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Melatonin: a pleiotropic molecule of natural origin. Evaluation of the different therapeutic activities in animal models and / or human patients and a study of the metabolic-biochemical pathways related to them.

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GENERAL INDEX

1.0. ABSTRACT

Background

Melatonin (MLT), a pineal gland hormone, seves as a bioclock and bio-calendar to mediate many receptor- or non-receptor functions. In addition to its immunomodulatory and neurological effects, MLT has a relevant oncostatic activity especially with respect to breast and prostate cancers, but the mechanism of action is still unclear. The growth of androgen-independent LNCaP prostate cancer cells has been demonstrated to be inhibited by MLT both in vitro and in vivo in a nude mice xenograft model. Clearly, the oncostatic effects of MLT may not be related to a single function, but rather to a complex interaction of several factors that involve the redox state, the immune system, the modulation of the endocrine system and membrane receptors.

MLT also increases sleepiness, decreases core temperature and increases peripheral temperature in humans. The role of MLT in the treatment of sleep disturbances, to prevent jet lag or as a part of the sepsis treatment is widely discussed; yet the role in critically ill patients still deserves further investigation. Critically ill patients suffer from severe sleep disturbances during their stay in an Intensive Care Unit (ICU). Moreover, these patients require high levels of antioxidants due to their critical illness.

Aims of the thesis

The main object of my PhD thesis was to confirm the pleiotropy of MLT molecule by testing its activity in two of the most promising clinical applications: the cure of prostate cancer and the regulation of the sleep/wake rhythm as adjuvant in the sedative therapy in critically ill patients.

Spcific Aims:

- To evaluate the oncostatic effect of MLT administered intraperitoneally (i.p.) by saline solution on human prostate tumor. To this purpose I have selected an *in-vivo* experimental model of nude mice (athymic), xenografted subcutaneously with tumor cells of a human prostatic line (LNCaP).
- Using the same animal model and the same administration route (i.p.) and treatment schedule of MLT administered in saline, to investigate the efficacy of a novel and promising pharmaceutical formulation: MLT included in a solid lipid nanoparticles system (SLN-MLT).
- Using the same mouse model of human prostate cancer, to test whether MLT can be administered efficiently using alternative ways that are more sustainable for prolonged treatments than i.p. MLT, e.g., transdermal delivery through the skin barrier directly onto the tumor *via* a novel and patented technique named cryoRx.
- To focus on the underlying action mechanism of MLT at the tumor cellular micro-environment and the possible influence on such a mechanism of the lipid nanocarrier employed.
- To evaluate in a cohort of ICU patients, if the circadian rhythm of MLT secretion is disrupted and to which extent MLT administration by different routes and different drug formulations (MLT as a tablet administered os, MLT encapsulated in SLN administered os as a suspension and MLT encapsulated in SLN applied transdermally as a suspension with the aid of a patch) is feasible in terms of absorption efficiency and adequacy in achieving and maintaining nocturnal peak plasma hormone.
- To evaluate if the restoration of the melatoninemia by the different ways of drug delivery in critically ill patients may be useful to restore the pleiotropic function of this hormone: facilitate the resolution of sleep-wake cycle disorders, improve the quality of sleep, reduce the number of episodes of anxiety, confusion and agitation, and reduce the amount of sedatives used, especially at night.

Materials and Methods

We used an in *vivo* model of human prostate tumor LNCaP cells xenografted into nude athymic mice. MLT has been administered i.p. as saline (n=13) and by SLN (n=13) or transdermally by cryoRx (n=14). For each treatment controls were also included. Each group received the same administration schedule: 3 treatments per week, for 6 week. At the end the animals were sacrificed and along the treatment period the mice weight were recorded as well as the tumor volume was measured. MLT concentration was assessed in plasma and tissues by ELISA test and tumors were evaluated for morphology, MLT content and HIF-1α expression.

The clinical effects of MLT administration as well as the pharmacokinetics profiles as a function of different administration ways (oral as MLT, oral as SLN and transdermal as SLN) have been studied in ICU patients. During the 2nd day of the ICU stay, serial withdrawal were taken to determine the endogenous MLT secretion, and then after MLT administration, additional plasma samples were obtained during the 3rd day to evaluate the exogenous plasma MLT content, for a total of 20 withdrawal for each patient. Each blood sample was centrifuged and the plasma stored at -20°C. To determine the MLT concentration we used an ELISA kit that includes a pre-purification of the sample by SPE (solid phase extraction) cartridges.

Results

Tumors developed slowly in all the MLT-treated (topical and i.p.) groups and at the end of the treatment, the mean volume was significantly lower *vs* control. Both tumoral and plasma MLT levels were significantly higher in treated (topical and i.p.) *vs* not-treated animals. Harvested tumor showed a strong inflammatory reaction which seemed to surround and infiltrate the tumor cells. In SLN-MLT treated animals, in addition to a strong lymphocyte infiltration, the tumor appeared limited also by the presence of fibroblast type cells. Preliminary results showed HIF-1α expression increased in both treatment groups (topical and i.p.) *vs* Ctrl.

In the clinical study, we have seen that MLT administration, is safe, reduces need for analgesic and sedative drugs restoring the normal circadian rhythm. In patients who received MLT or SLN-MLT by os, the absorption was rapid: the peak plasma concentration had a median of 30 min and after only 5 min, the MLT levels were significantly higher than physiological ones. The AUC of SLN-MLT was significantly higher than when MLT was administered by saline solution. SLN-MLT by transdermal route, presented a delayed peak plasma concentration (4 h) and a lower bioavailability but MLT plasma levels reached however the pharmacological concentration able to restore the pleiotropic function of this hormone and facilitate the resolution of sleep-wake cycle disorders.

Conclusions

We have confirmed the positive effects of MLT on tumor growth and we have focused on its effect on hypoxia. The possible role as anti-tumor drug candidate deserves to be further investigated. We demonstrated that different alternative and novel ways to deliver MLT are effective as well. This would accelerate the transferability of obtained data towards a therapy. on MLT oncostatic activity.

In the clinical study, we have proved that MLT is able to normalize the sleep-wake cycle, to ameliorate the sleep quality and to reduce the number of sedative drugs used in ICU pts. We proved also that transdermal administration by SLN is effective in rising plasma MLT levels as well as enteral administration and is more practicable in clinical setting.

Abbreviation's list

4P-PDOT	4-phenyl-2-propionamidotetralin
AANAT	arylalkylamine N-acetyltransferase
ADCC	antibody dependent cellular citotoxicity
AFMK	N^1-acetyl-N^2-formyl-5-methoxykynuramine
ALT	alanine aminotransferase
Akt	serine-threonine kinase
AMK	N^1-acetyl-5-methoxykynuramine
aMT6s	6-sulfatoxyMLT
AR	androgen receptor
AST	aspartate aminotransferase
AUC	area under the concentration curve
BCLC	Barcelona clinic liver cancer
BK_{Ca}	Ca^{2+}-activated large conductance potassium channels
BP	blood pressure
BSA	bovine serum albumin
BSP	bone sialo protein
cAMP	cyclic AMP
CAT	catalase
CE	capillary electrophoresis
CIN	contrast induced nephropathy
C-LOS	conditional length of stay
COX	cyclooxygenase
C-PAP	continuous positive airway pressure
CREB	cAMP response element-binding protein
CryoRx	cryotherapy
CsA	cyclosporine A
CV	coefficient of variation
DLMO	dim light MLT onset
DNR	do not resuscitate
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalograph
EHS	Engelbreth-Holm-Swarm
ELISA	enzyme-linked immunosorbent assay
EP	E-type prostaglandin
EP1	E-type prostaglandin receptor
ER	estrogen receptor
ESI	electrospray ionization
ETC	electron transport chain
G6PDH	glucose-6-phosphate dehydrogenase
GABA	γ-aminobutyric acid

GC-MS	gas chromatography-mass spectrometry
GI	gastrointestinal
GITS	gastro-intestinal therapeutic system
GPCR	G protein-coupled receptor
GPx	glutathione peroxidase
GSH	glutathione
GSSG	oxidized glutathione
Hb	hemoglobin
HBV	hepatitis B virus
HETE	hydroxyeicosatretanoic acid
HIF-1	hypoxia inducible factor-1
HIOMT	hydroxyindole-O-methyltransferase
HPH	high pressure homogenization
HPLC	high performance liquid chromatography
HPLC-MS	HPLC-mass spectrometry
ICU	intensive care unit
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
i.m	intramuscular
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
i.v.	intravenous
I/R	ischemia/reperfusion
LC-MS	liquid chromatography-mass spectrometry
LD	light-dark
LL	continuous light
LO	lipoxygenase
LOS	length of stay
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor
MEL	MLT
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MRM	multiple reaction monitoring
MtPTP	mitochondrial permeability transition pore
NA	noradrenaline
NAS	N-acetylserotonin
NE	norepinephrine
NF-kB	nuclear factor kappa B
NK	natural killer
NO	nitric oxide

NOS	nitric oxide synthase
OCT	optimal cutting temperature
PCa	prostate cancer
PGs	prostaglandins
PSA	prostate-specific antigen
PSV	pressure support ventilation
REM	rapid eye movement
RIA	radio immune assay
RZR/ROR	retinoid-related orphan nuclear receptor family
SAD	seasonal affective disorder
SAPS II	simplified acute physiology score
s.c.	subcutaneous
SCA	salvage cryoablation
SCF	stem cell factor
SCN	suprachiasmatic nucleus
SCT	spray CryoRx
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIM	selected ion monitoring
SIRS	systemic inflammatory response syndrome
SLN	solid lipid nanoparticles
SOD	superoxide dismutase
SOFA	sequential organ failure assessment
TACE	transcatheter arterial chemoembolization
TdT	terminal deoxynucleotidyl transferase
TGF	transforming growth factor
Th	T-helper
TMR	tetramethylrhodamine
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
WBC	white blood cell

2.0 INTRODUCTION

2.1. MLT

Melatonin (MLT), is a natural substance that has been identified in all major living species, including bacteria and other unicellular microorganisms, plants and animals, as well as in humans (Pandi-Perumal et al., 2006; Paredes et al., 2009). It is possible that the first function of MLT in phylogeny was related to its activity as direct and indirect antioxidant.

MLT is normally synthesized and secreted during the dark phase of daily photoperiod. Though it is produced primarily in the pineal gland, MLT is also synthesized in other organs like the retina, skin and lymphocytes. Reports on plasma MLT levels among subjects of different ages reveal a decrease in MLT production with advanced age (Pandi-Perumal et al., 2005)

Among the various functions attributed to MLT in the control of the immune system, antitumor defense assumes a primary role (Lissoni et al., 1996; Maestroni and Conti, 1990; Maestroni et al., 1988; Martins et al., 1998). The nighttime physiological surge of MLT in the blood or extracellular fluid has been suggested to serve as a "natural restraint" for tumor initiation, promotion and/or progression (Blask et al., 2005)

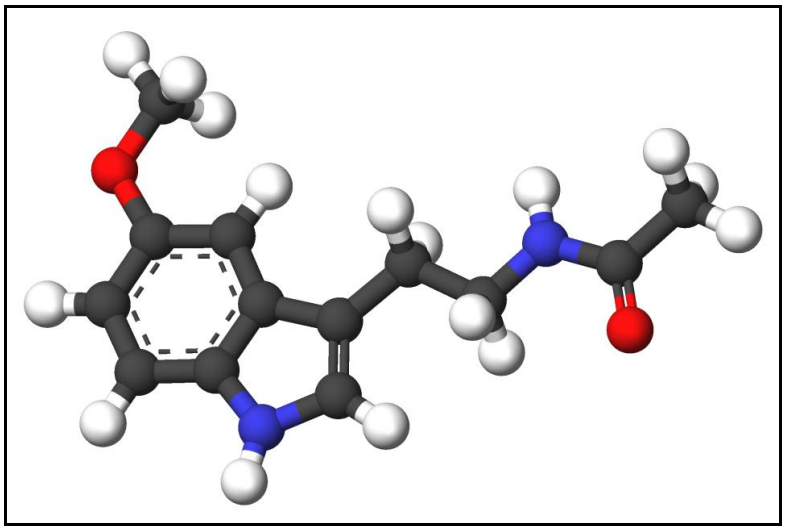

Fig. 1 *- MLT structure.*

2.1.1. MLT biosynthesis

MLT is synthesized from the amino acid tryptophan, taken up from blood, *via* its conversion to serotonin(Pandi-Perumal et al., 2006). Serotonin is then acetylated to form N-acetylserotonin (NAS) by the enzyme arylalkylamine N-acetyltransferase (AANAT), which, in most cases, represents the rate-limiting enzyme. NAS is converted into MLT by the enzyme hydroxyindole-O-methyltransferase (HIOMT). The enzymatic machinery for MLT biosynthesis was first identified by Axelrod et al. in the pinealocytes (Axelrod, 1974). The enzymes of MLT biosynthesis have recently been indentified in human lymphocytes (Carrillo-Vico et al., 2004), and locally synthesized MLT is probably involved in the regulation of the immune system. Among various other extrapineal sites of MLT biosynthesis, the gastrointestinal (GI) tract is of particular importance as it contains amounts of MLT exceeding by several hundred fold those found in the pineal

gland. GI MLT can be released into circulation, especially under the influence of high dietary tryptophan levels (Bubenik, 2002).

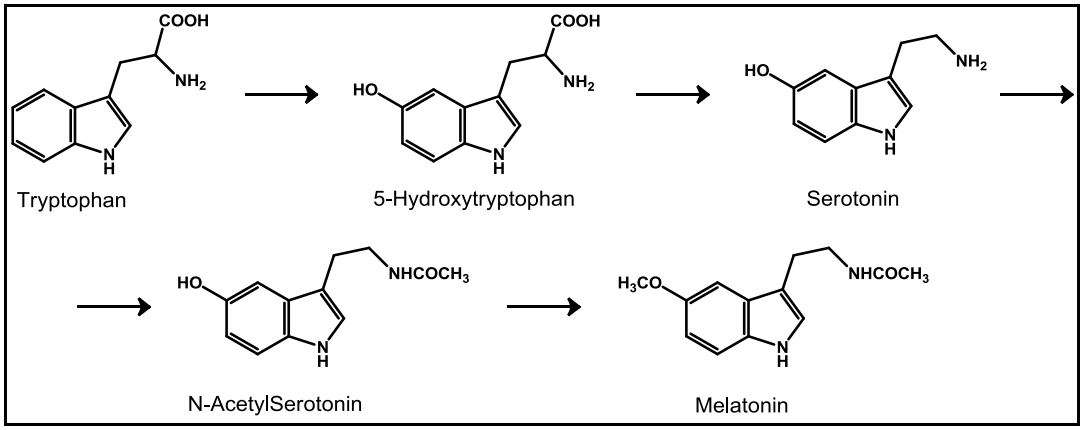

Fig. 2 *- MLT biosynthesis.*

2.1.2. *The control by endogenous circadian clock and environmental light as a chemical expression of darkness*

Pineal MLT production exhibits a circadian rhythm with low levels during daytime and high levels during night. This circadian rhythm occurs in all living organisms irrespective of whatever they are diurnally or nocturnally active. An exception to this “high-at-night” rule is the retina of some salmonoid fish, where MLT levels are high during the day or not significantly different (Besseau et al., 2006). These species-specific variations in MLT rhythm profiles may have developed as a result of changes in regulatory mechanisms during the course of evolution (Iigo et al., 2007)

In mammals, the regulation of pineal MLT biosynthesis, by ambient illumination is mediated by the retinohypothalamic tract that projects from the retina to the suprachiasmatic nucleus (SCN), the major circadian oscillator (Moore, 1997). Special photoreceptive retinal ganglion cells are the origin of the retinohypothalamic projection (Berson et al., 2002). These ganglion cells contain a special photosensitive pigment, known as melanopsin, which is involved in the phototransduction mechanism (Brainard et al., 2001).

