)

DBM - treatment period and RESULT					SURVIVAL IN YEARS WITH DBM						
File no.	DBM	CONDITIONS upon arrival	STAGE	RESULT	efficacy	Current Conditions	1 YEAR	3 YEARS	5 YEARS	Starting Stage	
438	1998	pulmonary metastases	IV B	Complete remission	R	Absence of disease	yes	yes	yes	III	
N1	2005	inoperable retroperitoneal relapse	IV	Progression	P	deceased (2008)	yes	yes	yes	II	
N2	1998	pulm. nodules - cardiopathy caused by chemotherapy	IV B	Remission	R	in 2007 was in remission	yes	yes	yes	II B	
779	2007	after surgery	I	Remission	R	Treated for 1 year - then surgery again	yes	yes	yes	I	
2653	2010	bilateral pulmonary metastases	IV	Remission then progression	RP	deceased (12/2010)	yes	NO	NO	III	10 months
783	2007	pulm. nodules Abdominal	IV	Complete remission	R	Absence of disease	yes	yes	yes	IV	
NO	1998	after surgery	II B	Complete remission	R	Absence of disease	yes	yes	yes	II B	
480	2006	lung metastasis relapse	IV B	Complete remission	R	Absence of	yes	yes	yes	II B	

						disease					
33	2004	lung metastasis	IV A	Complete remission	R	Absence of disease	yes	yes	yes	III	
42	1998	after the first surgery	III B	Remission/Progression	RP	deceased (10/2010)	yes	yes	yes	III B	13 years
1716	2001	relapse	III	Complete remission	R	Absence of disease	yes	yes	yes	II A	
1718	2001	after surgery	I	Complete remission	R	Absence of disease	yes	yes	yes	I	108 months
368	2005	cranial recurrence because treatment was suspended	IV	Stability	S	stable	yes	yes	yes	III	alive
L. DI BELLA	1999	6 months of life - 4 cm mass	III	Complete remission	R	Absence of disease	yes	yes	yes	III	alive
1981	2009	subclavicular axillary lung metastases	IV	Progression	P	deceased (1/2010)	yes	NO	NO	III	17 months
2066	2009	abdominal mass/iliac lymph nodes	IV	Remission	R	remission	yes	yes	yes	IV	
936	2007	abdominal relapse	IV	Stability (then stopped)	S	deceased (2008)	yes	yes	yes	I	12 months
6536	2015	after surgery	I	Complete remission	R	Absence of disease	yes	yes	yes	I	72 months
6640	2015	lung metastases	IV	absence of disease	R	Absence of disease	yes	yes	yes	II	
9846	2018	after surgery	II B	absence of disease	R	absence of disease	yes	yes	not assessed	II B	
10413	2019	after surgery	I	absence of disease	R	Absence of disease	yes	not assessed	not assessed	I	
10420	2019	after surgery	IV	progressing	P	progressing	yes	not assessed	not assessed	IV	
10457	2019	6 months after surgery	IV	partial remission	R	reduction of nodules	yes	not assessed	not assessed	IV	

10585	2019	after surgery	I	absence of disease	R	Absence of disease	yes	yes	not assessed	I	
10853	2019	after surgery	I	absence of disease	R	Absence of disease	yes	not assessed	not assessed	I	
11115	2020	absence of disease	I	absence of disease	R	Absence of disease	yes	yes	yes	I	
3674	2011	after surgery	III	absence of disease	R	Absence of disease	yes	yes	yes	III	
3694	2011	lymphadenopathies in various body districts	IV	Progression	P	deceased (2013)	yes	yes	NO	IV	
4345	2012	after several relapses	IV	Progression	P	progression, incl. the liver	yes	yes	yes	I	
9093	2017	relapse of cranial Ewing's sarcoma	IV	Progression	P	ask for information	yes	yes	yes	I	
9135	2017	after surgery	I	Stable (thymic residue)	S	Stability	yes	yes	not assessed	I	
9671	2018	leiomyosarcoma, pelvic relapse	IV	Stable	S	Stability	yes	yes	yes	IV	

DISCUSSION

The aetiology of sarcomas remains largely unknown; they are sporadic, idiopathic and related to genetic defects and environmental factors.^[6,5] Genetic defects leading to sarcoma development are divided into simple karyotypic defects and complex karyotypic defects.^[7] Simple karyotypic defects consist of disease-specific chromosomal translocations that lead to abnormal gene (and protein) function, which facilitates the development of sarcoma. Sarcomas associated with simple karyotypic defects include Ewing's sarcoma, alveolar rhabdomyosarcoma and synovial sarcoma. On the contrary, complex karyotypic defects, such as complex chromosomal rearrangements, intervene on cell cycle genes causing genetic instability. Sarcomas generated in this way tend to manifest in older patients and have a high frequency of mutations in the signalling pathways of p53 and retinoblastoma.^[7,8] Leiomyosarcoma, liposarcoma, angiosarcoma and osteosarcoma are examples of such cancers.^[7]

There is no evidence regarding the effectiveness of a sarcoma screening programme because of the different manner in which they present, their ubiquitous diffusion in the different anatomical areas, and the lack of an effective mass diagnostic test.^[5] A radiologic and pathological diagnosis is recommended, while staging is through cytological examination, cutting needle biopsy, incisional biopsy or excisional biopsy. The classification by stage allows a prognostic evaluation.^[5]

Survival at 5 years for stage I is approximately 90%, stage II 70%, stage III 50%, and stage IV 10%.^[5]

Sarcomas with complex karyotypic defects may be secondary to radiotherapy.^[6,9,10,11,12] A study in patients diagnosed with radiotherapy-induced sarcoma showed that they are unique in their epidemiology and tumour characteristics. They have an unfavourable prognosis and need new innovative strategies, because with conventional oncology the survival rate at 5 years is only 32%.^[13] It is not by chance that a significant percentage of aggressive bone sarcomas occurs in children and young people of prepubertal and pubertal age, because in this population the positive GH peak and negative Melatonin peak coincide. Cells in the bone growth zones have the highest expression of GH receptor.^[14] An increase in the incidence of sarcomas is also documented in taller than average subjects.^[4] There is clear and increasing confirmation of the primary role of GH increase in osteosarcomas, including the statistical study by Lisa Mirabello *et al.*, which documented that, compared to subjects with average

birth weight (2,665-4,045 g), individuals with high birth weight ($\geq 4,046$ g) had an increased risk of osteosarcoma (OR 1.35, 95% CI 1.01-1.79). Taller-than-average individuals (51-89th percentile) and very tall individuals ($\geq 90^{\text{th}}$ percentile) had an increased risk of osteosarcoma (OR 1.35, 95% CI 1.18-1.54 and OR 2.60, 95% CI 2.19-3.07, respectively; $P_{\text{trend}} < 0.0001$).^[4,15]

The significant decrease in melatonin after 3 to 5 years of age, with particularly low levels in the age groups most affected by osteosarcomas, coinciding with a GH increase peak, is widely documented.^[16,17,18]

In various sarcomas, the increasing percentage of tumour stem cells compared with these tumours' different neoplastic phenotypes is most likely the main reason these tumours rapidly acquire resistance to chemo-radiotherapy, become very aggressive and progress rapidly.

Uncontrolled proliferation phenomena and loss of differentiation, although to varying degrees, are common denominators to all cancers.

Protein synthesis and cellular proliferation (normal and neoplastic) are closely dependent on the interaction of Prolactin with the greatest growth inducer, GH^[19;20;21;22;23], and on mitogenic molecules, GH-dependent growth factors that are positively regulated by it, such as EGF, FGF, HGF, IGF1, VEGF, PDGF^[24,25,26,27,28], as well as gastrointestinal growth factors such as VIP, CCK, G.^[29]

The GH and PRL receptors are co-expressed on cell membranes and dimerise, amplifying the transduction of proliferative signalling pathways.^[30] Numerous studies indicate how these pituitary hormones play a crucial role in the development and progression of human tumours. Their receptor expression is ubiquitous^[19,31,32,33] and particularly high in cancerous tissue, with a dose-dependent relationship between GH-PRL receptor expression and tumour induction and progression processes, detected histochemically and through immunohistochemistry techniques, Western Blot, in situ hybridisation and qPCR techniques. The documentation of much higher GHR concentrations in tumour tissues compared to normal and peritumoural tissues confirms its powerful mitogenic role.^[15,21,20,34,35]

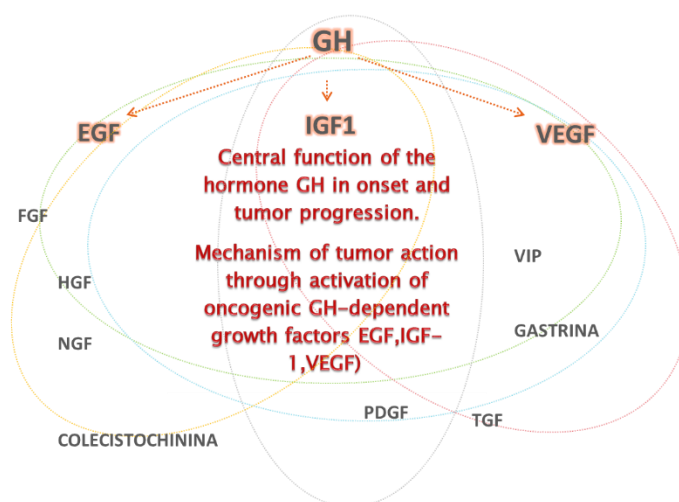


Fig. 1: Central function of GH.

This evidence explains the reason why the DBM uses somatostatin, analogues and prolactin inhibitors to prevent proliferation and the remaining components of the Method to achieve a differentiating, immunomodulating and anti-oxidising function. The DBM supports and enhances vital reactions and anticancer homeostasis, helping them counteract the onset of neoplasia and its progression.^[36] The DBM pursues this objective through innovative formulations and criteria for the use of MLT (complexed with adenosine and glycine), of retinoids solubilised in Vitamin E, as well as Vitamins C, D3, and ECM components. Inserting apolar components such as Beta-carotene and Vitamin E between the phospholipids of a cell membrane stabilises it, preserving it from oxidative damage and free radicals.^[37,38,39,40,41,42,43,44]

Sarcomas, therefore, represent a large group of heterogeneous malignant diseases, with a single common characteristic, i.e. mesenchymal origin. The heterogeneity of sarcomas is manifested in tumour mass but also intratumorally, which implies the existence of sarcoma stem cells that may derive from mesenchymal stem cells. Mesenchymal stem cells have, in fact, been used to establish several crucial experimental models of sarcoma and to trace the respective stem cell populations. Mesenchymal stem cells are heterogeneous and may develop differently. The different origin of cells determines substantial heterogeneity in the possible initiation of sarcoma. Genetic and epigenetic changes associated with sarcomagenesis can produce sarcoma stem cells. In the case of paediatric sarcomas characterised by discrete reciprocal translocations and essentially stable karyotypes, the oncogenes activated by translocation could be crucial factors that confer stemness, mainly modifying the transcriptome and interfering with normal epigenetic regulation. The most

studied examples of this process are myxoid/round cell liposarcoma, Ewing's sarcoma and synovial sarcoma. In adult sarcomas, which typically have complex and unstable karyotypes, stemness could be defined more operationally, as a reflection of the actual assembly of stem factors genetically and epigenetically conditioned and/or induced by the microenvironment. The molecular mechanisms of stemness could be significantly similar in different types of sarcoma, such as the silencing of the pRb and p53 oncosuppressors, the activation of Sox-2 or the inhibition of Wnt/ β -catenin canonical signalling. In addition, there is a homology with markers of stem cells of various carcinomas or leukaemias. Understanding the biology of sarcoma stem cells can improve therapy.^[45] Consistently with this strategy, without observing toxicity at the administered doses, the doses of active DBM components such as MLT, ATRA, vitamins D and C, Glucosamine, Somatostatin and Octreotide analogue have been increased and molecules active on stem cells have been added, such as Disulfiram and Acetazolamide, which will be rationally contextualised below.

In this study, 37 cases of sarcoma (of bone and soft tissue) treated with the Di Bella Method (DBM) were analysed. The treatment significantly improved the survival and quality of life in sarcoma patients compared to patients with sarcomas bearing similar immunohistochemical, histologic and clinical characteristics (oncological statistics).

The multitherapy includes, to prevent proliferation, Somatostatin and the Octreotide analogue, with D2R agonist prolactin inhibitors, whereas ATRA, Beta-Carotene, Axerophthol, solubilised in vitamin E, in addition to vitamins D and C, water-soluble Melatonin, Glucosamine and Chondroitin Sulphate were included for their differentiating, cytostatic, immunomodulating and antimetastatic action. Hydroxyurea was used at low metronomic doses to induce apoptosis.

MELATONIN

Melatonin (N-acetyl-5-methoxytryptamine, MLT) has antioxidant, anti-aging and immunomodulatory properties. It plays a significant role in blood composition, medullary dynamics, in platelet formation, in the protection of vascular endothelium, in platelet aggregation, in the regulation of leukocyte ratios and haemoglobin synthesis, in perfusion and blood-tissue exchanges. The considerable and non-toxic apoptotic, oncostatic, anti-angiogenic, differentiating and antiproliferative properties of this indole on all neoplastic diseases, both solid and liquid, are also documented.^[40,46,47,48,49,50,51,52,53,54,55,56,57,58,59,60,61,62,63,64,65,66,67,68]

Thanks to its remarkable functional versatility, MLT can have a direct and indirect anti-tumour effect in a factorial synergism with other differentiating/antiproliferative/immunomodulating molecules of the DBM. The interaction of MLT with the DBM molecules combats the multiple processes that characterise the neoplastic phenotype, mutation, proliferation, progression and/or dissemination. All these characteristics suggest the use of this molecule in cancer pathologies.^[69,70,71,72] MLT also plays a significant role in perfusion and blood-tissue exchanges, preventing tissue ischaemia, acidosis and hypoxia of the neoplastic environment and the consequent overexpression of oncogenic genes, including HIF-1 α . The anti-cellular expansion effect is also achieved through the reduction of intracellular reactive oxygen species and the increase of antioxidants.^[46]

Because there is now evidence of the correlation between the decline in melatonin levels, the increase in GH and the incidence of juvenile osteosarcomas, melatonin has been studied for its anti-osteosarcoma action, as a coadjuvant to conventional chemotherapy for osteosarcoma to improve the prognosis of the disease, which in most cases is poor.^[73] In addition to the multiple and above-mentioned anticancer mechanisms of action of MLT, some of its particular properties in osteosarcoma are emerging. This cancer occurs most frequently in adolescents, with peak incidence between 11 and 15 years. The decline of MLT to minimal levels in the same age groups, coupled with an increase in the level of GH (and related factors such as IGF1, VEGF, EGF, FGF) and subsequent bone growth, explains the osteosarcoma peak in these age groups. The most frequent initial site of sarcomas is the metaphysis of long bones (distal femur and proximal tibia) whose cells have the highest expression of GH receptors and therefore a high proliferative index. The relationship between the incidence of osteosarcoma and the bone growth rate^[74] is evident. The rationale for using melatonin and somatostatin against sarcomas is therefore logical and scientifically documented.