Nerve fibers from the SCN project to a multisynaptic descending pathway that passes through the paraventricular nucleus, medial forebrain bundle and reticular formation and makes synaptic connections with intermediolateral cells of the cervical spinal cord. From there, preganglionic fibers project to the superior cervical ganglia where postganglionic sympathetic fibers innervating the pineal gland are located, regulating pineal MLT synthesis by releasing norepinephrine (NE) at their postganglionic nerve terminals (Moore, 1997)

The release of NE from pineal nerve terminals occurs during nighttime. NE, by binding to β-adrenergic receptors at the pinealocyte membrane, activates G-protein subunits to stimulate adenylate cyclase and the subsequent cyclic AMP (cAMP) production. The increase of cAMP promotes the synthesis of enzymes involved in MLT biosynthesis (Klein, 2004)

Circulating MLT derives almost totally from the pineal gland, as shown by the fact that undetectable MLT level are found after pinealectomy. After its release, MLT binds to albumin (Cardinali et al., 1972) and reaches all tissues within a very short period (Cardinali and Pevet, 1998). MLT half-life is biexponential with a first distribution half-life of 2 min and a second of 20 min (Claustrat et al., 2005). MLT released to the

cerebrospinal fluid *via* the pineal recess attains, in the third ventricle, concentrations up to 20-30 times higher than in the blood. These concentrations, however, rapidly diminish with increasing distance from the pineal gland (Tricoire et al., 2003) thus suggesting that MLT is taken up by brain tissue. MLT production exhibits considerable inter-individual differences (Macchi and Bruce, 2004). Some subjects produce more MLT than others, during their lifetime, but the significance of this variation is not known. Studies of twins suggest that these differences may have a genetic base (Griefahn et al., 2003).

2.1.3. *MLT catabolism and secretion regulation*

MLT produced by the pineal gland is released into the circulation and gains access to various fluids, tissues and cellular compartments. Because this highly lipophilic hormone is not stored in the pineal gland, the profile of its plasma levels reflects pineal activity . MLT catabolism occurs mainly in the liver, where it is first hydroxylated in the C6 position (6-hydroxyMLT) by the hepatic cytochrome P450, then conjugated with sulfate and, to a lesser extent, with glucuronic acid, and finally excreted in urine (Skene et al., 2006). In some mouse strains, MLT has been shown to be metabolized to 6-glucuronylMLT rather than to 6-sulfatoxyMLT (aMT6s) (Ma et al., 2008). Very small amounts of free 6-hydroxyMLT are excreted unchanged in the urine; other minor metabolites have also been identified. Urinary aMT6s excretion closely reflects the plasma MLT profile and is frequently used for the evaluation of MLT rhythm, especially in humans (Arendt, 2006). The metabolism of MLT is rapid, and its half-life in humans following exogenous administration is short, ranging between 10 and 60 minutes. MLT is also metabolized into kynuramine derivates (Hirata et al., 1974). It is interesting to note that the antioxidant properties of MLT are shared by some of their metabolites like N^1-acetyl-5-methoxykynuramine (AMK) and N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) (Hardeland et al., 2009). Thus MLT gives rise to a cascade of antioxidant molecules that multiply the free radical scavenger effect. Metabolic breakdown of retinal MLT is different from that of MLT synthesized by the pineal gland. Initially, aryl-acylamidase (aryl-acylamide amidohydrolase) catalyzes the deacetylation of MLT to 5-methoxytryptamine. Subsequently, 5-methoxytryptamine is metabolized *via* the same pathway of indoleamines and catecholamines, with deamination by monoamine oxidase to form 5-methoxyindole acetaldehyde, and further oxidation to 5-methoxyindoleacetic acid or reduction to 5-methoxytryptophol (Grace et al., 1991).

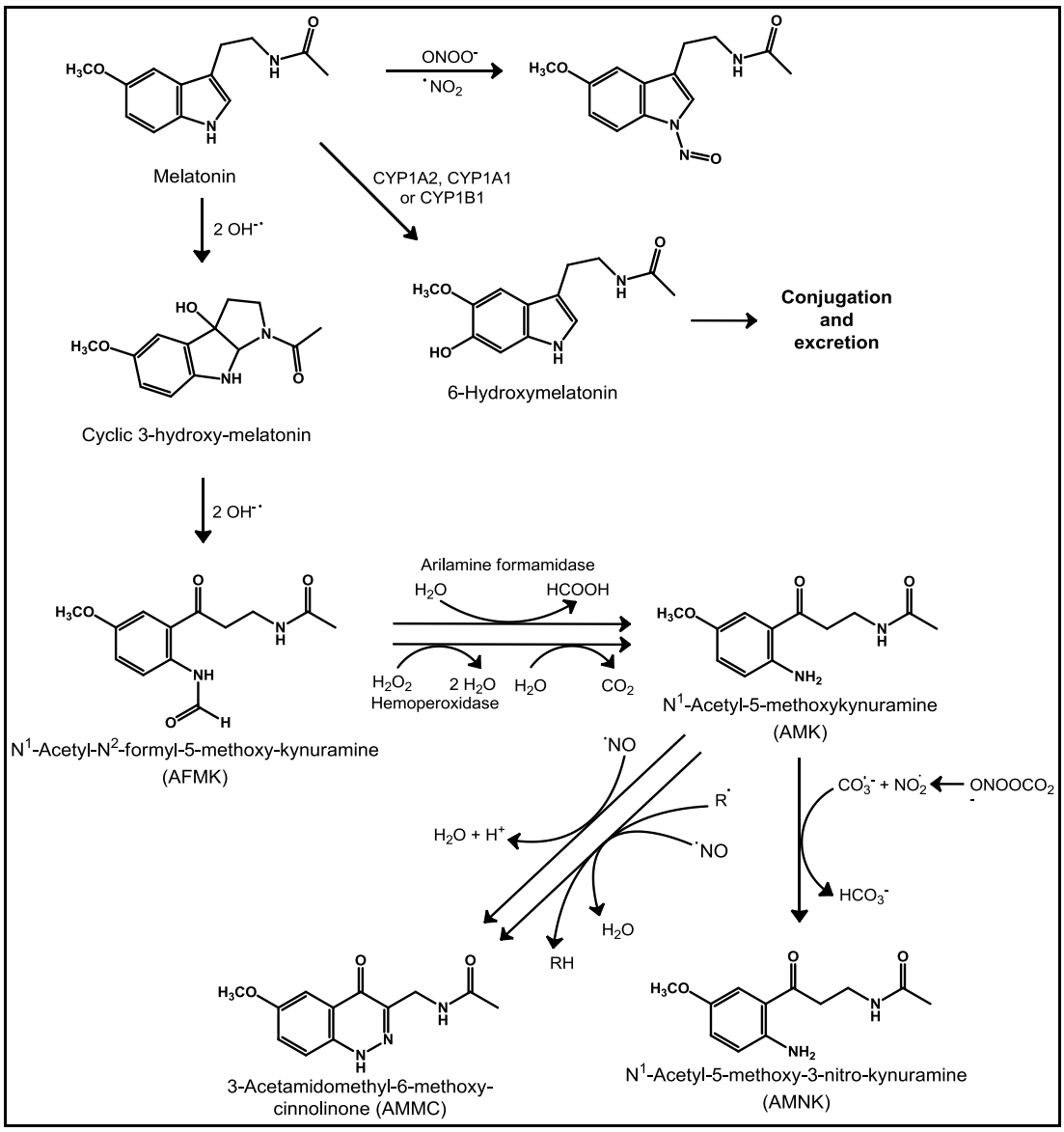

Fig. 3 *- MLT metabolism.*

2.1.4. *MLT receptors, other binding sites and signaling mechanism*

MLT exerts some of its actions through interaction with MT_1 and MT_2 receptors (Dubocovich et al., 2010; Dubocovich and Markowska, 2005). These two receptors are members of the 7-transmembrane G-protein-coupled receptor (GPCR) family (Fig. 4). A third binding site, identified initially as MT_3, was subsequently characterized as the enzyme quinone reductase 2 (Nosjean et al., 2000). Many G-protein-coupled receptors, including MT_1 and MT_2 receptors, exist in living cells as dimers. The relative propensity of the MT_1 homodimer and MT_1/MT_2 heterodimer formation are similar whereas that of the MT_2 homodimer is 3-4 fold lower (Daulat et al., 2007). Another MEL-related receptor, named GPR50, has also been found in different species, including humans. This receptor does not bind MLT, thus may have a role in MLT function by altering binding to the MT_1 receptor.

MLT also acts by binding to cytoplasmic proteins like the calcium binding protein calmodulin (Benitez-King, 2006) or tubulin and to nuclear receptors like RZR/ROR (Wiesenberg et al., 1998). The MLT receptor present in the skin has been identified as MT_1 (Slominski et al., 2005). MT_2 receptors have been detected in neonatal keratinocytes, and in cutaneous melanoma cells as well as in normal and malignant uveal melanocytes (Roberts et al., 2000).

The decrease in cAMP production caused by MLT via MT_1 and MT_2 receptor interaction reduces the uptake of linoleic acid, an essential fatty acid by affecting a specific fatty acid transporter (Blask et al., 2002). Linoleic acid can be oxidized to 13-hydroxyoctadecadienoic acid by 15-lipoxygenase, serving as an energy source for tumor growth and tumor growth-signaling molecules. Inhibition of linoleic acid uptake by MLT is regarded as a mechanism of its antiproliferative effects (Blask et al., 2002).

Some studies have also suggested that modulating the expression and function of nuclear receptors, RZR/ROR, as the mechanism for biological effects of MLT. By binding to nuclear receptors, MLT alters the transcription of several genes that play a role in cellular proliferation (i.e., 5-lipoxygenase , p21 or bone sialoprotein) (Carlberg, 2000).

Another mechanism of action of MLT may be its ability to modulate intracellular calcium and calmodulin activity. Calcium-activated calmodulin is involved in the initiation of the S and M phases of the cell cycle, in the cell cycle-related gene expression regulation and in the reentry of quiescent cells from G_0 back into the cell cycle MLT has been shown to increase calmodulin degradation trough a direct binding as well as trough redistributing it, thereby inhibiting cell cycle progression (Benitez-King, 2006)

MLT also serves as a potent modulator of gene transcriptional activity. MLT has been shown to target a large number of genes, in central or in peripheral tissues. It has been hypothesized that MLT mediate seasonal photoperiodic control *via* phasing the expression of clock genes in the pars tuberalis .

In addition, MLT down-regulates gene expression of integrin and integrin-associated protein-encoding genes in rat retina, while up-regulates the cAMP response element binding protein (CREB) gene in retinal pigmentary cells (Wiechmann, 2002).

Notably, MLT has also demonstrated a pronounced effect on the expression of genes related to oncogenesis (e.g. *Mybl1, Rasa1, Mllt3* and *Enigma homolog 2*) and calcium metabolism (*Kcnn4* and *Dcakl1*) (Anisimov et al., 2006).

MLT shows a significant effect on mitochondrial genes expression, like genes encoding 16S ribosomal RNA (*mt-RNr2*), cytochrome C oxidase subunits I and II (*mt-Co1*, *mt-Co3*) and NADH dehydrogenase 1 (*mt-Nd1*) (all-upregulated) and ATP synthase subunit 6 (*mt-ATP6*; down-regulated) (Anisimov et al., 2006).

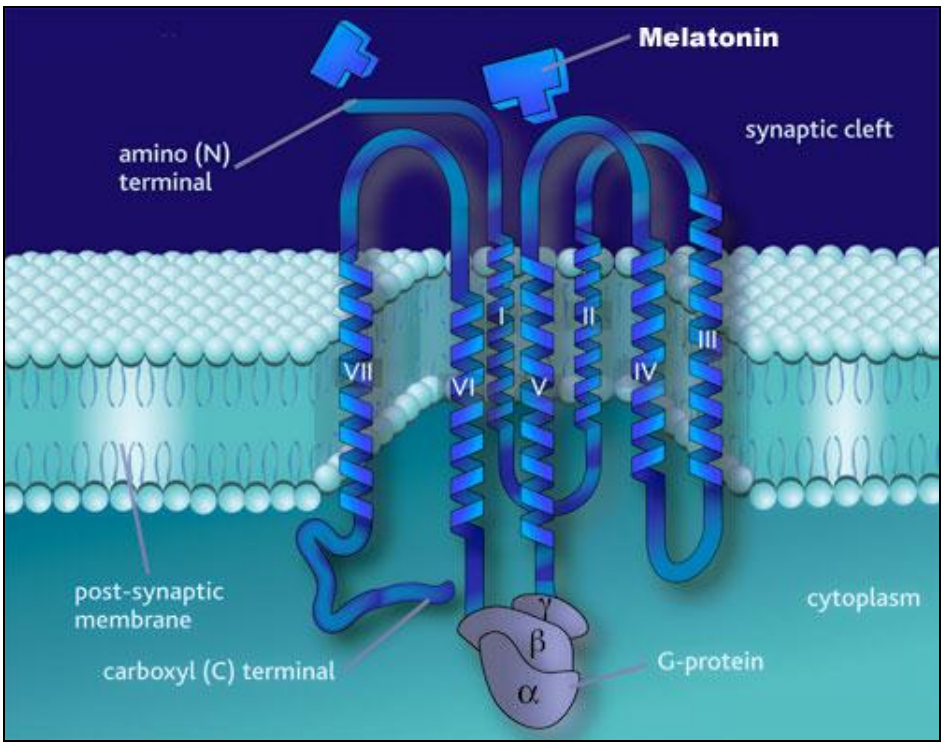

Fig. 4 *– MLT receptor structure Picture modified from: http://www.hivehealthmedia.com/melantonin-drug-potential-cure-insomnia-invented/.*

2.2. THE PLEIOTROPY OF MLT: ITS MAJOR CELLULAR AND PHYSIOLOGICAL FUNCTIONS

MLT is a pleiotropic molecule that mediates many seasonal physiological, immunological and other receptor- or non-receptor mediated functions (Fig. **5**).