Melatonin blocks the proliferation of osteosarcoma MG-63 cells by reducing the D1 and B1 cyclins, CDK4 and CDK1, blocking the cell cycle in the interphase, with an increase in the cells in the G0/G1 phase. In carcinosarcoma cells, MLT administration resulted in a reduction in Bcl-2 expression and a decrease in tumour volume.^[75] In Ewing's sarcoma, melatonin induces cell death in SK-N-MC cells by increasing the expression of the Fas receptor and the respective FasL ligand through the caspase 8 pathway. Fas/FasL expression is controlled by the activation of the NF-kB nuclear factor, increased by melatonin, in relation to the

intracellular redox state.^[76] In leiomyosarcomas, MLT inhibits tumour growth by blocking the absorption and metabolism of linoleic acid, which would lead to the production and release of the 13-HODE mitogen, ERK1/2, MEK and Akt, as well as the suppression of cAMP production in tumours.^[77]

The differentiation of mesenchymal stem cells is regulated by the action of mechanical and molecular signals coming from the extracellular environment. Melatonin can also be an important regulator of the commitment and differentiation of cell precursors. Adipogenesis and osteogenesis are known to be reciprocally related in the bone marrow. On human mesenchymal stem cells, melatonin directly inhibits adipogenic differentiation towards the adipocyte lineage and simultaneously promotes osteogenic differentiation by suppressing the expression of the γ receptor (PPAR γ) activated by the peroxisome proliferator and improving the Runt-related transcription factor 2 (RUNX2). MLT improves differentiation of human mesenchymal stem cells into osteoblasts via MT2 receptors and the mitogenic/kinase signalling cascade regulated by the extracellular (MEK)/kinase signal regulated by the extracellular signal (ERK).^[18]

Melatonin, with its immunomodulating, myeloprotective, differentiating and antioxidant properties, increases the anticancer effects of chemotherapy while reducing its toxicity. Through the radioprotective and radiosensitising effect, it improves the therapeutic response to radiation therapy by reducing radiotoxicity and therapeutic failures due largely to the refractoriness of mesenchymal tumour cells of sarcoma to cytolytic and radiation therapies.

A significant therapeutic property of melatonin is the differentiation and reprogramming of mesenchymal stem cells, multipotent progenitors, osteocytes, chondrocytes, myocytes and adipocytes.

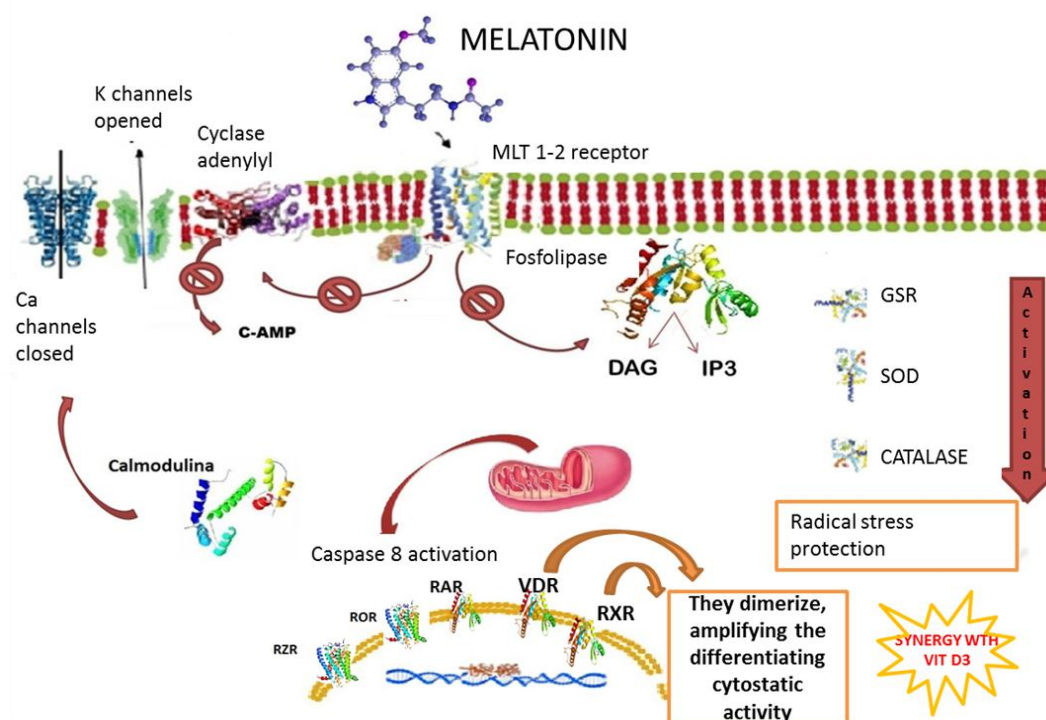


Fig. 2: Role of Melatonin.

RETINOID SOLUTION IN VITAMIN E

Retinoids are a family of molecules that derive from the metabolism of vitamin A, or retinol.^[78] Their function is mediated by the respective receptors on the plasma membrane and nuclear membrane which act on cell growth, differentiation, tissue homeostasis and apoptosis via the caspase 9 pathway, with a major, documented effect in the prevention and treatment of tumours.^[75] Retinoids are the most powerful non-hormonal activators of solely orderly, functional growth aimed at optimal biological balance, while, at the same time, with differential toxicity, they inhibit aimless and disorderly neoplastic growth with their antiproliferative and cytostatic effect. Together with Melatonin, retinoids are the only biological molecules with differential toxicity, with a cytostatic and apoptotic effect on cancer cells alone and not on healthy cells in which, on the contrary, survival and functionality are increased. Retinoids can preserve and enhance the vitality and efficiency of normal cells while at the same time inhibiting neoplastic ones, which have a tendency to mutate. Retinoids intervene in two critical aspects of neoplastic biology: fast proliferation and the resulting mutations, common denominators of all cancer types. They play a crucial role both in the prevention and therapy of cancer, limiting the consequences induced by cancer and conventional anticancer therapies.^[23,79,80,81,82,83,84,85,86,87,88,89,90,91,92,93,94,95]

FUNCTIONS OF THE COMPONENTS OF THE RETINOID SOLUTION

1) BETA-CAROTENE

Type of effect

- Protective on cell membranes^[36];
- Directly antiproliferative (regardless of conversion to ATRA) on cancer cells, suppressing their mobility (as measured by tetrazolium “MTT” assay), DNA synthesis (controlled through the uptake of 3H-thymidine) and proliferation (measured by cell count).

2) RETINOIC ACID

- Works by inducing redifferentiation in blasts and tumour cells^[96];
- Suppresses the transcription of oncogenic factor genes and promotes the antiproliferative effect^[97];
- Has anti-angiogenic action^[98];
- Decreases the potential for neoplastic proliferation and plays an important role in cell differentiation, apoptosis and adhesion^[99,100];
- Makes the neoplastic cells particularly sensitive to chemotherapeutic agents, also inducing an increase in intercellular communication in the junction spaces^[101];
- Counteracts the hepatotoxic effect of chemotherapy.^[102]

3) VITAMIN A

- Causes the apoptosis of neoplastic cells through the activation of proteolytic cell enzymes, the Caspases, and the degradation of the general transcription factor Sp-1.^[103,104]

4) VITAMIN E

Among the tocopherols, Alpha-Tocopherol has the highest biological activity. Commonly called vitamin E, it has a high antioxidant and anti-free radical activity;

As a constituent of enzyme systems, vitamin E directly affects a key step in energy exchange and life itself, the transport of electrons in the respiratory chain;

- It inhibits the growth of various tumour cell lines^[105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130],
- It enhances the anticancer action of various chemotherapy drugs such as Adriamycin, cisplatin and tamoxifen^[116, 120];
- It protects bone marrow cells from the lethal effects of doxorubicin^[107];

- It enhances the anticancer effect of chemotherapeutic agents, protecting healthy cells from toxic effects^[117];
- It has anti-angiogenic activity.^[37,109,112,113,123]

It has been observed that, in people with sarcoma, the number of granulocytic MDSC cells is higher than in healthy conditions, resulting in reduced T-cell efficiency. Retinoic acid has the ability to regulate the differentiation of MDSC cells and, in combination with GD2-CAR T cells, has greater antitumour power and better combats the immunosuppressive action associated with MDSC cells in sarcoma.^[131]

When human osteosarcoma cells are treated with ATRA, the expression of vitamin D3 receptors has been observed to be greater; in addition, synergistic treatment with ATRA and calcitriol is associated with an increase in the efficacy of the antiproliferative effect on these cell lines.^[132]

Retinoic acid acts during the initial stages of osteosarcoma associated with macrophages, inhibiting the polarisation of M2 TAMs and the activity of neoplastic cells.^[133] It can stimulate cell differentiation in osteosarcoma cells and thus negatively act on cell proliferation and induce apoptosis; these properties have been observed not only in cases of osteosarcoma, but also in Kaposi's sarcoma, neuroblastoma and breast cancer.^[134] Studies on Kaposi's sarcoma cell lines treated with retinoic acid have shown that it acts by negatively regulating cell growth, reducing IL-6 and TNF- α levels and increasing the expression of the RAR α receptor for retinoic acid; increased cellular adhesion^[135] has also been observed.