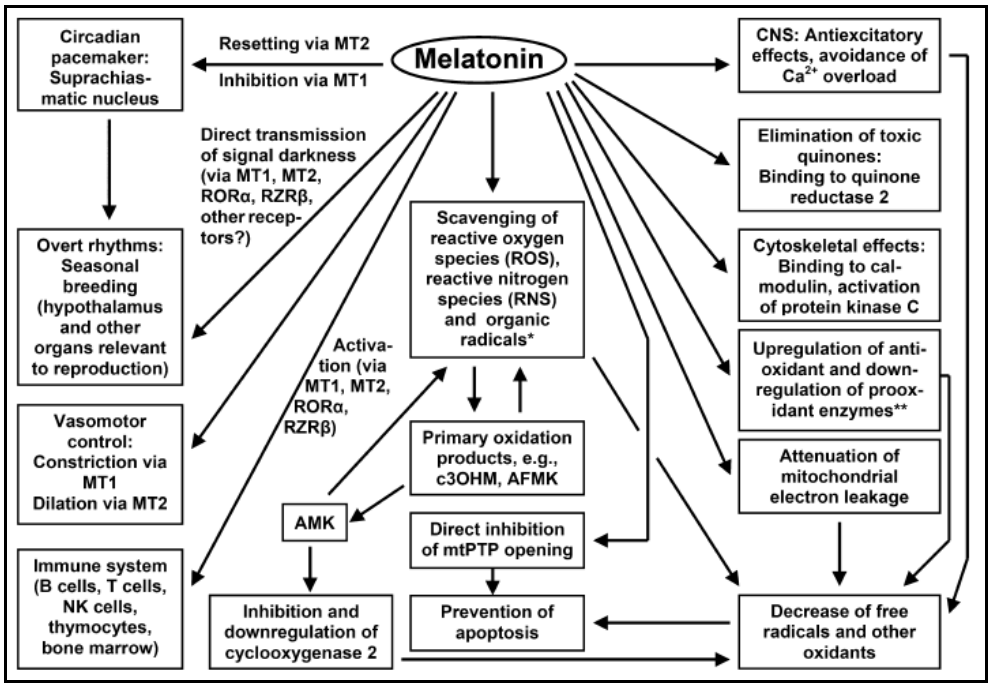

Fig. 5 – *The pleiotropy of MLT. Picture modifed from Pandi-Perumal-2006.*

2.2.1. *Circadian rhythmicity and actions on SCN: antiexcitatory effects, avoidance of Ca^{2+}*

In mammals, MLT appears to have a more modest role in the organization of adult circadian physiology. In contrast to seasonal physiology, MLT appears to be mostly associated with sleep propensity and the core temperature rhythm. MLT may be more important in the perinatal period. However, there is convincing evidence that MLT can indicate the time of day to the circadian system. For example, sleep is worse and the core temperature rhythm amplitude is blunted in the absence of MLT during the night compared to when it is present (Scheer and Czeisler, 2005).There is also evidence for an influence of MLT on the circadian aspects of systems such as glucose homeostasis (la Fleur et al., 2001), the immune system (Maestroni et al., 1988) and cardiovascular function (Scheer et al., 2004).The most direct link between MLT and the circadian system was shown by *in vitro* experiments on the SCN. In the mammalian SCN, MLT acutely inhibits neuronal firing (van den Top et al., 2001). This effect appears to be mediated through stimulation of MT_1 MLT receptors (Jin et al., 2003) and is thought to result from the activation of Kir3 potassium channels and an increase in potassium conductance with subsequent neuronal hyperpolarization (van den Top et al., 2001). In addition, MLT applied at certain circadian times phase advanced the peak of the circadian rhythm of neuronal firing and other measured SCN outputs (Hunt et al., 2001). Initially, this phase shifting effect of MLT was attributed solely to MT_2 receptors. However, more recently it appears that there is redundancy between MT1 and MT2 receptors in terms of the regulation of the circadian activity (Jin et al., 2003).

2.2.2. *Regulation of the cardiovascular function and temperature*

In rat caudal arteries, stimulation of the MLT MT_1 receptor produced vasoconstriction while activation of the MT_2 receptor resulted in vasodilatation (Masana et al., 2002). The vasoconstrictive action of MLT appears to be mediated by inhibition of Ca^{2+}-activated large conductance potassium channels (BK_{Ca}). It is suggested that MLT-induced vasodilatation of arteries and an increase in blood flow in the distal parts of skin regions that are important for heat loss regulation may underlie the hypothermic effects of the hormone (Krauchi et al., 1997).

2.2.3. *Inhibition and downregulation of cyclooxygenase 2*

Cyclooxygenase 2 (COX-2) is the key enzyme that catalyzes the two sequential steps in the biosynthesis of prostaglandins (PG)s from arachidonic acid. COX-2, the inducible isoform of COX, plays a critical role in the inflammatory response, and its overexpression has been associated with several pathologies including neurodegenerative diseases and various types of cancer. Mayo et al. have investigated the suppressive effect of MLT and its metabolites on the activities of COX-2 and inducible nitric oxide synthase (iNOS), using LPS-activated RAW264.7 macrophages as model (Mayo et al., 2005). In addition, Deng et al. have shown that MLT, but not tryptophan or serotonin, time- and concentration-dependently inhibits the LPS-induced protein levels and promoter activities of COX-2 and iNOS in RAW264.7 cells (Deng et al., 2006). Noteworthy MLT, like serotonin and tryptophan, is an indole derivative. Furthermore, Noguchi et al. have suggested that COX-2-dependent exogenous PGE2 downregulates IL-1α induced production of matrix metalloproteinase-13 (MMP-13) *via* E-type prostaglandin (EP) receptor 1 (EP1)

in human periodontal ligament cells (Noguchi et al., 2005). Endogenous PGE2 may be involved in regulating the destruction of extracellular matrix components in periodontal lesions. However, exogenous PGE2 may act as an anti-inflammatory agent *via* the inhibitory prostanoid receptor(s) EP receptor (Meja et al., 1997). Therefore, MLT may prevent various oral diseases including periodontitis, even neoplastic diseases such as precancerous leukoplakia, lichen planus, and oral cancer (Gomez-Moreno et al., 2010). Murakami et al. (Murakami et al., 2012) in a recent study, have found that MLT significantly inhibits Pg fimbria-induced expression of the COX-2 gene through suppression of NF-kB activation in RAW264.7 cells and thus may help prevent Pg-induced oral diseases and chronic infections in the body.

2.2.4. *Regulation of the immune system*

2.2.4.1. B cells, T cells, NK cells, thymocytes and bone marrow

It is important to note that MLT is produced not only by the pineal gland, but also in the retina, kidneys and digestive tract (Jaworek et al., 2005). This suggests that the immune system might be affected by MLT originating from different organs of the body. Additionally it was found that human peripheral blood mononuclear cells synthesize biologically relevant amounts of MLT (Carrillo-Vico et al., 2004). This indicates a potential intracrine and paracrine role of MLT in immune regulation.

It is believed that MLT influences cells of the immune system *via* MLT receptors. Both membrane and nuclear receptors have been identified on leukocytes. Membrane receptors were found mostly on $CD4^+$ T lymphocytes, but also on CD8 T and B cells (Maestroni, 2001). Through these receptors, MLT modulates the proliferative response of stimulated lymphocytes. On the other hand, MLT induces cytokine production by human peripheral blood mononuclear cells via the nuclear MLT receptor.

The immunoregulatory activity of MLT was determined with the use of following experimental models: surgical or functional pinealectomy, *in vivo* treatment with MLT or *in vitro* treatment of immune cells with MLT. Some studies demonstrated an immunoenhancing activity for MLT. Daily afternoon injections of MLT induced an increase in thymus weight in the gerbil and spleen hypertrophy in the Syrian hamster. Treatment with MLT also increased the mitogenic response of mouse spleen cells to concanavalin A and lipopolysaccharide (LPS). The mechanism by which MLT acts to enhance the immune response is not fully understood. It is believed that, in part, it may act to increase phagocytosis and antigen presentation (Maestroni, 2001). Indeed it was shown that treatment with MLT enhanced antigen presentation by splenic macrophages to T cells with a concurrent increase in MHC class II expression and synthesis of the pro-inflammatory cytokines IL-1 and TNF-β. Additionally, MLT was observed to induce IL-12 production to drive T cell differentiation towards the Th1 phenotype. The activating effect of MLT on the immune system is also mediated through the regulation of gene expression of cytokines in the spleen, thymus, lymph nodes and bone marrow. It was shown gene expression of M-CSF, TNF-α, TGF-β and SCF was increased in peritoneal macrophages, while IL-1β, IFN-γ, M-CSF, TNF-α and SCF was increased in spleen cells of mice treated with MLT.

Other studies have shown that MLT administration increases NK cell activity in humans. Similar observations were made in mice where treatment with MLT increased antibody dependent cellular cytotoxicity (ADCC) (Vermeulen et al., 1993). Aside from activation of immune cells by MLT, this hormone

also enhances production of NK cells and monocytes in the bone marrow of mice (Currier et al., 2000). MLT seems also to promote the survival of precursor B cells in mouse bone marrow(Yu et al., 2000).

To summarize, MLT is considered as a modulator of hematopoiesis and of immune cell production and function. MLT as been demonstrated to stimulate cytokine production, enhanced phagocytosis, increased NK cell activity and skewing of the immune response toward a helper T cell type 1 profile.

MLT has been shown to aggravate Th1 dependent inflammatory response in animal models of multiple sclerosis and rheumatoid arthritis. Additionally, it was found in rats that MLT is important in controlling cell recruitment from the bone marrow and their subsequent migration to the lung. It may suggest that MLT is involved in allergic lung inflammation (Martins et al., 2001)This observation in line with human studies showing that elevated serum MLT is associated with the nocturnal worsening of asthma (Sutherland et al., 2003) Moreover, it is suggested that MLT may play a role in the etiology and treatment of several dermatoses e.g. atopic eczema, psoriasis and malignant melanoma (Kimata, 2007).

Importantly, while many studies have implicated MLT as a positive regulator of immune responses, a number of other reports have suggested that MLT may act as an anti-inflammatory agent, inhibiting immune responses in some cases. It is believed that the anti-inflammatory action of MLT is at least partly due to the induction of Th2 lymphocytes that produce IL-4, thereby inhibiting the function of Th1 cells (Shaji et al., 1998). Indeed, MLT has been shown to be protective in septic shock (Escames et al., 2006), an animal model of ulcerative colitis (Nosal'ova et al., 2007) and experimental pancreatitis (Leja-Szpak et al., 2004).

2.2.5. Upregulation of antioxidant enzymes

2.2.5.1. GSH peroxidase; GSSG reductase; γ-glutamylcysteine synthase; G-6-P dehydrogenase; hemoperoxidase/catalase, Mn- and Cu-Zn-SODs

Besides its actions in direct free radical scavenging and membrane stabilization, MLT acts on enzymes that either generate or metabolize reactive oxygen intermediates, thereby further increasing its protective activity toward free radicals. Superoxide dismutase (Abulencia et al.), of which there are several isoforms (i.e. Mn- and Cu-Zn-SODs) is considered a major antioxidant enzyme, because it dismutates $O_2^{\bullet-}$ to H_2O_2, thereby not only removing the anion but also reducing the formation of $ONOO^-$ (Rodriguez et al., 2004). MLT has also been shown to influence antioxidant enzyme gene expression. As first reported by Antolin et al. (Antolin et al., 1996) MLT increases mRNA levels for both Cu-Zn-SOD and Mn-SOD in the Harderian gland of female Syrian hamsters after its exogenous administration. Increases in antioxidant enzyme gene expression following MLT injections were later confirmed by the same group in rat brain cortex.

Once generated, H_2O_2 can be easily converted into the highly reactive and destructive $^{\bullet}OH$ in the presence of ferrous ion (Fe^{2+}) via the Fenton reaction. Two enzymes participate in the removal of H_2O_2 from the cellular environment, peroxidases and CAT. The most abundant peroxidase is the glutathione peroxidase (GPx), which is present in both the cytosol and mitochondria. This enzyme has the transition metal selenium at its active site and uses reduced glutathione (Appeltans et al.) as a substrate to transfer electrons to H_2O_2 (and other peroxides) thereby converting it into two molecules of water. The second H_2O_2 metabolizing enzyme is catalase (Sjogren et al.); being mainly present in the peroxisomes, it contains a molecule of ferric ion at its active site and converts two molecules of H_2O_2 into one molecule of water and diatomic oxygen (Mates, 2000).

Pharmacologically, and possibly physiologically as well, MLT stimulates the activity of GPx to remove H_2O_2 from cells; in doing so glutathione (Appeltans et al.) is converted to oxidized glutathione (GSSG); GSSG is reduced back to GSH in the presence of the enzyme GSSG reductase (Fig. ***6***).

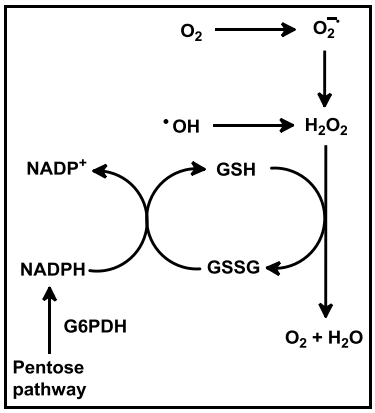

Fig. 6 *- H_2O_2 is metabolized to nontoxic products by glutathione peroxidase (GPx); in the process glutathione (Appeltans et al.) is oxidized to form oxidized glutathione (GSSG). GSSG is converted back to GSH by the enzyme glutathione reductase (GR); this reaction requires the cofactor NADPH, which is generated by the enzyme glucose-6-phosphate dehydrogenase (G6PDH). GPx, GR, and G6PDH are all reportedly stimulated by MLT, thereby lowing the concentration of H_2O_2 and reducing $^{\bullet}OH$ formation. Picture modified from Reiter-1998.*

The activity of this enzyme is also stimulated by MLT, thereby replenishing the important antioxidant GSH. GSSG reductase requires the cofactor NADPH, which is generated from NADP in a reaction catalyzed by G6PDH (Fig. ***6***); the activity of this enzyme is also reportedly increased in the presence of MLT. Thus, several important anti-oxidative enzymes seem to be stimulated by MLT, protecting cells from oxidative damage.