Treatment with retinoic acid, which acts through the increased release of lysosomal enzymes, may be a potentially effective therapy against chondrosarcoma.^[136] In dermatofibrosarcoma protuberans, the RAR β retinoic acid receptor is implicated in the reduced aggressiveness of neoplastic growth, in relation to the COX-2 cyclooxygenase, on which it has a negative effect.^[137]

VITAMIN E

Vitamin E (Tocopherol) is fat soluble and present in eight isoforms, known for their action against the formation of reactive oxygen and nitrogen species in neoplastic tissues following alterations in the oxidative systems. Vitamin E also performs important inhibitory functions

in angiogenesis, in the NF- κ B signalling pathway and on the HMG CoA reductase enzyme.^[138]

In several tumours, including sarcoma, thymic involution has been observed, leading to the death of thymocytes and thus a decrease in T-cell production, resulting in decreased immune system efficiency.^[139]

In a preclinical study in mice with fibrosarcoma, the mechanism of action of vitamin E was evaluated in combination with cyclophosphamide, an alkylating agent used in various anticancer therapies, both alone and in combination with other cytotoxic drugs. However, it may cause hyperlipidaemia in patients, and so was administered in combination with α -tocopherol that reduces hyperlipidaemia. The results showed that lipid metabolism returned to normal levels in treated mice and hyperlipidaemia was significantly reduced compared to the values observed in the untreated control groups.^[140]

Vitamin E also reduces damage from oxidative stress, regulates osteocartilaginous homeostasis and promotes tumour apoptosis both in vitro and in vivo.^[141]

VITAMIN D3

Vitamin D3 is synthesised in the skin, starting from vitamin D, following exposure to ultraviolet rays. It can be introduced through dietary intake; low concentrations of vitamin D3 are associated with increased sensitivity to infections and tumours. The active biological form of vitamin D3, i.e. calcitriol, and its analogues perform important anticancer functions mediated by the nuclear receptor, VDR.^[142]

In Kaposi's sarcoma associated with Herpes virus, calcitriol was observed, both in vitro and in vivo, to negatively regulate the growth of endothelial cells that express the vGPCR receptor, by lowering cyclin D1 and the activation of p27 and p21. Calcitriol lowers vGPCR-induced NF- κ B action and induces cell death through increased VDR expression. It also reduces Akt and ERK 1/2, which are important in the carcinogenesis process, and acts in the angiogenic process through the dose-dependent down regulation of VEGF and HIF-1 α factors.^[143] Treatment of Kaposi's sarcoma with calcitriol also results in decreased production of IL-6 and IL-8, two important growth factors in this cancer.^[144]

A study conducted on seven human osteosarcoma cell lines showed that therapy with ATRA and calcitriol inhibits cell proliferation and increases sensitivity to inducers of differentiation

with greater efficacy compared to when the molecules are used alone. Additionally, ATRA indirectly increases the expression of VDR, at mRNA and protein level, in cells with high endogenous levels of RAR α and low endogenous levels of VDR.^[132]

By analysing the human osteosarcoma MG-63 cell line in vitro, a decrease in cell growth, saturation density and [3H]-thymidine uptake was observed following the administration of calcitriol; moreover, an increase in cell adhesion was observed in treated cells following an increase in fibronectin.^[145]

Metabolites of vitamin D3 also act on cell differentiation in osteosarcoma cells at the same doses at which they inhibit growth, as well as inducing osteocalcin synthesis.^[146] The antiproliferative effect of calcitriol has been studied on several soft tissue sarcoma cell lines, including rhabdomyosarcoma, fibrosarcoma, liposarcoma, leiomyosarcoma and synovial sarcoma, in relation to VDR expression. Cell growth following calcitriol treatment is inhibited more effectively in cells with a higher amount of VDR and vice versa. This study showed the correlation between VDR expression and the effect of calcitriol on soft tissue sarcomas.^[147]

FUNCTIONS OF VITAMIN D3

- Pro-differentiation activity, achieved not only through interaction with the receptor, but also through an extra-receptorial membrane-mediated mechanism^[148];
- Inhibition of angiogenesis, development and growth induced by VEGF (vascular endothelial growth factor), in a dose-dependent manner; inhibition of the formation of elongated endothelial cell networks in 3D collagen gels, promoting apoptosis^[149];
- Vitamin D3 activates a specific nuclear receptor, inhibiting proliferation, promoting differentiation of various types of tumour cells and adhesion of cells migrating from the basal membrane, due to the downregulation of alpha-6 and beta-4 integrins, laminin receptors associated with the greatest cellular migration and invasiveness of prostatic cancer cells in vivo^[150];
- Induction of the expression of mRNA for the BRCA1 protein, and transcriptional activation by the BRCA1 promoter. The sensitivity to the antiproliferative effects of Vitamin D3 is intimately linked to the ability to modulate the BRCA1 protein by means of transcriptional activation of the factors induced by VDR^[151];

- The activation of VDR, in addition to the antiproliferative effect, increases the expression of the protein-binding insulin-like growth factor, IGF^[152];
- It induces the phenotypic maturation of tumour cells into functionally mature, differentiated, physiologically normal cells, simultaneously inhibiting neoplastic cell proliferation by enhancing the antiproliferative effect of Trans-retinoic acid^[153];
- It inhibits the invasion of the extracellular matrix and metastases by blocking the degradation of the extracellular matrix barriers (ECM) by tumour cells through collagenolysis^[154];
- It exerts, also through non-receptor mechanisms, a powerful antiproliferative and pro-differentiating action^[155];
- It suppresses proliferation and migration in glioma cell lines expressing the human vitamin D receptor.^[158]

VITAMIN C

Ascorbic acid, or Vitamin C, has a great reducing activity, reacting directly with oxygen singlets, hydroxides and superoxide radicals^[159]; biologically, it acts as a hydrogen carrier in intermediary metabolism, including cellular respiration processes. Due to its key role, it was included in anticancer therapy.^[160,161,162,163] The antioxidant capacity and the natural role in immunity of Ascorbic acid are very relevant, in addition to its remarkable biological activity on cellular trophism and support structures.^[164,165] It is easy to guess that Vitamin C, jointly with melatonin, concurs to regulate these exchanges, leading to optimal function of the epithelium in terms of resistance and permeability to the transit of cancer cells, and therefore of metastasis.

The functions of Vitamin C in the DBM are various. Among them, the most relevant are:

- To prevent cellular damage induced by oxidative molecules, including free radicals^[166];
- It can have a preventive and therapeutic role in cancer^[167];
- It can inhibit the carcinogenic effects of mutagenic molecules^[168,169];
- It can preserve the integrity of connective tissue in terms of antiblastic function^[167];
- It can exert angiostatic activity on endothelial cell proliferation^[170];
- It can exert an antineoplastic activity through various mechanisms of action.^[33,171]
- It can have an antimetastatic activity through collagen synthesis^[172,173], the inhibition of hyaluronidase^[174] and by decreasing the permeability of endothelial cells to neoplastic cell populations^[175];

- It can reduce the toxicity of chemotherapeutic agents such as Adriamycin^[176,177];
- It reduces the risk of glioma^[178];
- The use of intravenous Vitamin C is a safe support intervention, decreasing inflammation and symptoms related to deficiency in antioxidants and the collateral effects of standard antitumoural treatments^[179,180];
- It can induce the degradation of the hypoxic inducible factor HIF-1, essential for tumour cell survival in hypoxic conditions.^[165]

Vitamin C, or ascorbic acid, is a water-soluble antioxidant that reacts with superoxide, hydroxyl radicals and oxygen singlets; in laboratory studies, ascorbic acid has been observed to protect plasma lipids and low-density lipoproteins (LDL) from peroxidative damage and degeneration associated with aging and diseases such as cancer. It is very important for the synthesis of glycosaminoglycan, by proteoglycans, and for the synthesis of collagen.