2.2.6. Downregulation of pro-oxidant enzymes

2.2.6.1. NO synthases

The activity of the enzyme nitric oxide synthase (NOS) determines the amount of $NO^{\bullet}$. As noted above, $NO^{\bullet}$ reacts with the $O_2^{\bullet-}$ to form the toxic agent $ONOO^-$. Thus, NOS can be considered a pro-oxidative enzyme, and any factor that reduces its activity would be considered an antioxidant. It has been shown that in both the cerebellum (Pozo et al., 1997) and hypothalamus physiological levels of MLT reduce NOS activity. Thus, inhibition of $NO^{\bullet}$ production may be another way used by MLT to reduce oxidative damage under conditions such as neural ischemia/reperfusion where $NO^{\bullet}$ seems to be important in terms of the resulting damage (Guerrero et al., 1997).

2.2.6.2. Lipoxygenases

Lipoxygenases catalyze the stereo-specific insertion of molecular oxygen into polyunsaturated fatty acids. In mammals, significant knowledge has accumulated about 5-lipoxygenase (5-LO), the enzyme responsible for the synthesis of inflammatory leukotrienes from arachidonic acid. In addition to their inflammatory action, which appears to be crucial for the pathophysiology of asthma, leukotrienes and the 5-LO pathway may play another important physiological role. It has been suggested that the 5-LO pathway is involved in the regulation of pineal MLT synthesis. On the other hand, the pineal hormone MLT, is capable of

regulating the expression of 5-LO gene. The latter mechanism was first described in human B lymphocytes and involves the binding of MLT to a nuclear receptor RZR/RORα, which in turn binds the promoter region of the 5-LO gene and suppresses its expression. In line with this mechanism is the recent observation that MLT deficiency created in rats by pinealectomy resulted in increased expression of 5-LO mRNA in the hippocampus.

Uz et al. (Uz and Manev, 1998) proposed that pineal 5-LO might represent a step in feedback control of MLT synthesis. This scheme could function as follows. During the period of light (Hum et al.), pineal RZR/ROR expression is low which, along with the low levels of MLT, favors the pineal expression of the 5-LO gene. This results in the synthesis of 5-LO protein that is not fully active. Namely, several factors, including intracellular calcium increase, are required for the activation of the 5-LO enzyme. At the onset of darkness, noradrenaline (NA) is released from pinealopetal fibers, whose perikarya are in the superior cervical ganglia. Interaction of NA with pineal adrenergic receptors during the night may increase intracellular calcium, activate 5-LO and lead to formation of leukotrienes, which in turn are needed for stimulation of MLT synthesis. Increased MLT levels, along with the circadian up-regulation of RZR/ROR, gradually suppress 5-LO mRNA expression and, with time, downregulate the 5-LO pathway and attenuate the effect of NA. At the onset of light, NA stimulation ceases, MLT levels drop, RZR/ROR expression decreases, and the 5-LO gene is again up-regulated.

In another study, conducted by Zhang et al. (Zhang et al., 1999) MLT has been seen to reduce, *in vivo*, 12-lipoxygenase (12-LO) expression. The MLT-induced reduction in 12-LO protein level was abolished in the presence of the MLT receptor antagonist luzindole, further establishing the role of MLT in this process. Incubation of pineal homogenates with exogenous MLT partially inhibited 12-LO activity. Taken together, an inverse relationship exists in the endogenous production of 12-hydroxyeicosatetranoic acid (12-HETE), 12-LO mRNA and protein with respect to MLT production in the rat pineal gland. MLT decreased both 12-LO mRNA and protein levels in addition to 12-LO enzyme activity, indicating that MLT is an endogenous modulator of pineal 12-lipoxygenation.

2.2.7. MLT's antioxidant action: clinical significance

2.2.7.1. Decrease of free radicals and other oxidants via scavenging of ($^{\bullet}OH$), ($O_2^{\bullet-}$), ($CO_3^{\bullet-}$), organic cation radicals, O_2 ($^1\Delta_g$), O_3

Since the discovery that MLT is oxidized by photocatalytic mechanisms involving free radicals, its scavenging actions have become a matter of particular interest (Hardeland et al., 1993). MLT's capability for rapidly scavenging hydroxyl radicals has stimulated numerous investigations into radical detoxification and anti-oxidative protection. Evidence has shown that MLT is considerably more efficient than the majority of its naturally occurring analogs (Poeggeler et al., 2002), indicating that the substituents of this indole moiety strongly influence reactivity and selectivity (Hardeland and Pandi-Perumal, 2005). Rate constants determined for the reaction with hydroxyl radicals were $1.2 * 10^{10}$- $7.5 * 10^{10}$ $m^{-1}* s^{-1}$, depending on the method applied. Regardless of the differences in the precision of determination, MLT has been shown independently, by different groups, to be a remarkably good scavenger for hydroxyl radicals.

Contrary to most of its analogs, MLT is largely devoid of pro-oxidant side-effects. Contrary to initial claims in the literature that almost all MLT is metabolized in the liver to aMT6S followed by conjugation and excretion, recent estimates attribute almost 30% of overall MLT degradation to pyrrole ring cleavage (Ferry

et al., 2005). The rate of AFMK formation may be even higher in certain tissues because extrahepatic P450 mono-oxygenase activities are frequently low and, consequently, smaller amounts of aMT6S are produced.

AFMK appears to be a central metabolite of MLT oxidation, especially in non-hepatic tissues (Hardeland, 2005). It should be noted that the kynuric pathway of MLT metabolism includes a series of radical scavengers with the possible sequence of MLT - cyclic 3-hydroxyMLT - AFMK - AMK. In the metabolic steps from MLT to AFMK, up to four free radicals can be consumed (Tan et al., 2003). However, the complete cascade should be only expected under high rates of hydroxyl radical formation. Otherwise, MLT forms AFMK directly and the conversion to AMK is, according to present knowledge, predominantly catalyzed enzymatically. Recent studies have shown a greater number of free radicals eliminated than predicted from the cascade, and many previously unknown products are now being characterized (Than-2006 and J. Rosen & R. Hardeland, unpublished results). The potent scavenger, AMK, consumes additional radicals in primary and secondary reactions (Than et al., 2006). Interestingly, AMK interacts not only with reactive oxygen but also with reactive nitrogen species (Guenther et al., 2005).

MLT has also the capacity of up-regulate and down-regulate enzymes as we have seen before. The attenuation of NO (nitric oxide) formation for example, by MLT, is significant as it limits the rise in the levels of the pro-oxidant metabolite, peroxynitrite, and of free radicals derived from this compound (ie. NO_2, CO_3^- and OH radicals). It also helps to reduce the inflammatory response (Hardeland, 2005).

Since mitochondria are the major source of free radicals, the damage inflicted by these radicals contributes to major mitochondria-related diseases. Electron transfer to molecular oxygen at the matrix site, largely at the iron–sulphur cluster N2 of complex I, is a main source of free radicals (Genova et al., 2004). This process also diminishes electron flux rates and therefore the ATP-generating potential. MLT increases mitochondrial respiration and ATP synthesis in conjunction with the rise in complex I and IV activities (Leon et al., 2005).

The effects of MLT on the respiratory chain may represent new opportunities for the prevention of radical formation, in addition to eliminating radicals already formed. A model of radical avoidance, in which electron leakage is reduced by single electron exchange reactions between MLT and the components of the electron transport chain (ETC), was proposed by Hardeland and his coworkers (Hardeland et al., 2003b). According to this model, a cycle of electron donation to the respiratory chain at cytochrome c should generate a melatonyl cation radical which can compete, as an alternate electron acceptor, with molecular oxygen for electrons leaking from N2 of complex I, thereby decreasing the rate of O_2^- formation. In the proposed model, not only are electrons largely recycled to the respiratory chain, but most of the MLT is also regenerated in the cycle. Inasmuch as the recycled electrons are not lost for the respiratory chain, the potential exists for improvements in complex IV activity, oxygen consumption and ATP production.

Similarly, the highly reactive MLT metabolite, AMK, may undergo single-electron transfer reactions (Ressmeyer et al., 2003). The mitochondrial protection by AMK was proposed (Hardeland et al., 2003b). and experimentally confirmed (Acuna-Castroviejo et al., 2003). In a manner similar to the action attributed to MLT, AMK exerts its effects on electron flux through the respiratory chain and seems to improve ATP synthesis.

2.2.8. Prevention of apoptosis

2.2.8.1. Direct inhibition of mtPTP opening and attenuation of mitochondrial electron leakeage

Numerous study have been conducted investigating the effects of MLT on mitochondria. A direct inhibition of the mitochondrial permeability transition pore (mtPTP) by MLT is observed at elevated concentrations (Andrabi et al., 2004) and presumably based on a low-affinity binding site, should be of importance in experiments on prevention of apoptosis. Interpretations concerning effects in the lower pharmacological range, as by administration via the drinking water, would require other interpretations. One idea had been that MLT might participate in a kind of redox cycling by interacting with components of the ETC and contributing to an electron shuttle (Hardeland et al., 2003a). This possibility was also discussed for the MLT metabolite AMK (Hardeland, 2005) which also exerts protective effects in mitochondria (Acuna-Castroviejo et al., 2003). This interpretation would require further substantiation. Another recent finding concerns the existence if a high affinity binding site with a K_d of 150 pM, localized according to inhibitor studies at the amphipathic ramp of complex I. These results have not yet been published in detail, but were cited a couple of times (Hardeland, 2009). If MLT binding to this site is not related to electron exchange reactions, as previously assumed (Hardeland, 2005) its presence would imply a regulatory role at the first control point of the ETC and should, therefore, be assumed to modulate electron flux. This possibility would go beyond classic mitochondrial protection by an antioxidant and antinitrosant agent, but could likewise contribute to anti-oxidative effects by metabolic adaptation.

Another kind of metabolic adaptation, with respective consequences for respiratory capacity, energy efficiency and electron leakage, might be achieved by stimulation of mitochondrial biogenesis. We had previously suggested a relationship between MLT and sirtuins an assumption that has recently gained some support. In SAMP8 mice, a few, rather preliminary data showed upregulation of SIRT1 by MLT (Gutierrez-Cuesta et al., 2008). More detailed studies have now demonstrated that MLT favors the hippocampal expression of SIRT1 in a model using sleep-deprived rats (Chang et al., 2009). In another investigation, effects of MLT were compared in neuronal cultures from young and aged rats (Tajes et al., 2009). MLT stimulated SIRT1 expression in the aged neurons to levels approximating those from young rats and caused enhanced deacetylation of various SIRT1 substrates, such as PGC-1α, FoxO1, NFκB, and p53, effects which were largely reverted by the SIRT1 inhibitor sirtinol (Tajes et al., 2009). Although this has not yet been demonstrated directly, the MLT-induced deacetylation of PGC-1α strongly suggests that a long-term treatment with the indoleamine would stimulate mitochondrial biogenesis. Finally, another connection between MLT and SIRT1 seems to exist, which may deserve further attention. SIRT1 was shown to modulate chromatin remodeling via the circadian clock gene protein CLK. Thereby, it seems to directly influence at least peripheral oscillators by interacting with the CLK/BMAL1 complex (Nakahata et al., 2009). Remodeling of chromatin represents a necessity of circadian gene expression and is also influenced by MLT, the major non-photic synchronizer. Relative MLT deficiency, as occurring during aging, causes circadian dysregulations (Jung-Hynes and Ahmad, 2009). Pronounced rhythms in metabolism exist in numerous organs including the brain, and consequently lead to periodic radical generation (Hardeland et al., 2003b). To reduce oxidative stress, a delicate internal coordination of rhythms is required Hence, the convergence and eventual interdependence of MLT and sirtuin pathways might be of high interest in terms of aging and radical avoidance. The effects on chromatin structure should lead to numerous secondary changes in circadian functions, including mitochondrial metabolism.

2.3. MLT: THERAPEUTIC AND CLINICAL UTILIZATION

2.3.1. Insomnia and phase shift conditions: jet lag and shift work

Evidence from studies in both day-active animals and humans that the circadian pacemaker promotes wakefulness at certain times of day, together with evidence that neuronal firing of the mammalian SCN is inhibited by SCN Mel1a receptor-specific MLT binding, has led to the hypothesis that MLT may act to facilitate sleep by inhibiting the circadian drive for waking that emanates from the SCN (Scheer and Czeisler, 2005). The direction in which MLT phase shifts the circadian system depends on its time of administration. When given late in the subjective day (at dusk), MLT phase advances the clock while its administration early in the subjective day (at dawn) phases delays circadian rhythms. Although results are still controversial, studies suggest that night-time MLT administration help induce sleep in people with disrupted circadian rhythms (such as those suffering from chronic insomnia or jet lag or poor vision or those who work the night shift) and those with low MLT levels (such as some elderly subjects) (Brzezinski et al., 2005). When used to improve sleep, i.e. to decrease sleep latency and/or cause more prolonged sleep, it is taken roughly 30 min prior to bedtime. MLT has been successfully used with various degrees of effectiveness to enhance sleep processes in elderly individuals with insomnia and in individuals with restless leg syndrome, REM sleep disorder behavior, delayed sleep phase syndrome, manic patients with insomnia and in patients with fibromyalgia. Recently, in a meta-analysis by Brzezinski et al. (Brzezinski et al., 2005) in which 17 studies were included to investigate the sleep-promoting potency of MLT, it was found that MLT has only a modest sleep-promoting effect, with an increase in sleep efficiency of 2–3%. Jet-lag is the result of long distance travel east/west crossing time zones at a rapid rate. Symptoms such as sleep disturbance, loss of appetite, reduced psychomotor efficiency and general malaise may occur. Circadian rhythms need about 1 day to adapt for each time zone crossed. In other words, 5-h time difference will require approximately 5 days of adaptation. In fact, a recent review of scientific studies found that MLT supplements help prevent phase-shift conditions: shift work and jet lag, particularly in people who cross five or more time zones (Arendt and Skene, 2005). Multi-centre clinical trials are needed to investigate whether chronic MLT administration may be beneficial for the treatment of phase-shift conditions and chronic insomnia. In addition to the potential beneficial influences on sleep, chronic night-time MLT administration may also be of clinical relevance in the treatment of hypertensive patients with an impaired BP rhythmicity (Claustrat et al., 2005).

2.3.2. Immunity

Circadian rhythmicity is revealed in circulating cells, lymphocyte metabolism and transformability, circulating hormones and other substances that may exert various actions on different targets of the immune system, cytokines, receptors, and adhesion molecules, cell cycle events in health and cancer, reactions to antigen challenge, and disease etiology and symptoms Interactions between MLT and the immune system have been known for nearly three decades, and in virtually all cases, MLT has been proven to have immune-enhancing effects. Currently, accumulated evidence shows that the pineal is able to play an important role in modulating the immune response (Guerrero and Reiter, 2002). MLT can stimulate the immune response and correct immunodeficiencies secondary to acute stress, viral diseases or drug treatment. Binding of MLT to its specific receptors resulted in an up-regulation of cytokine production and immune function. In humans, daily oral MLT administration increases natural-killer cell activity (Guerrero and Reiter, 2002). Additionally, MLT reportedly regulates gene expression of several immunomodulatory cytokines including tumor necrosis

factor-α, transforming growth factor-beta and stem cell factor by peritoneal macrophages as well as the levels of interleukin-1beta, interferon gamma, tumor necrosis factor-a and stem cell factor by splenocytes (Liu et al., 2001). The rise in blood MLT levels in humans at night stimulates associated rise in the thymic production of peptides including thymosin 1a and thymulin. Finally, MLT is a potent inhibitor of apoptosis in immune cells. In addition, interactions between the pineal gland and the immune system are bidirectional as interleukins and cytokines (interferon gamma) affect MLT synthesis and release. Also, there has been described MLT scavenging of nitric oxide or free radicals in lymphoid cells, which could explain the MLT-modulated circadian variation in the experimental chronic inflammation.