Ascorbic acid acts on various cancers in humans; it captures free radicals and reactive oxygen species and reduces nitrite, preventing oxidation effects and damage to DNA and cell membranes caused by free radicals associated with the tumour.

Studies have shown that, together with vitamin C, several other dietary nutrients, such as vitamin E, carotenoids and folic acid, participate in protective processes.^[159]

Vitamin C, in mice treated with MCA, significantly reduced the onset of sarcoma compared to the control groups, demonstrating the prophylactic effect of this component^[181]; moreover, at supraphysiological concentrations, it inhibits cell proliferation and promotes the differentiation of osteoblasts.^[182]

Cases of regression of sarcomas have been observed following continuous treatment with high doses of vitamin C^[183], and so has the inhibition of the angiogenesis process.^[184]

At physiological doses, ascorbic acid negatively regulates the migration of carcinosarcoma cells, thus inhibiting tumour metastasis.^[185]

SOMATOSTATIN AND SOMATOSTATIN ANALOGUES

Somatostatin is a 14-amino-acid peptide, which has documented, evident and generalised anticancer properties, as already stated in the publication by the Nobel Prize winner, Schally, in 1998, “Impressive antineoplastic activity of somatostatin analogues has been demonstrated

in many tumour models". Professor Di Bella had already published in 1979^[186] a study on the anticancer effects of somatostatin in synergy with other components of his method, such as a solution of retinoids in vitamin E, prolactin inhibitors and melatonin. In 1981^[187] he presented a report on more than a thousand cases of neoplasia favourably treated with somatostatin.

Regardless of the presence of SSSTR receptors in tumour cells, SST's mechanisms of action mean it acts directly through inhibition of cell growth, inducing apoptosis and preventing metastasis; indirectly, it acts by suppressing the production of growth and angiogenesis factors.^[188]

The use of somatostatin and analogues, by negatively regulating GH and GH-dependent growth factors, is a rational indication for its use in any cancer.^[15,21,62,90,186,189,190,191,192,193] It is evident that the PRL/GH/GF axis has a decisive influence on neoplastic development, hence the rationale for the synergistic use against cancer of anti-prolactin D2R agonists with biological antagonists of GH, such as Somatostatin and analogues, which extend their negative regulation to highly mitogenic GH-related growth factors, such as IGF1 - 2^[189,194], EGF^[195,196], FGF^[197], VEGF^[170,198,199], PDGF^[200] and the related signalling pathways, resulting in anti-proliferative, pro-apoptotic, differentiating and anti-angiogenic effects.^[196] This vision is slowly emerging through more and more basic research, although still rarely applied to humans. In many tumours, not only in neuroendocrine ones, the expression of a somatostatin receptor has been documented.^[21,75,196,200,201,202,203,204,205,206,207,208,209,210,211,212,213,214,215,216,217,218,219,220,221,222,223,224,225,226] Although absent in the cell membranes of some tumors, SST receptors are almost always expressed in the peritumoural vessels.

GFRs mitogenically respond to IGF, and the suppressive effect of SST and analogues on serum IGF1 levels is direct (by inhibiting the IGF gene) and indirect (by suppressing the GH and therefore its induction of IGF1 in the liver). The antiproliferative effect of somatostatin and its analogues in sarcomas, as in other malignancies, is therefore also achieved through mechanisms involving the suppression of the IGF system.^[227] Regression and long-term survival with somatostatin of a patient with primary gliosarcoma, a rare malignancy with short-term negative prognosis, confirms the efficacy and indication of Somatostatin.^[19,20,21,22,23,228]

Mitogenic GH molecules such as EGF, FGF (whose receptors are co-expressed on cell membranes), IGF, VEGF^[24,25,26,27,28] and growth factors produced by the gastrointestinal system (VIP, CCK, G)^[29], are all negatively regulated by Somatostatin and Octreotide. The mitogenic effects of GH on somatic cells are triggered by the signal transduction of several pathways including JAK-2/STAT, MAPK, PIK3. This evidence validates the rationality of using, in oncotherapy, somatostatin/octreotide that act independently of the presence of SSTR in neoplastic cells, given the evidence about them in the peritumoural vessels and the aforementioned indirect anticancer mechanisms.

Somatostatin receptors have been identified on cells of bone and vascular/perivascular tumours, indicating that these neoplasms are the target for SST therapy.^[229] In preclinical studies, somatostatin has been shown to inhibit the growth of Kaposi's sarcoma cells with SST receptors. In an *in vivo* model, somatostatin effectively inhibits angiogenesis, while *in vitro* it acts on both endothelial cells and monocytes, inhibiting growth and invasion. Its administration in these neoplasm, should be maintained over time as SST acts primarily as an angiostatic.^[198]

Suppression induced by somatostatin and its analogues on corticotropin levels is important as well, because chondrosarcoma cells are sensitive to these molecules.

In studies on human and rodent cancer xenografts, the TT-232 somatostatin analogue leads to a decrease in tumour growth, although this is dependent on the sensitivity of the tumour to somatostatin, with S-180 osteosarcoma growth slowing by 67-100%.^[230] Octreotide is an analogue of somatostatin with a longer half-life than the native peptide. *In vitro* and *in vivo* it inhibits the growth of cells expressing the receptors with high affinity for somatostatin, but also acts indirectly by reducing the concentration of GH and IGF-1.^[231] It has also been observed that octreotide has a strong VEGF-suppressing effect, resulting in an anti-angiogenic effect.^[232]

In a study in rats, following partial removal of the liver, the effect of octreotide on tumour growth in regenerating hepatic cells was studied and it was observed that, following the drug's administration, the proliferation of fibrosarcoma and colon adenocarcinoma cells was inhibited.^[233]

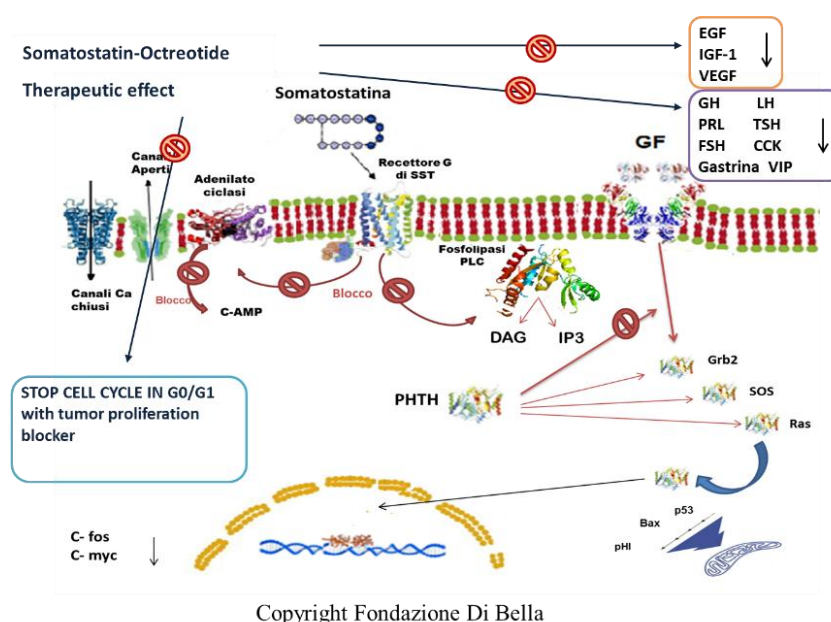


Fig. 3: Role of Somatostatin.