This kind of approach raises new questions regarding the mechanism of chronic inflammation, in disorders such as rheumatoid arthritis and nocturnal asthma, diseases that present rhythmic symptoms during a 24 h period (Sulli et al., 2002). It is clear that MLT provides a functional link between the neuroendocrine and immune-hematopoietic systems. The pineal neurohormone MLT has been widely shown to exert an immunostimulatory and anti-apoptotic role, mainly by acting on Th cells and on T- and B-cell precursors respectively. The increased prevalence of autoimmune diseases, such as rheumatoid arthritis, which is seen in northern Europe also may be related to the increased immunostimulatory effects that are exerted during the night by MLT and to a reduced neuroendocrine modulation during the light phase of the photoperiod (cortisol) (Maestroni et al., 2005). Nocturnal asthma is associated with elevation and phase delay of peak serum MLT levels (Sutherland et al., 2003). Elevated MLT levels might contribute to the pathogenesis of nocturnal asthma. However, MLT can improve sleep in patients with asthma Further studies looking into long-term effects of MLT on airway inflammation and bronchial hyper-responsiveness are needed before MLT can be recommended in patients with asthma(Campos et al., 2004). However, for individuals with chronic sarcoidosis who do not respond to corticosteroids, MLT may be an effective alternative therapy. In one study, individuals with sarcoidosis who did not respond to corticosteroid therapy experienced the following improvements after taking MLT for 4–12 months: improved breathing; decreased lymph node swelling and normalization of blood tests. Once the MLT supplements were discontinued, however, these improvements disappeared. Given that MLT is generally considered to be immunostimulatory, the question as to whether it should be taken by individuals with an autoimmune disease has been raised. To date, the information is meagre on this issue, although in one case of Crohn's disease, a condition of excessive immune reactivity of the gut wall was reported; MLT did aggravate the condition (Calvo et al., 2002). Whether this will be a general finding in autoimmune diseases, however, remains to be established.

2.3.3. Cardiovascular diseases

Cardiovascular diseases (coronary heart disease, stroke, etc.) remain the major cause of death in most developed countries. The clinical importance of circadian biological rhythms has been strengthened by a number of studies showing a circadian distribution of cardiovascular events such as myocardial infarction, stroke, complex arrhythmia or sudden cardiac death. Incidence of cardiovascular events showed a maximum during the early morning hours after awakening from sleep. In addition, a number of pathophysiological mechanisms have been identified to coincide with this peak including blood pressure (BP) and heart rate surges, decreased endothelial dilatory capacity of peripheral and coronary arteries, enhanced sympathetic activity, decreased cardiac electrical stability and increased platelet aggregation. This time window of high risk for the incidence of cardiovascular events has been identified as a target for new treatment and

prevention strategies including new release forms of antihypertensive and anti-ischemic drugs. Decreased MLT production was found in several cardiovascular diseases (Altun et al., 2002). The use of MLT as an antihypertensive, antioxidant and anti-ischemic drug has been explored and opens new opportunities for the management of cardiovascular dysfunction and disease from a circadian perspective (Altun and Ugur-Altun, 2007).

A role of MLT in the control of cardiovascular rhythmicity is supported by animal and human studies. Pinealectomy of laboratory rats results in hypertension while the hypertensive effect of pinealectomy was blocked by administration of exogenous MLT. There is an inverted relationship between plasma MLT concentrations and acrophase of the BP rhythm in man, as high MLT level coincides with lower BP values. In humans, administration of exogenous MLT decreases BP in normotensive patients, in patients with essential hypertension (Scheer et al., 2004) and in patients with diabetes mellitus type 1(Cavalli et al., 2003). Single exogenous MLT intake can lower BP, but only when MLT is taken during the daytime, when general SCN neuronal activity is high and endogenous MLT levels are low. On the contrary, repeated nighttime MLT intake supports the endogenous MLT rhythm, improving circadian rhythmicity (Sharkey and Eastman, 2002).

Synthesis and release of MLT are stimulated by NE via beta1-adrenoceptors, and this process is further potentiated by stimulation of alpha1-adrenoceptors. Accordingly, beta-blockers have been shown to reduce the production of MLT. Carvedilol is an effective adrenergic alpha1- and beta1-antagonist. However, Stoschitzky et al. (Stoschitzky et al., 1999) reported that carvedilol does not decrease nocturnal MLT production. Verapamil does not alter MLT release . Lacidipine treatment in hypertensive patients increases endogenous MLT production (Escames et al., 2004). However Lusardi et al. (Lusardi et al., 2000) showed that the chronic evening ingestion of MLT in hypertensive patients well-controlled by nifedipine GITS induces a BP increase and a heart-rate acceleration. Kinetic or pharmacodynamic interaction between MLT and nifedipine, is able to impair the antihypertensive efficacy of the calcium-channel blocker. This suggests caution in uncontrolled use of MLT in hypertensive patients. As the pineal hormone might interfere with calcium-channel blocker therapy, it cannot be considered simply a dietary supplement. Zaslavskaia et al. (Zaslavskaia et al., 1998) showed that combination losartan and MLT-reduced BP more noticeably than losartan alone. Recently, this group also showed that combination moxonidine and MLT is more effective on hemodynamic parameters in patients with arterial hypertension than moxonidine alone. MLT (1–5 mg) has been widely used as a nutritional supplement in the United States for several years, without any serious adverse side effects being reported. Daytime exogenous MLT intake may result in sleepiness and hypothermia during the day and should thus be avoided. MLT taken at night could thus be a gentle alternative or supplement to regular antihypertensive medication.

The increased formation of cardiac malondialdehyde and serum nitric oxide, and the decreased activity of cardiac antioxidant enzymes (i.e. superoxide dismutase, glutathione peroxidase and catalase) were found on chemotherapeutic drug-induced oxidative damage in the heart tissue. Paskaloglu et al. (Paskaloglu et al., 2004) showed that MLT or insulin alone can provide limited protection against hyperglycemia-induced oxidative damage in diabetes. Combined treatment with insulin and MLT can suppress hyperglycemia, prevent oxidative damage and can restore endothelial function completely, implying that treatment of diabetes mellitus with this combination would be beneficial. Zaslavskaya et al. (Zaslavskaya et al., 2004) studied MLT effects on contractile myocardial function; patients with post-myocardial infarction and heart failure, assessed as stage II-III by New York Heart Association (NYHA). They found MLT associated anti-anginal and anti-ischemic effects, indicating improvement of contractile function.

The ejection fraction increased; the anti-anginal effect appeared by the fifth day of treatment. Thus O'Rouke (O'Rourke et al., 2003) showed that additional anti-ischemic effect of MLT, acting via specific MLT receptors, inhibits nitrate tolerance in coronary arteries and that this effect is dependent on the presence of vascular endothelium. MLT has been found to protect heart tissues against oxidative damage induced by other free radical-generating agents and processes.

Disturbances in renal hemodynamics and direct cytotoxicity have been identified as key factors in the pathogenesis of contrast induced nephropathy (CIN). Contrast agents markedly aggravate this physiological hypoxia of the outer medullary layer because they cause enhanced metabolic activity and oxygen consumption as a result of osmotic diuresis and increased salt delivery to the distal nephron. The result of the hemodynamic changes is hypoxia followed by oxidative stress and repair. Recently, we experimentally demonstrated for the first time that pre- and post-treatment with MLT did prevent and protect renal function as measured by Fe-Na, serum Cr and Cr clearance in rats with CIN (Gazi et al., 2006). MLT protects renal function against the development of CIN and opens a new era in the management of CIN. This study revealed that only pretreatment with MLT was not sufficient to prevent renal deterioration completely and improve renal function in CIN. However, rats pre- and post-treated with MLT showed significant improvement in their renal function possibly related to ongoing continuous MLT effect.

Several recent publications present evidence that MLT has significant protective actions against the cardiac damage and altered physiology that occurs during ischemia/reperfusion (I/R) injury (Sahna et al., 2002). Sahna et al. (Sahna et al., 2005) showed that physiological concentrations of MLT were important in preventing I/R-induced cardiac infarct size. The results showing increased I/R-induced cardiac injury after reduction in physiological levels of MLT have implications for elderly people in as much as in old individuals endogenous levels of MLT are significantly lower than in young individuals. Several studies have reported that humans with cardiovascular diseases have noticeably lower circulating MLT levels than do age-matched subjects without significant cardiovascular deterioration. Similarly, patients suffering from cardiac syndrome X have an attenuated nocturnal peak in serum MLT levels relative to those of age-matched individuals with no cardiac pathology (Altun et al., 2002). It remains unknown, however, whether the reduced endogenous MLT levels in patients with cardiovascular disease is a cause, an effect or even related to the compromised cardiovascular function.

2.3.4. Neurological disorders

A large number of individuals suffer from primary headache (migraine and cluster headache). Migraine and cluster headache can be viewed as transient disturbances of the body adaptive response to internal or external environmental changes. Among these factors, light is a major precipitating or aggravating factor of attacks (Claustrat et al., 2004). Abnormalities in the secretion of MLT and cortisol in patients with migraine and cluster headache have been documented (Peres, 2005). MLT mechanisms are related to headache pathophysiology in many ways, including its anti-inflammatory effect, toxic free radical scavenging, reduction of pro-inflammatory cytokine upregulation, nitric oxide synthase activity and dopamine release inhibition, membrane stabilisation, γ-aminobutyric acid (GABA) and opioid analgesia potentiation, glutamate neurotoxicity protection, neurovascular regulation, serotonin modulation and the similarity of chemical structure to that of indomethacin. Treatment of headache disorders with MLT is promising, particularly in cluster headaches, hypnic headaches, indomethacin-responsive headaches and migraine (Peres, 2005).

Recent studies showed disruption of nocturnal surge of MLT in ischemic stroke patients and patients with acute cerebral hemorrhage. Endogenously produced and exogenously administered MLT may reduce the degree of tissue damage and limit the biobehavioral deficits associated with ischemia/reperfusion injury in the brain (i.e. stroke). MLT's protective actions against ischemia/reperfusion injury are believed to stem from its direct free radical scavenging and indirect antioxidant activities, possibly from its ability to limit free radical generation at the mitochondrial level (Reiter et al., 2005). Recently, a meta-analysis demonstrated a marked efficacy of MLT in animal models of focal cerebral ischemia, identified priority areas for future animal research, and suggested MLT as a candidate neuroprotective drug for human stroke (Macleod et al., 2005).

The decline in MLT production in aged individuals has been suggested as one of the primary contributing factors for the development of age-associated neurodegenerative diseases. Parkinsonism is the second most common neurodegenerative disorder after Alzheimer's disease. MLT not only plays an important role in the regulation of circadian rhythms, but also acts as an antioxidant and neuroprotector that may be of importance in ageing and neurodegenerative diseases. MLT has been shown to be effective in arresting neurodegenerative phenomena seen in both in vivo and in vitro studies of Alzheimer's disease and Parkinsonism (Srinivasan et al., 2005).

Decreased MLT levels have also been reported in patients with some forms of epilepsy. Some authors suggest a potential use of MLT as an adjunct to anti-epileptic therapy because of its diverse spectrum of action as an antioxidant, neuroprotector and free radical scavenger, thus offering the advantage of reducing oxidant stress and subsequent damage. The beneficial effects of MLT on sleep, its wide safety window and its ability to cross the blood–brain barrier have the potential to improve quality of life in pediatric epilepsy (Gupta et al., 2004). Molina-Carballo et al. (Molina-Carballo et al., 1997) showed that high doses of MLT as adjunctive anti-epileptic therapy in a child with severe myoclonic epilepsy improved both the frequency of seizures and the EEG tracing. MLT could be beneficial in combination with other anti-epileptic medications (Gupta et al., 2004). However, Sheldon (Sheldon, 1998) showed that proconvulsant effects of MLT in neurologically disabled children. Although MLT had a positive effect on sleep disorders, four of six children with severe neurological disabling conditions and seizures had increased seizure activity after MLT treatment. Seizure frequency returned to baseline after MLT was discontinued and re-challenge resulted in recurrence.

2.3.5. Psychiatric diseases

In normal subjects, the secretion of MLT, the pineal hormone that regulates the rhythm of many functions, exhibits a circadian pattern synchronized with the day–night cycle. An alteration of this secretory pattern has been found in various psychiatric disorders (seasonal affective disorder (SAD), bipolar disorder, unipolar depression, bulimia, anorexia, schizophrenia, panic disorder, obsessive compulsive disorder and delirium) (Pacchierotti et al., 2001). Numerous studies have reported low MLT secretion in depression, but other studies have suggested no deficit or an increase (Crasson et al., 2004). Recent studies evidence conflicting results (normal MLT peak, normal or phase delay rather than phase advanced peak) which could be explained by methodological differences (size of samples, duration of drug wash-out, selection of patients and comparison of patients with not strictly matched controls) and seniority of the disease(Crasson et al., 2004). Seasonal affective disorder is a condition of regularly occurring depressions in winter with a remission the following spring or summer. In addition to depressed mood, the patients tend to experience increased appetite and an increased duration of sleep during the winter. SAD is a relatively common condition,

affecting 1–3% of adults in temperate climates, and it is more prevalent in women. SAD patients' circadian rhythms are delayed relative to the sleep/wake or rest/activity cycle (Magnusson and Boivin, 2003). MLT levels in the SAD patients were found to be on average 2.4 times as high as in the controls (Karadottir and Axelsson, 2001). Heterogeneous results were also observed for MLT profiles in schizophrenia and anorexia nervosa . Delirium is a common syndrome among hospitalized elderly patients. In humans, sleep and circadian rhythms are disturbed during delirium, and both are influenced by the hormone MLT. Recently, a study showed that urinary MLT metabolite during delirium was higher in hypoactive and lower in hyperactive patients (Balan et al., 2003). At present, it is not known if such alterations have an etiological role or are secondary to the dysfunctions underlying various psychiatric disorders. An understanding of the role of the MLT and of its alterations in psychiatric diseases could help to identify the biological mechanisms underlying such disorders.

2.4. MLT: USE IN SLEEP DISRUPTION IN CRITICALLY ILL PATIENTS

Sleep disruption in critically ill patients is a well-recognized phenomenon. Indeed, one intensive care unit (ICU) reported that none of their mechanically ventilated patients displayed a normal sleep pattern (Cooper et al., 2000). The consequences of inadequate sleep are catabolism induction and impaired cellular and humoral immunity, which may lead to delayed healing. Additionally, Eveloff and Gabor et al. (Gabor et al., 2001) have reviewed the consequences of sleep disruption in the ICU and concluded that it can cause respiratory dysfunction, which could prolong mechanical support. This type of respiratory dysfunction is due to increased respiratory muscle fatigue and decreased ventilator responsiveness to hypercapnia (Gabor et al., 2001). Patients themselves perceive their sleep in the ICU as worse than usual and that this does not improve during their ICU stay . Difficulty in sleeping is a common symptom identified by cancer patients receiving intensive care (Nelson et al., 2001). Patients also report that sleep disruption is also one of the most stressful components of their time in the ICU.

Some patients will be predisposed to sleep disturbances in the ICU due to chronic illness. Patients with chronic obstructive pulmonary disease have increased sleep latency, reduced total sleep time and experience increased arousals. Cheyne-Stokes respiration is common in patients with chronic heart failure whose ejection fractions are less than 40% and is associated with sleep fragmentation and reduced sleep efficiency (Quaranta et al., 1997). Patients with acute neurological disorders (e.g. intracerebral hemorrhage, meningitis) may also suffer from Cheyne-Stokes respiration. Asthmatic patients are known to experience sleep disorders including early awakening (Janson et al., 1990).

The causes of disrupted sleep in critically ill patients have been reviewed extensively (Gabor et al., 2001). Effects of environmental factors such as excess noise and lighting, the patient's acute illness itself, patient care activities and mechanical ventilation are detrimental to quality sleep in the ICU. Noise is often regarded as the most disruptive on sleep function. However, environmental noise was not found to be a major determinant of sleep disruption in mechanically ventilated medical patients (Freedman et al., 2001). Another study found that only 30% of sleep arousals and awakenings were due to noise and patient care activities (Gabor et al., 2003) which suggests that other environmental or patient related factors are important in the etiology of this condition.

The effect of medication on sleep disturbances in critically ill patients has not been documented systematically. Polypharmacy, increased use of the intravenous drugs and the particular drugs commonly used in ICU increase the risk of drug induced sleep disturbances in this group of acutely ill patients. This review outlines pharmacological considerations relating to sleep disruption in critically ill patients, and the various treatments available.

2.4.1. Normal sleep architecture

Sleep occurs in two distinct phases, involving rapid eye movement and non-rapid eye movement. Non-rapid eye movement comprises four subdivisions (1–4) with increasing sleep depth. The more restful sleep of stages 3 and 4 represent slow wave sleep. Rapid eye movement (REM) is also a restful period of sleep but has a lower threshold for awakening than slow wave sleep. Dreaming normally occurs during periods of rapid eye movement sleep. Normal sleep architecture is described by a continuous cycle during the night between non-rapid eye movement and rapid eye movement sleep, the sleep cycle lasts approximately 90 min. Rapid eye movement periods become more prolonged the further into the total sleep episode.

The sleep-wake cycle and sleep stages are regulated by a complex interplay of numerous neurotransmitters including NE, serotonin, acetylcholine, dopamine, histamine and γ aminobutyric acid Adenosine has a role in the initiation of the more restful sleep phases (Porkka-Heiskanen et al., 2002). Pituitary hormones may affect sleep and be of functional significance in the maintenance and quality of sleep The neurohormone MLT is also important in regulating the sleep-wake cycle in humans (Sack et al., 1997). Drugs that have an effect on these neurotransmitters and hormones may influence normal sleep architecture. Sleep stage classification and spectrum analysis using polysomnography (continuous polygraph of multiple physiological variables during sleep) can provide detailed information on the adverse effects of drugs on sleep (Dietrich, 1997). However, few data are available, especially in relation to critically ill patients. Hence data forming the basis of this review is drawn from studies related to sleep disorders in healthy subjects and non-critically ill patients.

2.4.2. Sleep disorders associated with critical care

The article by Bourne et al. (Bourne and Mills, 2004) focuses on the common sleep disorders in critically ill patients on ICU, principally insomnia (an intrinsic sleep disorder (dyssomnia)) and nightmares (a parasomnia). Drugs that suppress rapid eye movement sleep can cause nightmares, possibly due to increased rapid eye movement intensity over shorter periods. Insomnia and nightmares comprise some of the characteristic symptoms of the post-traumatic stress disorder, whose importance after ICU discharge is becoming more recognized. It has been suggested that post-traumatic stress disorder related to ICU care may be associated with periods of amnesia (Jones et al., 2001). Drug related reduction in slow wave sleep might affect memory formation and predispose the patient to post-traumatic stress disorder (Jones et al., 2001). Rundshagen and colleagues found that in sedated and ventilated patients discharged from ICU, 9.3% could recall nightmares and 6.6% hallucinations (Rundshagen et al., 2002). Follow-up clinics indicate that continued sleep disturbance occurs in patients after ICU care (Eddleston et al., 2000). ICU patients are not deprived of sleep over a 24-h period, but demonstrate sleep fragmentation, with increased stage 1 and

reduced stage 2, 3 and 4 and little rapid eye movement sleep (Freedman et al., 2001). This sleep pattern may also predispose to post-traumatic stress disorder.

2.4.3. Pharmacokinetics of MLT in health subjects

MLT secretion in humans increases considerably with the fading of the light, reaching peak plasma concentrations between the hours of 2:00 and 4:00 (Brzezinski, 1997) then decreased gradually in the second half of the night. The age of the subject significantly influences hormone secretion by the pineal gland: in infants it is practically absent while it reaches the highest levels and the characteristic circadian pattern in children from one to three years and then decrease with age. In the young adult, mean values of plasma MLT during the day and at night-peak are, respectively, 10 and 60 pg/ml (Liu et al., 2000).

The secretory rate of hormone epiphyseal follows the alternating light-dark cycle by integrating the signals from the SCN; it is proved, however, that a certain periodicity is retained even in individuals not exposed to light for 24 hours (Czeisler et al., 1995).

Overhead lighting is not directly responsible for the production of MLT secretion but affects the circadian secretion managing to completely block the release if the exposure is short but considerable intense. In healthy volunteers the inhibition of hormone secretion by the light is dose-dependent: at intensities equal to 200 - 400 lux (ordinary fluorescent light) there is an initial inhibition which becomes maxima after exposure to 600 lux for an entire hour. Further increases in light intensity, as well as duration of exposure, no additional effects (Brzezinski, 1997). Curious is the observation that, in blind subjects with no pupillary reflex at direct light, this still exerts an inhibiting action on MLT secretion, suggesting the existence of two different photo-receptive systems: one regulator of hormone epiphyseal secretion and the other one responsible for the conscious perception of light exposure (Czeisler et al., 1995).

In the same subjects the bioavailability of the hormone administered orally varies considerably depending on the dose used: 1 to 5 mg of MLT induce the achievement of plasma concentrations 10-100 times higher than the physiological night peak with prolonged plasma half-life from 4 to 8 hours (Brzezinski, 1997). In healthy subjects, no side effects were observed taking MLT, although it lacks an accurate assessment of the intensity of the physiological effects of the hormone (hypothermia, increased sleep, decreased attention span and alertness, alteration of the cycle sex hormone) in the subject that takes, for long time, large quantities of the same substance (Liu et al., 2000). Despite MLT do not have an endocrine strong action, it's well-known a decrease in the plasma concentration of luteinizing hormone and an increase in that of prolactin, in the subject that assumes pharmacological doses of the pineal derivative (Brzezinski, 1997).

2.4.4. Pharmacokinetics of MLT in ICU patients

The clinical study carried out by Shilo e al. (Shilo et al., 1999) at the Intensive Care Unit of the Sapir Medical Center in Israel is an important starting point in the approach to the regulation of sleep and daily secretion of MLT in critically ill patients. The work of Shilo is intended to demonstrate an alteration in the production of circadian epiphyseal hormone and a correlation between this data and the unequivocal observation of decreased quality of sleep in patients in the ICU (Krachman et al., 1995). The research team conducted an observational study, involving fourteen conscious patients (eight women and six men) admitted to the ICU, with the following characteristics: mean age 61 years ± 11 years, hospitalization higher than 4 days , normal renal function, abstention by drugs whose use interferes with the secretion of MLT (beta-blockers, beta-agonists and opioids).

The results obtained in the group of critically ill patients were compared with those of six patients hospitalized in a department of internal medicine with similar characteristics to those of the first group. Actigraphy was chosen as the method of detection of sleep time in critically ill patients because it correlates precisely with the data coming from the surveys polysomnographic (Cole et al., 1992). During the study period, the fourteen patients have worn on the wrist an actigraph, a small tool, like a watch, capable of detecting the subject's movements along the arc of twenty-four hours. The results produced by reading the traces of actigraph have confirmed the suspicion that the sleep of critically ill patients is not only poor but also of poor quality when compared with the patients 'control' (Fig. **7**).

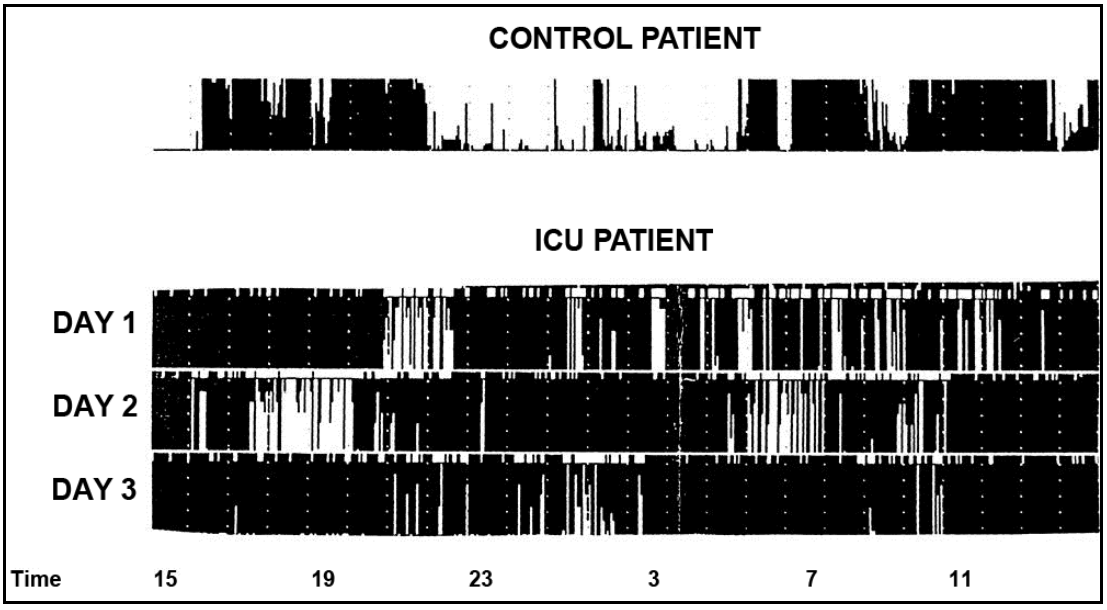

Fig. 7 – *Actigraph recordings in control and in ICU patients. Picture modified from Shilo-1999.*

The analysis of the traces presented above it can be seen in critically ill patients completely lacking a resting phase, and then to abstain from voluntary movement, which characterizes the central hours of the night (from about 22:00 to 7:00 the next morning) in the group of patients "control".

During the study period, the urine of the fourteen patients was collected every three hours for 24 consecutive hours and from each sample 5 ml were taken for the determination of the metabolite 6-sulfatoxyMLT (aMT6s) content using an radioimmunoassay (RIA) method.

The results of the study conducted by Shilo and colleagues have been fundamental: patients admitted to the ICU, sleep for very short periods during the day and during the night, confirming the fact that there must be a deregulation of the internal biological clock. During the night, in fact, even in the ICU, the noises are reduced as well as the overall level of activity of the department theoretically allowing a rest which, in fact, is not realized. Everything appears to be closely related to the frequent occurrence, in these critically ill patients, of delirium and ICU syndrome (Ely et al., 2001).

The pattern of aMT6s secretion proved to be abnormal in all fourteen patients enrolled with a reduction in the concentration of the hormone peak and an abolition of the same in twelve of them when compared with the sample of six patients in general medicine (Fig. 8).

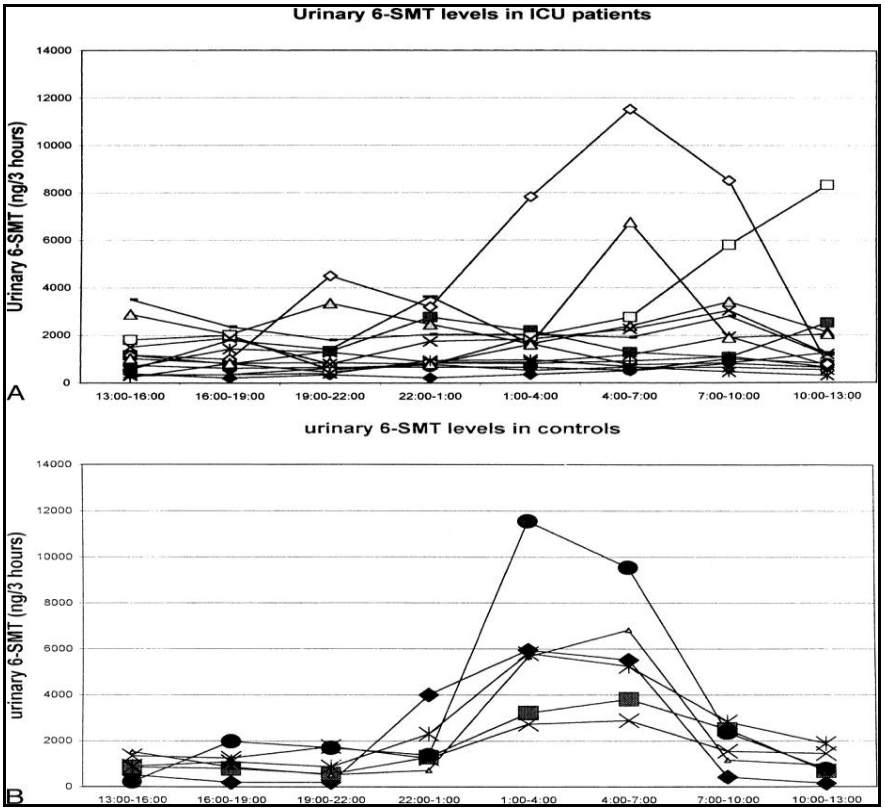

Fig. 8 *– (A) Urinary aMT6s levels in ICU patients. (B) Urinary aMT6s levels in control subjects. Picture modified from Shilo-1999.*

Sepsis is a clinical condition is very common in patients admitted to the Intensive Care Unit, contribute to its development several factors including immunosuppression, iatrogenic or self-induced. In this context, the loss of the regulation of circadian MLT secretion, with its immunomodulator action, constitutes a possible key factor in the worsening of the clinical case. The study conducted by Mundigler et al. (Mundigler et al., 2002) investigates the relationship between the septic condition and the daily alteration of the epiphyseal hormone regulation. To reach this aim, the daily urinary excretion of the major metabolite of MLT has been studied in a group of septic patients (Group A) compared with a group of critically ill patients with sepsis (Group B) and a group of healthy volunteers (Group C). Group A included 17 patients with severe sepsis, defined according to the following criteria: body temperature > 38.5°C or < 35.5°C, leucocytes (WBC) > 12000/mm^3 or < 4000/mm^3, heart rate > 100 beats / min, tachypnea > 20 breaths / min or hypocapnia ($PaCO_2$ <32 mmHg), mean arterial pressure < 60 mmHg or need of vasoactive amines, infection with bacterial isolation. In Group B included seven patients admitted to the ICU for reasons other than sepsis (7

post-anoxic coma). Group C includes 21 healthy volunteers. Exclusion criteria from the study were: hepatic failure, renal failure, use of beta-blockers. In order to avoid accidental exposure to light at night, patients belonging to groups A and B are fitted with a mask that covers the eyes and the lights of the department are lowered. As the study made by Shilo et al., patients are monitored every 4 hours starting at 6:00 in the morning for 24 hours; 5 ml of urine from each of the patients is dosed for the determination of the aMT6s content. The results of the study showed a total lack of circadian rhythm related to the production of epiphyses in 17 septic patients, whereas in the other seven critically ill patients with sepsis and in the group of healthy volunteers this phenomenon does not occur (Fig. 9).