CHONDROITIN SULFATE

The proteoglycans of chondroitin sulphate participate in the modulation of various cellular functions, including adhesion and migration. The role of the chondroitin sulphate chain in adhesion, chemotaxis and migration has been studied in fibrosarcoma cells. The cleavage of CS chains associated with cells and the specific inhibition of endogenous CS production severely compromised these fibrosarcoma cellular functions. This result shows that the reduction of chondroitin sulphate proteoglycans, e.g. via the cleavage of CS chains, inhibits cell motility, migration and adhesion in fibrosarcoma. CS chains increase cell motility through the MAP kinase pathway.^[234]

In oestrogen-mediated bone anabolism, chondroitin sulphate-E plays an essential role by increasing the differentiation and maturation of osteoblasts. The control of chondroitin sulphate-E expression in bone metabolism therefore has an important therapeutic potential to improve the loss of bone mineralisation.^[235]

In patients recovered from osteosarcoma, the risk of developing osteoporosis is higher, particularly if there are three additional risk factors: being male, having a diagnosis of osteosarcoma at a young age and having a low amount of lean mass.^[236] Other elements that may determine the onset of osteoporosis following cancer are chemotherapy, radiotherapy and hormonal decompensation, which cause a loss of bone mineral density. In addition,

patients with osteosarcoma may have reduced physical activity during therapy or after surgery and this results in a decrease in muscle mass, vitamin D and bone mineral density.^[237]

Osteoporosis associated with diabetes is a bone disease, which can impair bone microstructure, reduce resistance and increase bone fragility and the risk of fracture. This disorder is associated with oxidative stress (which causes a decrease in the proliferation and differentiation of osteoblasts) and with the inflammatory process: in people with diabetes, the level of pro-inflammatory cytokines is considerably higher than in healthy people, resulting in increased oxidative stress, the proliferation of osteoclasts and the absorption of the bone matrix, causing osteoporosis. In addition, a high expression of biochemical markers of bone formation and resorption has been observed in mice with type 1 diabetes, indicating that the bone element turnover is much greater in diabetes cases. The study of chondroitin sulphate therapy in mice with type 1 diabetes had positive effects in the treatment against osteoporosis, leading to the reduction of cytokines and inflammation, to the inhibition of oxidative stress and bone metabolism while enhancing the repair of bone microstructures. This therapy is also very safe, with adverse effects limited to modest and transient gastrointestinal disorders such as nausea and vomiting.^[238]

GLUCOSAMINE SULPHATE

The anticancer properties of glucosamine are documented by scientific evidence that has identified the anticancer mechanism of action in the inhibition of cancer stem cells (CSC), which are the subpopulation of tumour cells responsible for maintaining the tumour and for relapse, due to their very high ability to resist various anticancer treatments.^[239,240] Glucosamine induces autophagic cell death through stress stimulation of the endoplasmic reticulum (ER) in human glioma cells. ER stress induced by glucosamine was manifested by the induction of the expression of BiP, IRE1alpha and phospho-eIF2alpha. Glucosamine treatment reduced cell viability by increasing the autophagic cell death of glioma cells. This information provides new insights into the potential anticancer properties of glucosamine.^[241]

According to some studies, glucosamine suppressed the proliferation of the DU145 human prostate cancer cell line by inhibiting STAT3 signalling. In DU145 cells, glucosamine reduced the N-glycosylation of gp130, decreased the binding of IL-6 to cells and altered the phosphorylation of JAK2, SHP2 and STAT3. The glucosamine-mediated inhibition of N-glycosylation was neither specific for proteins nor cells. The sensitivity of DU145, A2058 and PC-3 cells to glucosamine-induced inhibition of N-glycosylation is directly correlated

with glucosamine cytotoxicity in these cells. Global inhibition of glucosamine-induced N-protein glycosylation could therefore be the mechanism underlying its multiple biochemical and cellular effects.^[242]

Glucosamine may also be a promising candidate for the prevention and/or treatment of some other diseases due to its antioxidant and anti-inflammatory activities. Most of its function is exerted through the modulation of inflammatory responses, especially through the nuclear factor κ B (NF- κ B) which can control the production of inflammatory cytokines and cell survival.^[243]

The conjugation of D-glucosamine with the lipophilic fraction can facilitate its application in the superficial modification of liposomes.^[244]

Pohlig F. carried out a study in which glucosamine sulphate was found to have a pronounced suppressive effect, in particular on MMP-3 and also on the levels of MMP-9 mRNA and proteins in the osteosarcoma cell lines in vitro.^[245,246,247]

Below is a wider literature review on the differentiating, cytostatic, immunomodulatory, homeostatic properties of Retinoids, vitamins E, C, D, Proteoglycans:

- Di Bella G et al.,^{2015[75]}
- Di Bella G., 2010
- “The Di Bella Method” Mattioli ED., 2005^[40]
- “La scelta antitumore” Macro-Uno Edizioni, 2019.^[47]

BROMOCRIPTINE and CABERGOLINE

Bromocriptine and cabergoline are two alkaloids derived from the ergot fungus; they are used in preventive and anticancer therapy because they suppress prolactin synthesis, reduce cell growth and tumour size and inhibit angiogenesis. Numerous studies have shown the effectiveness of anticancer therapies with bromocriptine, suggesting that the administration of high doses leads to a reduction in cancer^[248] and also plays an important role in chemotherapy and in the phenomenon of multidrug resistance, in which cancer cells develop resistance to several drugs with cytotoxic action. Studies have shown that it can convert a tumour's pharmacological resistance to a normal condition, restoring the anti-tumour effect of drugs such as doxorubicin.^[249]

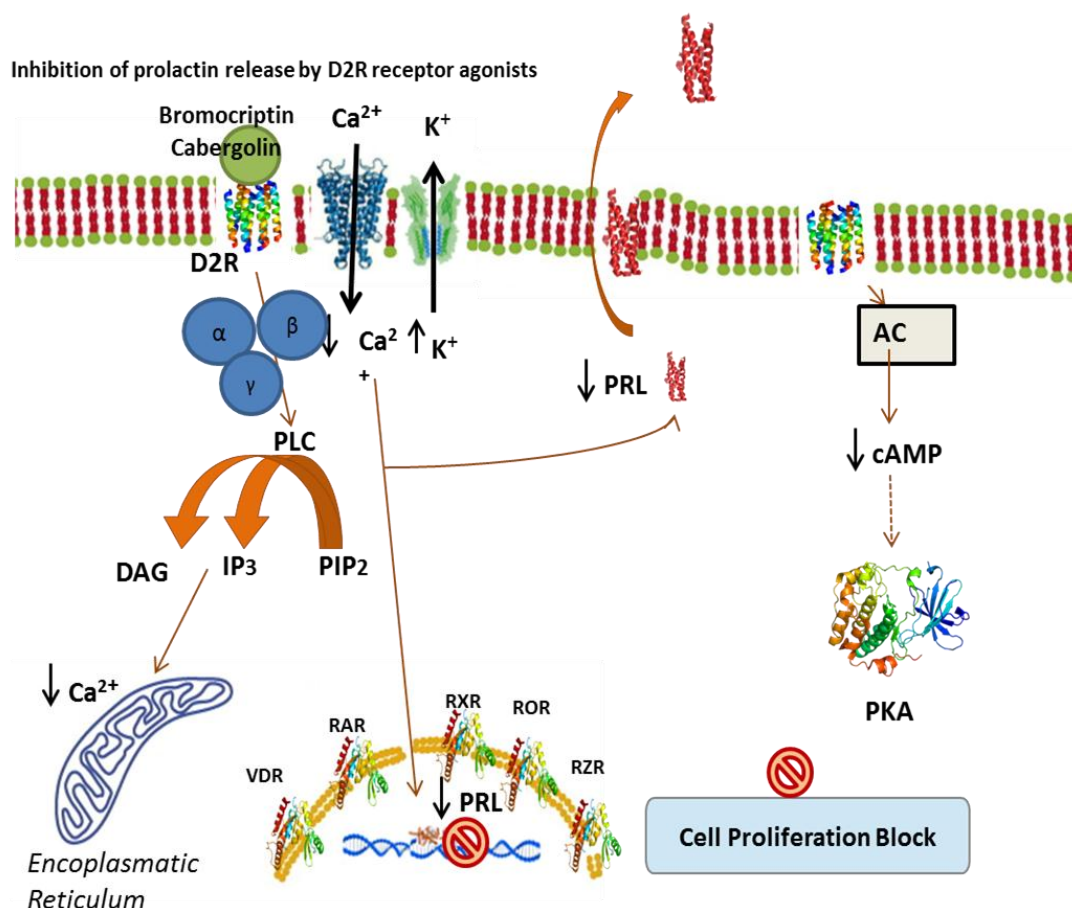
Cabergoline also has an antitumor effect by suppressing growth factors that promote tumour growth and expansion.^[250]