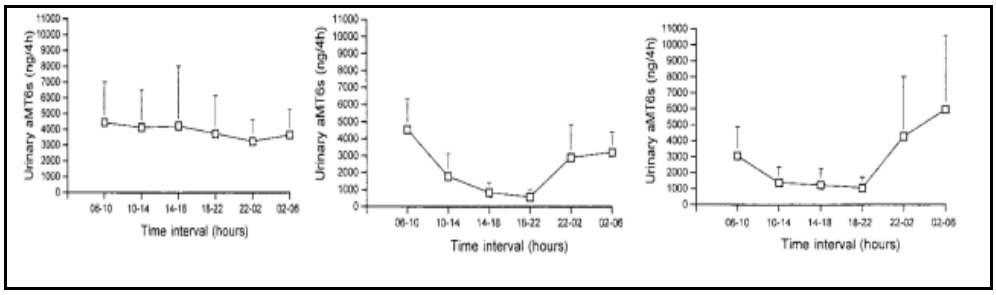

Fig. 9 *– 24-hour profiles of urinary aMT6s excretion in different patient groups. Values are means ± SD. (a) septic patients (group A), (b) non-septic patients (group B), (c) control patients (group C). Picture modified from Mundingler-2002.*

It is the severe sepsis to play a key role in this scenario: note the progressive tendency to restore circadian rhythm in the 9 patients who gradually went off the septic process (Fig. 10).

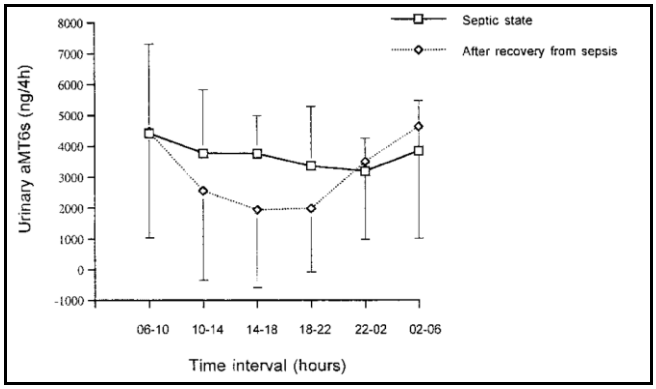

Fig. 10 - *24-hour profiles of urinary aMT6s excretion of sepsis survivors (n=9) at study entry and after recovery from sepsis. Values are means ± SD. Picture modified from Mundingler-2002.*

At the end of the work, Mundigler emphasizes the large number of possible therapeutic implications of MLT as immunostimulant substance; hormone receptors are present at the level of CD4+ T lymphocytes and B lymphocytes (Gonzalez-Haba et al., 1995) while it has been proved its role in promoting the activity of monocytes and natural killer cells together with the antioxidant action (Reiter et al., 2002). Considering the importance of immunodepression and oxidative stress in the development and maintenance of the septic state, the role of MLT, with all its properties clinics, emerges with increasing importance.

2.5. MLT AS AN ONCOSTATIC SUBSTANCE

2.5.1. Aging, immune function and cancer

That the levels of immunity is a predictor of individual longevity in human beings has been suggested by several epidemiological studies like OCTO and NONA which revealed the existence of "immunological risk phenotypes" that can predict the life span in the elderly (Pawelec et al., 2004; Pawelec et al., 2002). Longer life in centenarians has been associated with high natural killer (NK) cell number, augmented interferon (IFN)-γ production and phagocytosis (Miyaji et al., 2000). The age-associated increases in NK cells were interpreted as a compensatory response to overcome the decreased immune function that could otherwise trigger neoplastic growth (Srinivasan et al., 2005).

Studies of knockout mice have shown the important role of the immune system in controlling the spontaneous generation of tumors. Nearly 50% of aged IFN-γ -/- or perforin -/- mice developed lymphomas, lung adenocarcinoma or sarcoma (Street et al., 2002). Immune changes during aging may result in tumor growth since the incidence of metastatic cancer at autopsy peaks 75-90 years and has been shown to decline in 95-99 years old and centenarians (Stanta et al., 1997). That the personality and the emotional state of the individual can influence the course of illness by altering the immune function has been well documented (Segerstrom, 2005).

The understanding of the immune changes in the elderly can provide new insights into the complexes relationship between immunity and cancer (Hegde et al., 2009). In this respect, the decline in the production of MLT with aging was suggested to play an important role in triggering immunoscence, especially age-associated neoplastic diseases (Miller et al., 2006).

Any search for therapeutic agent that can improve the quality of life in the elderly depend upon the identification of substances that have both antioxidant and immunoenhancing qualities. As MLT has been identified as a natural antioxidant with immunoenhancing properties, it has the potential of becoming an effective therapeutic substance in preventing or arresting neolpastic growth.

2.5.2. MLT in immune mechanism

There are many natural mechanisms that protect against carcinogenesis and they fall into two main categories, immune and non-immune. Among the former, immunosurveillance has been suggested as one of the major processes by which cancerous cells are detected and eliminated. The activation of lymphocytes and monocytes/macrophages by MLT can be one of the major mechanisms in preventing tumor development (Martins et al., 1998). MLT has a significant immunomodulatory role in the immunocompromised state (Cardinali et al., 2008). The age-related impairment of the immune system first appears around the sixth decade of age coinciding with a normal decrease in plasma MLT concentration. Aging is associated with a decline in immune function that predisposes to increased incidence of cancer and infectious and neurodegenerative diseases like Alzheimer's disease.

The diurnal and seasonal changes in the immune function correlate with MLT biosynthesis and secretion (Skwarlo-Sonta, 2002). In addition, the synthesis of MLT by human lymphocytes (Carrillo-Vico et al., 2004) lead to support the hypothesis that MLT has a role in the regulation of immune function. Other studies demonstrated that the MLT synthesized by human T cells contributes to regulation of interleukin (IL)-

2 production acting as intracrine, autocrine and/or paracrine substance . The presence of high levels of MLT in cultured rat thymocytes and expression of mRNAs encoding for AANAT and HIOMT in the rat and human thymus cells support that MLT is also synthesized by thymocytes (Naranjo et al., 2007).

Seasonal changes of MLT secretion are observed in human beings (Ueno-Towatari et al., 2007)and it is suggested that MLT has significant role in immune modulation during different seasons of the year (Srinivasan et al., 2008). The role of MLT as a possible mediator of seasonal changes effects on immune function has been well documented (Nelson, 2004; Nelson and Drazen, 2000).

MLT receptors are detectable in the monocyte/macrophage lineage (Garcia-Maurino et al., 2000). Administration of MLT increases the production of both monocytes and NK cells in bone marrow and spleen within 7-14 days of treatment (Currier et al., 2000). As both cell types are components of the non-specific immune system, the findings suggest that MLT can be effective in arresting neoplastic growth and in destroying virus infected cells. MLT's stimulatory action on monocyte production could be due either to its direct action on MLT receptors in monocytes or to its sensitizing action on monocytes to stimulants like IL-3, IL-4 or granulocyte-macrophages-colony stimulating factor (Currier et al., 2000). By this action MLT was able to rescue hematopoiesis from the toxic effect of cancer chemotherapy in several experimental models. This evidence actually poses the basis for the therapeutic use of MLT as an adjuvant in combination with myelotoxic anticancer therapeutic protocols.

NK cells play an important role in immunosurveillance against neoplasia and virus infected cells (Chaplin, 2010). Acute administration of MLT increased NK cell responsiveness to IFN-γ while its chronic administration not only augments NK cell activity but also increases the number of NK cells in circulation. The increased NK cell number brought out by MLT is attributed to an increased production of cytokines like IL-2, IL-6, IL-12 and IFN-γ from T helper (Th)-1 lymphocytes and from monocytes (Currier et al., 2000). The presence of MLT receptors on T lymphocytes explains MLT's action in releasing cytokines that enhance the NK cell activity and augmented NK cell number. By activating Th-1 cells MLT enhances the production of IFN-γ . MLT's immunoenhancing effect depends not only upon its ability to enhance production of cytokines but also upon its antiapoptotic actions.

2.5.3. MLT and T lymphocytes

Th lymphocytes play an important role for the protection against malignancy, by recruiting cells of the immune system and by activating antigen-specific effector cells (Knutson and Disis, 2005). Importance has been given to the stimulation of $CD4^+$ Th cell in cancer chemotherapy. $CD4^+$ lymphocytes secrete IFN- and tumor necrosis factor (TNF)-α that activate and regulate cytotoxicT cell responses. MLT treatment augmented $CD4^+$ cells in lymph nodes of rats (Castrillon et al., 2000). Th-1 cells directly kill tumor cells by releasing cytokines that activate "death" receptors on the tumor cell surface (Knutson and Disis, 2005). MLT also favors Th-2 responses: it not only stimulates the release of IFN-γ and IL-2 but also IL-10 (Raghavendra et al., 2001).

In immune-depressed states, MLT's immunoenhancing action is restricted to T-lymphocytes (Maestroni, 2001). Suppression of nocturnal MLT rise in mothers with mastitis was highly correlated with increased TNF-α secretion from immunocompetent cells in calostrum (Pontes et al., 2006). Since the proinflammatory cytokine inhibits nocturnal pineal MLT production (Fernandes et al., 2006) the results

suggest that during the response to an injury the production of MLT can e transiently shifted from an endocrine (pineal) to a paracrine (immunocompetent cells) (Markus et al., 2007).

2.5.4. MLT in melanoma

MLT acts as a protective agent against damage induced by UV radiation in the human skin (Fischer et al., 2006). MLT is also radioprotective against X-ray induced skin damage in the albino rat . The Bradioprotective action of MLT is attributed to its antioxidant properties via direct radical scavenging properties and stimulation of antioxidant enzymes as demonstrated in human skin fibroblasts (Kim et al., 2001).

MLT has oncostatic properties in melanomas and tumors of epithelial origin (Oh et al., 2001). The ability of MLT to stimulate IL-2 production and to enhance its antitumor activity has been tested both in experimental animals and in clinical trials. MLT on its own exerted a significant antitumor effect but when combined with IL-2 it potentiated the antitumor effect of IL-2 in an additive manner. In cancer patients both T and Nk cells are generally depressed, and since MLT administration can augment the production of T lymphocytes and NK cells via IL-2 increase, MLT administration could be a useful adjuvant therapy to impair tumor growth (Bartsch et al., 2000).

MLT administration along with IL-2 and naltrexone in patients with untreatable metastatic melanoma increased Th-1 and suppressed Th-2 responses, a reportedly favorable result in anticancer treatment (Lissoni, 2007; Lissoni et al., 1993a). In the studies by Lissoni et al. it was found that advanced neoplasms resistant to IL-2 responded well to il-2 therapy after the concomitant administration of MLT (Lissoni et al., 2008). Patients who received both IL-2 and MLT exhibited a significantly higher number of lymphocytes, T lymphocytes, NK cells and $CD4^+$ cells than those receiving IL-2 alone. A further study using IL-2 along with MLT and cisplatin demonstrated that it was the most effective immunotherapeutic way for treating metastatic melanoma. In that study the combination of MLT with IL-2 was proved to be successful after failure of a first line therapy with decarbazine and IFN-α. MLT not only suppressed tumor growth but also suppressed significantly the toxicity of chemotherapeutic drugs and potentiated their anticancer cytotoxic (Brivio et al., 2010).

In a study aiming to determine location and intensity of expression of MT_1 MLT receptors and of Ki-67 proliferation-associated antigen in dermal melanoma, material from 48 cases of dermal melanoma, including 38 primary tumors and 10 metastatic lymph nodes was examined (Danielczyk and Dziegiel, 2009). Expression of MT_1 receptor was more pronounced in primary tumors than in related metastatic lymph nodes. Depth of tumor infiltration demonstrated a moderate positive correlation with the intensity of MT_1 expression and a strongly positive correlation with the expression of Ki-67 antigen. In both primary tumors and metastatic lymph nodes, a weak correlation was found between the expression of MT_1 receptor and the expression of Ki-67 antigen (Danielczyk and Dziegiel, 2009).

MLT was effective in inhibiting cell proliferation of S-91 murine melanoma cells, under both i*n vitro* and *in vivo* conditions (Kadekaro et al., 2004). MLT exerted its antiproliferative action by increasing the expression of MT_1 receptor and also by increasing the activity of antioxidant enzymes. Early studies demonstrates that MLT can act directly at the cellular level to inhibit the proliferation of PG 19 and B16BL6 mouse melanoma cells in culture (Cos et al., 2001). The antiproliferative action of MLT is dose-dependent (Yerneni and Jayaraman, 2003). With the highest MLT concentration employed (19356 pg/cell) the cancer

cells became undetectable at day 5 of treatment. The total elimination of cancer cells observe in this study was the first of this kind reported in the scientific literature.