In one clinical case of angiosarcoma, the high concentration of prolactin, together with IL-6 and osteocalcin, was associated with rapid tumour progression.^[251]

Tumours whose growth is sensitive to prolactin concentration also include Swarm chondrosarcoma.^[252]

These two components are therefore important as they lead to a decrease in the levels of prolactin, which is involved in the development and sensitivity of some tumour cells. They act through two different mechanisms: bromocriptine stimulates apoptosis through the ERK/EGR1 pathway, while cabergoline induces autophagia by inhibiting the AKT/mTOR signalling pathway.^[253]

PROLACTIN INHIBITORS



URSODEOXYCHOLIC ACID

Ursodeoxycholic acid is a secondary bile acid that is particularly important in maintaining the integrity of the intestinal barrier and in lipid metabolism. It increases the proportion of non-toxic hydrophilic bile acids and decreases hydrophobic endogenous bile acids; it also increases the hepatocellular excretion of bile acids, has cytoprotective, immunomodulatory actions and inhibits apoptosis.^[254] It is indicated to counteract the cholagogue and choleretic inhibition effect of somatostatin and octreotide.

SODIUM BUTYRATE

The use of Sodium Butyrate in patients with Sarcoma is contextualised epigenetically in the relaxation of chromatin. According to the study performed by Singh, butyrate blocks the generation of dendritic cells from bone marrow stem cells, without affecting the generation of granulocytes. This effect depends on SLC5A8, the sodium-coupled monocarboxylate transporter that transports butyrate into the cell and allows it to inhibit the deacetylases of histone acetate, which is also a substrate for SLC5A8 but not an inhibitor of histone deacetylases. It therefore does not affect the development of dendritic cells, indicating the essential role of histone deacetylase inhibition in the process.^[255]

In the study performed by Gupa N. et al., SLC5A8 is identified as an oncosuppressor gene in colorectal cancer. The subsequent definition of the functional identity of SLC5A8 as a Na(+)-coupled transporter for short-chain monocarboxylates provides a mechanism for the transporter's function as tumour suppressor. Butyrate, a substrate for the transporter, is an inhibitor of histone deacetylase. This fatty acid is produced in the lumen of the colon by bacterial fermentation of the dietary fibre. SLC5A8 mediates the concentration-based entry of butyrate into the cell. Consequently, the transport function of SLC5A8 has the ability to influence the acetylation status of histones.^[256]

SLC5A8, therefore, is a candidate oncosuppressor gene that is silenced in colon cancer, gastric cancer and possibly other cancers in humans. This gene encodes for a transporter belonging to the family of the Na(+)/glucose (SLC5) co-transporter gene. The silencing of the gene associated with cancer involves hypermethylation of the CpG islands present in exon 1 of the gene. The protein encoded by the gene mediates the electrogenic Na(+)-coupled transport of a variety of monocarboxylates, including short chain fatty acids, lactate and nicotinate. It can also carry iodide. One of the short chain fatty acids serving as a substrate for SLC5A8 is butyrate. This fatty acid is an inhibitor of histone deacetylases and is known to

induce apoptosis in a variety of tumours. Since high concentrations of butyrate are produced in the colon lumen, as previously mentioned, by the bacterial fermentation of dietary fibre, we hypothesise that the ability of SLC5A8 to mediate the entry of this short-chain fatty acid into the colon epithelial cells underlies the potential oncosuppressor function of this transporter.^[257]

Sodium butyrate affects the proliferation and differentiation of different cell types, including osteoblasts. In the study performed by Perego S. et al., the effects of different doses of butyrate on the differentiation and functionality of osteosarcoma cells in vitro and the expression of a pro-inflammatory phenotype in a normal or inflammatory environment were evaluated. SaOS-2 osteosarcoma cells were induced to differentiate and simultaneously treated for 24 h, 48 h or 7 days with sodium butyrate 10^{-4} , 5×10^{-4} or 10^{-3} M in the presence or absence of tumour necrosis factor alpha (TNF α) 1 ng/mL, a pro-inflammatory stimulus. Despite the mild effects on proliferation and alkaline phosphatase activity, butyrate induced the dose- and time-dependent expression of a differentiated phenotype (RUNX2, COL1A1 gene expression, and gene and protein expression of osteopontin). This was associated with a partial inhibition of nuclear factor κ B activation (NF- κ B) and the induction of histone deacetylase 1 expression. The net effect was the expression of an anti-inflammatory phenotype and the increase in the ratio of osteoprotegerin and the receptor activator of NF- κ B ligand (RANK-L). In addition, butyrate, especially at the highest dose, counteracted the effects of the pro-inflammatory stimulus of TNF α 1 ng/mL.^[258]

DISULFIRAM (INHIBITOR OF ALDEHYDE DEHYDROGENASE)

Aldehyde dehydrogenases are a family of enzymes that oxidise aldehydes to carboxylic acids. These enzymes are important in cellular homeostasis during oxidative stress for the elimination of toxic by-products of aldehyde from various cellular processes. In osteosarcoma, aldehyde dehydrogenase 1A1 has been described as a marker of cancer stem cells. Its activity has been found to be related to metastatic potential and metastatic phenotype.^[259] Disulfiram is an ALDH inhibitor, produced for the clinical purpose of treating alcoholism; it has recently also been considered a drug that can be used to suppress sarcoma stem cells.

Sarcoma tumour cells spread by local invasion and distant metastases, which depend on the extracellular matrix. The expression of matrix metalloproteinases (MMP) has been implicated in the invasion and metastases of the tumour cells. Cho HJ performed a study on the effects

of disulfiram on the suppression of tumour invasion, as well as its effects on MMP-2 and MMP-9 activity in human osteosarcoma cells (U2OS). The anticancer effects of disulfiram have been demonstrated in an invasion test (that uses U2OS cells) and its inhibitory activity on type IV collagenase, which inhibits the expression of genes and proteins responsible for cellular and non-cell mediated invasion on pathways. Disulfiram inhibited the expression of MMP-2 and MMP-9 and regulated the invasion of human osteosarcoma cells, making it a clinically usable drug for the inhibition of cancer invasion.^[260]

CARBOANHYDRASE INHIBITORS

Carbonic anhydrases (CAH), zinc metalloproteins, are enzymes that can have a clinical relevance in cancer therapy, because their cell-surface specific isoform, CA9, is almost exclusively associated with cancers and is involved in tumorigenesis. Its competitive inhibition mediated by Acetazolamide (AAZ) is part of the development of new therapies against cancer. Based on this evidence, we added AAZ to the DBM.

Hypoxia is associated with malignant progression and poor outcome in several human tumours, including soft tissue sarcoma. The study performed by Måseide K. suggested that CA IX is a potential intrinsic marker of hypoxia and a predictor of unfavourable prognosis in patients with large, high-grade, deep soft tissue sarcoma.^[261] CA IX is often overexpressed in human osteosarcoma (OS) but not in normal tissues, and its expression levels correlate with prognosis. In the study performed by Perut F., the therapeutic potential of newly synthesised CA IX sulphonamide inhibitors in OS was studied. CA IX expression levels were significantly higher in OS than bone marrow stromal cells (BMSC) after drug administration. Consequently, CA IX inhibitor 3 induced significant cytotoxicity on OS cells without affecting the proliferation of BMSC. This activity increased in hypoxia and was mediated by stopping the cell cycle and modulating the cytosolic and extracellular pH. In vivo, CA IX inhibitor 3 reduced tumour growth, leading to significant necrosis.^[262]

CONCLUSIONS

Patients treated with DBM, although experiencing rare and temporary modest toxicity, had a clear improvement in survival, objective response and quality of life compared to patients with sarcomas in the same stages, with the same histochemical characteristics, treated with conventional oncology protocols.

The rare cases in which the DBM was applied as first-line therapy, both neoadjuvant and adjuvant, had the best responses.

In the progression of sarcomas, as in most tumours, the percentage of tumour stem cells, compared to other components of the neoplastic population, increases until it is almost total and is associated with chemo-radio-resistance and rapid progression. For this reason, we have gradually increased the doses of molecules that in the scientific literature are documented to negatively regulate CSCs, such as Melatonin, ATRA, Glucosamine, which improve the differentiation and reprogramming of tumour stem cells by negatively regulating proliferation, invasiveness and resistance. This objective was also achieved by modifying the criteria and methods of administration of alkylating drugs, such as hydroxyurea. Unlike in oncology protocols, their metronomic administration in the DBM allowed better control of the proliferation and invasiveness of tumour cells in the absence of myelotoxicity, thanks to the myeloprotective properties of 100 mg of daily water-soluble MLT and about 25 ml of retinoid solution in vitamin E, divided into 3 daily administrations.^[46,48,263] The antiproliferative and antiangiogenic effect was increased by the regular combination of 4 mg of 14-amino-acid SST administered with a 12 hour-adjusted timer (because of the short half-life of SST, 3 minutes) and delivered between 19.00 and 20.00, with the intramuscular administration of 20 mg slow-release octreotide every 20 days, enabling receptorial and temporal saturation. An improvement in the therapeutic response was achieved by administering SST 14 intravenously instead of subcutaneously, always in 12 hours, using the timer. The DBM, unlike the oncological conception, shifts the therapeutic axis from cytolytic toxic and immunodepressive mechanisms to fighting neoplastic proliferation by negatively regulating the blood level of GH, which activates oncogenesis through multiple mechanisms.^[15, 264] Current oncological paradigms are beginning to admit that cancer may be considered a pathological recapitulation of growth processes.

The concomitant administration of Na-Butyrate creates an epigenetic context of chromatin relaxation, essential for the interaction with transcription factors of the Zinc Finger and Homeodomain family, the RXR, VDR, RZR, ROR receptors, co-expressed on nuclear membranes and involved in differentiation processes. The differentiating components of DBM, such as the solution of retinoids in vitamin E, Vitamins C, D and MLT, therefore counteract the mutagenic capacity of cancer cells based on a defence system, and it is this survival programme that allows them to effectively and quickly repair DNA damage induced

by chemo-radiotherapy. The first forms of life, prokaryotes, survived to the present day because, as they evolved, they became equipped with a defence system based on a programme of mutations, which allowed them to repair DNA damage caused by various adverse events. The prokaryotes transmitted the survival programme to bacteria, which in turn transferred it to somatic cells. Radman, a molecular biologist, has identified and studied this programme of survival and defence transferred from prokaryotes to eukaryotes, and from the latter to somatic cells. Because of its functions and survival purpose in emergency conditions, he called it the "SOS programme", which somatic cells access to overcome critical situations.^[265] Israel L. confirmed that these defence mechanisms are activated by tumour cells that implement the same procedure as germs, selecting and retaining for each mutation a number of benefits much more quickly and efficiently than bacterial cells.^[266] Tumour cells in an acute stress situation implement DNA repair systems and express or silence genes according to their needs, selecting and retaining for each mutation a series of advantages much more quickly and efficiently than bacterial cells. Israel studied the SOS system, identifying numerous gene homologies between neoplastic and bacterial cells. The SOS system allows neoplastic populations to become progressively refractory to various oncotherapeutic treatments through DNA repairs and genetic recombinations. In our body, under stable conditions and biologically balanced, the SOS system is silenced and inactive, blocked by a transcriptional repressor, the LEX-A protein. When the DNA of a somatic cell is severely damaged, to access the SOS survival path and repair the DNA, the cell deactivates the LEX-A transcriptional repressor using the REC-A positive regulator. The expression of SOS thus initiates a series of mutations that repair but at the same time modify the DNA, initiating the carcinogenesis process. The cell, thus mutated, begins a tumoural involution, continuously selecting and retaining, with a progression predefined by the SOS programme, a series of advantages, as confirmed by Lambert^[267] and, more recently, other authors, including Russo^[268], who showed that various mechanisms against tumour cells, even beyond chemo- and radiotherapy, such as monoclonal antibodies and inhibitors of ligand mitogenic signalling pathways such as EGFR, VEGF, IGF1, FGF, etc., can very rapidly activate the SOS system and an extremely large number of multiple survival mechanisms. The two strategic objectives of DBM, not yet pursued by oncology, are proliferative-invasive, neoplastic angiogenic inhibition using SST/Octreotide, DR2 agonist analogues, together with fighting the mutagenic capacity of the neoplastic phenotype by silencing the SOS survival system through the distinguishing components of the DBM, which, with their trophic, immunomodulating and antioxidant activity, improve the vitality and efficiency of healthy

cells while, simultaneously, reducing the efficiency, vitality and functionality of neoplastic cells. The same molecules exert opposite effects on healthy and neoplastic cells. The metronomic use of apoptotic, non-cytotoxic doses in a biological context unfavourable to the viability, proliferation and migration of neoplastic cells, slowly but progressively causes their extinction through apoptosis. In a tumour, the chemo-radiotherapy-resistant stem cells also play a crucial role in maintaining tumour growth and the initiation of the metastatic process. Chemotherapy and radiation therapy act on actively proliferating tumour cells, the “cancer transit amplifying cells”. On the contrary, tumour stem cells proliferate slowly and are not affected by chemo- and radio-therapy. For this reason, the current therapeutic strategies cannot maintain long-term control of the tumour process.

Today, in the absence of valid therapeutic alternatives, sarcomas are characterised by particularly high proliferative indices and metastatic potential, resulting in a high mortality rate.

Three causes contribute to this dramatic therapeutic failure:

1. Total unresponsiveness to all cancer protocols of sarcoma populations consisting mainly of tumour stem cells on which, in contrast, the efficacy of the DBM molecules discussed here has been demonstrated.
2. Non-administration of Melatonin.
3. Failure to administer somatostatin, a physiological inhibitor of GH (and related growth factors) and, therefore, of neoplastic proliferation and dissemination.

The DBM, by extending its activity to fight multiple vital reactions of the neoplastic biology, shifts the therapeutic axis from a pure cytotoxic-cytolytic conception and the illusory and utopistic eradication of all cancer cells, to the gradual physiological reconversion of the vital functions deviated by the cancer, to the recovery of immuno-neuro endocrine homeostasis, to the differentiation of tumour cells and reprogramming of tumour stem cells. No cytotoxic chemotherapy treatment (or monotherapy) exists (or will ever exist) that can heal a solid tumour. There is only a method, a rational and biological multitherapy, a complex of synergistic and factorially interactive substances, individually equipped with non-toxic antitumour activity, which sequentially or simultaneously act centripetally on the myriad biological reactions of tumour life, gradually restoring to normality the vital reactions altered by the cancer.

Abbreviations

ATRA - All Trans Retinoic Acid
CCK – Cholecystokinin
C.M. - GH-induced chemotaxis of Monocytes
CSC - Cancer Stem Cells
DBM - Di Bella Method
EGF - Epidermal Growth Factor
EGFR - Epidermal Growth Factor Receptor
FGF - Fibroblastic Growth Factor
GF - Growth Factor
GH - Growth Hormone
GHR - Growth Hormone Receptor
HDAT - Histone deacetylase
HGF - Hepatocyte Growth Factor
HIF-1 α - Hypoxia-Inducible Oncogenic Factor 1-alpha
IGF1-2 - Insulin-like Growth Factor 1-2
IGFR - Insulin-like Growth Factor Receptor
IL8 - Interleukin 8
MRI - Magnetic Resonance Imaging
MLT - Melatonin
NGF - Nerve Growth Factor
NHL - Non-Hodgkin's Lymphoma
eNOS - Endothelial Nitric Oxide Synthase
PDGF - Platelet-Derived Growth Factor
PET - Positron Emission Tomography
PG2 - Prostaglandin 2
PRL - Prolactin
PRLR - Prolactin Receptor
NMR - Nuclear Magnetic Resonance
SSN - National Health Service
SST - Somatostatin
SSTR - Somatostatin Receptor
TGF - Transforming Growth Factor
TRK - Tyrosine-kinase

VEGF - Vascular Endothelial Growth Factor

VIP - Vasoactive Intestinal Peptide

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