The disruption of circadian rhythmicity becomes significant as a tumor progresses, whereas the incidence of cancer augments after disruption of the circadian system. In a study to test whether body temperature rhythms are impaired by tumor progression, and to what extent exogenous MLT restricts tumor growth and restores circadian rhythmicity, C57 mice were subcutaneously inoculated with melanoma cells (Otalora et al., 2008). Animals were then submitted to 12:12 light-dark (LD) cycles or to continuous light (LL), with or without MLT administration (2 mg/kg/day). Under LD light conditions, the body temperature rhythm exhibited a marked reduction and increased phase instability a the tumor progressed. MLT administration increased the body temperature rhythm amplitude and phase stability, reduced tumor weight and prevented i.p. dissemination when administered in the subjective night (Otalora et al., 2008).

The effect of MLT on the growth of uveal melanoma cells has also examined. Hu and his coworkers (Hu-1998) by using cultured human uveal melanoma cells, found that MLT (0,1-10 nM) inhibited the growth cells in a dose-dependent manner. Growth inhibition occurred at a concentration of 2 nM, the physiological levels found in aqueous humor. High affinity MLT binding sites occurred in SK- Mel 28 human melanoma cell lines. In these cells use of luzindole, a selective blocker of MT_2 receptors reversed the antiproliferative and melanogenic effects of MLT. In human melanoma cells SK-Mel 1, the antiproliferative effects of MLT were associated with an alteration in the progression of the cell cycle and also with an increase in tyrosinase activity, a key regulatory of melanogenesis (Cabrera et al., 2010). Antagonists for MLT membrane receptors (luzindole and 4P-PDOT) and the general G-protein-coupled receptor inhibitor, pertussis toxin, did not prevent the MLT-induced cell growth arrest; this suggest a mechanism independent of G-protein-coupled membrane receptor. The p38 mitogen-activated protein kinase signaling pathway seems to play an important role in cell growth inhibition by MLT (Cabrera et al., 2010)

2.5.5. MLT in breast cancer

MLT is oncostatic and antiproliferative in breast cancer (Hill et al., 2009). Studies using MCF-7 human breast cancer cells demonstrated that physiological concentration of MLT inhibit cell proliferation. As the MLT's growth inhibitory effect was abolished by MT_1 receptor antagonism, the MT_1 receptors detectable in MCF-7 cells were identified as functional receptors responsible for transducing growth inhibitory effect of MLT (Ram et al., 2002). As the antiproliferative effect of MLT is also a serum dependent phenomenon, the interaction of MLT with a factor in the serum has been postulated for its antiproliferative action.

MLT not only blocks the mitogenic effects of estradiol but it is also able to counteract the estradiol-induced invasiveness of MCF-7 cells . *In vitro* experiments with the ER-positive MCF-7 human breast cancer cells demonstrated that MLT at physiological concentration (1 nM) inhibited the cell proliferation in the presence of serum or estradiol and increased the expression p53 and p21WAF1 proteins, which modulate the length of cell cycle. There is indication that MLT could exert its antitumoral effects on hormone-dependent mammary tumors by down-regulating the sulfatase pathway of the tumoral tissue (Gonzalez et al., 2010). Since MLT binds to calmodulin in a Ca^{2+} dependent fashion, calmodulin was implicated in the antiestrogenic effects of MLT. MLT acts as a calmodulin antagonist inducing conformational changes of the Erα-CaM complex thus impairing binding of the Erα-CaM complex to DNA and thereby transcription(del Rio et al., 2004). This has been suggested as the mechanism by which MLT exerts oncostatic and antiproliferative actions.

In recent years increased breast cancer risk in women associate with work at nightshifts has been attributed to the low MLT level following light-induced inhibition of MLT synthesis (Flynn-Evans et al., 2009). The protective role of MLT in mammary carcinogenesis was also suggested by studies in postmenopausal women with advanced breast cancer who have diminished urinary levels of MLT as compared to controls. The inhibitory action of MLT on mammary carcinogenesis has been attributed to effect of MLT on immune modulation (Vijayalaxmi et al., 2002). Indeed, disturbances of immune mechanisms have been documented in experimental models of mammary cancer. For example, the absence of the cytosolic protein Nod1 in MCF-7 cells correlated with tumor growth, an increased sensitivity to estrogen induced cell proliferation, and a failure to undergo Nod1-dependent apoptosis.

IL-2 and chemotherapy are employed for treatment of metastatic breast cancer (Burns et al., 2003) IL-2 used to achieve an increased efficacy of increasing NK cells and cytolytic function and, in combination with IFN-α and chemotherapy, as an adjuvant treatment in high-risk breast cancer. The link between MLT and immune system in cancer has been explored in phase II studies with MLT causing increase of some cytokines and amplification of objective responses to cytokine in patients (Abrial et al., 2005).

A correlation between tumor size and the nocturnal amplitude of MLT secretion was noted in some studies. Peak nocturnal amplitude of MLT was reduced in 50% of patients with primary breast cancer and was inversely correlated with tumor size. The nocturnal amplitude of the aMT6s concentration was found to be lower in patients with primary breast cancer. The circadian rhythm of nocturnal MLT production may represent a “regulatory shift” for the carcinogenesis process; it may exert a “natural restraint” on tumor initiation, promotion, and/or progression (Blask et al., 2009).

2.5.6. MLT in ovarian, endometrial, and other cancers of the female reproductive tract

Low MLT secretion has been reported in patients with endometrial cancer, but not in those with non-invasive cancer or squamous cervical cancer. *In vitro*, an ovarian adenocarcinoma cell line (BG-1) exposed to MLT (1-100 nM) showed a 20-25% reduction in cell number. In another study application of MLT to ovarian carcinoma cell cultures revealed that three out of seven ovarian cell cultures were affected by MLT in different ways (Bartsch et al., 2000)Cells of one tumor were inhibited by 90% at 10 nM, while in another the growth inhibition was by 30% at a concentration of 0,1-1000 nM; a third specimen was stimulated up to 30% by 100 nM. The variability in the response was attributed to the presence of some unknown tumor condition likely to modify the MLT response (Bartsch et al., 2000).

MLT did not exert antiproliferative effects on ovarian cancer cell lines at 0,001 nM – 1 μM concentrations but enhanced the sensitivity to cisplatin in two ovarian cell lines (Futagami et al., 2001). Results were interpreted as indicating that MLT may play a role in the control of telomerase activity and the suggestion was made that the resistance of ovarian cancer to cisplatin could be overcome by the administration of MLT.

In ovarian cancer patients, IL-2 treatment has been employed (Zwirner et al., 2010). For example, in the analysis of six studies of i.p. immunotherapy in ovarian cancer, 21 individual responses to IL-2 treatment were reported out of 69 patients showing a 22% of clinical efficacy (Grande et al., 2006). Since MLT increases the production of IL-2, the prospective therapeutic role of MLT in cancer is that it may well acts as a modulator of IL-2 and IFN-γ production by Th1 cells. MLT has the possibility of being used as a novel oncostatic adjuvant agent (Ramos et al., 2010; Regodon et al., 2005).

2.5.7. MLT in hepatocellular carcinoma

Hepatocellular carcinoma is the cancer of the liver found after hepatitis B and hepatitis C infection, as well as in conditions associated with alcohol abuse (Zerbini et al., 2006). Many immunotherapeutic procedures were employed for treating hepatocellular carcinoma, like the use of cytokines or transfer of autologous-activated lymphocytes. Intratumoral injection of recombinant adenoviral vectors that induce the local release of IL-2 has been employed (Sangro et al., 2004). Improvement in overall recurrence-free survival was seen in 155 hepatocellular carcinoma patients after immunotherapy with IL-2 and a CD3 (Takayama et al., 2000). In another study carried out on stage III and IV inoperable patients, IL-2 administered along with IFN-γ and transarterial chemotherapy brought about tumor size reduction in 14 out 20 patients (Reinisch et al., 2002).

MLT induces cell cycle arrest and apoptosis in hepatocarcinoma HepG2 cell line (Ozdemir et al., 2009). In 100 patients with inoperable advanced primary hepatocellular carcinoma, transcatheter arterial chemoembolization (TACE) was used alone or associated with MLT.

2.5.8. MLT in colorectal carcinoma

Epidemiological studies of nurses engaged in night-shift work indicated an increased incidence of colorectal cancer, a finding interpreted as supporting the cancer-promoting effect of MLT inhibition by environmental light (Schernhammer et al., 2010). Indeed, many *in vitro* and *in vivo* studies have shown that MLT exerts antiproliferative effects on intestinal cancer. In a study on CT-26 a murine colon carcinoma-derived cell line, MLT inhibited growth in a dose-dependent manner (Farriol et al., 2000). A statistically significant correlation was found between the decrease in DNA synthesis and the doses of MLT used. The growth inhibitory effect found was 22% (1 nM MLT), 25% (2 nM MLT) and 47% (3 nM MLT) (Farriol-2000). High MLT binding sites were demonstrated in human colonic mucosa and a MLT concentration of 467 $\pm$ 99 pg/g of wet tissue of human colon has been reported. The oncostatic action of MLT appears to depend on both MT_2 and nuclear RZR/ROR receptors. Luzindole (a MT_1 and MT_2 antagonist) but not 4P-PDOT (a specific MT_2 antagonist) diminishes the inhibitory effect of MLT on murine colon 38 cancer cell growth in vitro.

The inhibitory effect of exogenous MLT on colon oncogenesis was investigated using the azoxymethane/dextran sodium sulfate rat model (Tanaka et al., 2009). At week 20, the development of colonic adenocarcinoma was significantly inhibited by the administration with MLT in a dose-dependent manner. MLT exposure decreased mitotic and apoptotic indices in the colonic adenocarcinomas and lowered the immunohistochemical expression of nuclear factor k B, TNF-α, IL-1β and STAT3 in the epithelial malignancies. These results may indicate the beneficial effects of MLT on colitis-related colon carcinogenesis and a potential application for inhibiting colorectal cancer development in the inflamed colon.

Early studies on the effects of MLT in colorectal carcinoma were based upon the immunoneuroendocrine and synergistic relationship between MLT and IL-2 (Barni et al., 1992). In a study on 24 patients with advanced cell tumors (non-small cell lung cancer, 9 patients; colorectal cancer, 7 patients; gastric cancer, 3 patients; breast cancer, 2 patients; cancer of pancreas, 1 patients; hepotocarcinoma, 1 patient; unknown tumor, 1 patient) who did not respond to previous chemotherapies, IL-2 plus MLT was given. MLT was administered starting 7 days before IL-2 injection. While progress was reported in 6/24 patients, stability was reported in 14/24 patients. IL-2 combination with MLT seemed useful to control tumor

growth n patients with advanced neoplasms (Lissoni et al., 1993b). In another study on 35 patients with advanced neoplasm of the digestive tract, immunotherapy with a low-dose of IL-2 plus MLT was a well-tolerated and effective therapy. Complete response was obtained in patients with gastric carcinoma and hepatocarcinoma. The overall response rate was 8/35 (23%) (Lissoni et al., 1993b). Similarly, in another study a low subcutaneous dose of IL-2 plus MLT was found to be a second-line therapy for tumor regression and for prolonging survival of patients with metastatic colorectal cancer (Barni et al., 1995)

The distinct MLT rhythm with higher concentrations during the darktime was found in plasma of both control patients and patients with colorectal carcinoma (Vician et al., 1999).

Daytime MLT concentrations in gut tissue of colorectal carcinoma patients was found to be 317 $\pm$ 87,8 pg/g, nearly 1 times higher than the day time levels in circulation. An increased level of MLT in the gut has been found after surgery and it was suggested that they play a protective role against the development of colorectal cancer (Vician et al., 1999).

The interrelationship between MLT and immune function was studied in patients with advanced GI cancer (42 patients with colorectal, gastric and pancreatic cancer) (Muc-Wierzgon et al., 2003). The circadian rhythm of MLT was altered with peak MLT level reaching at 08:00-09:00 h, with a 5-7 h-delay respecting average peak time in healthy humans. The rhythm in TNF-α and soluble TNF-α receptors (type I and type II) also indicated the existence of complex self-regulatory mechanisms between the neuroendocrine system and the cytokine network in those patients (Muc-Wierzgon et al., 2003). Suppression of nocturnal MLT rise in mothers with mastitis was highly correlated with increased TNF-α secretion from immunocompetent cells phagocytes in calostrum (Pontes et al., 2007).

Besides interacting with cytokines, MLT induces apoptotic cell death in cancer cells. In a study on HT-29 human colon cancer cells, MLT potentiated flavones-induced apoptosis (Wenzel et al., 2005). The role of MLT as pro-apoptotic agent s a new field of investigation. The pro-apoptotic action of MLT has been documented not only in colon cancer cells but also in breast cancer (Cos et al., 2002). The mechanisms underlying the pro-apoptotic action of MLT is still not clear. The findings that MLT induces apoptosis uniformly in all cancer cells may have important clinical significance. It could involve free radical scavenging properties and other intracellular pathways. Indeed, the antioxidant and anti-inflammatory actions of MLT counteracting the oxidative status and reducing the production of nitric oxide by cultured HT-29 cells seem to be directly involved in its oncostatic properties (Garcia-Navarro et al., 2007).

2.6. MLT IN PROSTATE CANCER

2.6.1. Prostate cancer

The prostate is a gland (Fig. 11) found only in men, positioned in front of the rectum and that produces a part of the seminal fluid released during ejaculation. Under normal conditions, the size of a walnut, but with the passing of time or because of certain diseases can swell up to give mainly affects urinary type. This gland is very sensitive to the action of hormones, particularly male ones, such as testosterone, which affect their growth. Prostate cancer has its origin from the cells inside the gland begin to grow uncontrollably. In the prostate are several types of cells, each of which can be transformed and become cancerous, but almost all prostate cancers diagnosed arise from cells of the gland and are accordingly called adenocarcinomas (as all cancers that originate from cells of a gland). In addition to adenocarcinoma in the prostate can be found in rare cases sarcomas, small cell carcinomas and squamous cell transition. Much

more common are the benign conditions that affect the prostate, especially after the age of 50, and sometimes cause symptoms that may be confused with those of the tumor. In benign prostatic hyperplasia the central portion of the prostate gland swells and the excessive growth of this tissue compresses the urethra - channel that carries urine from the bladder through the prostate that, compressed, creates problems in passing urine.

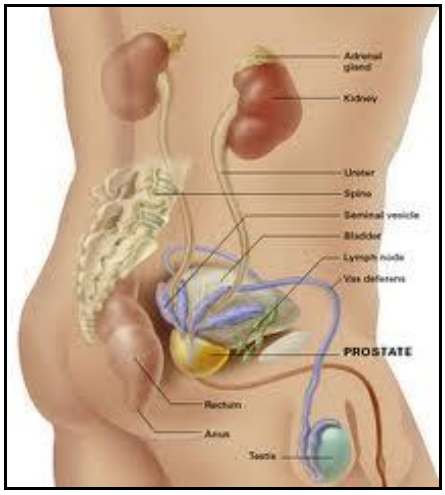

Fig. 11 – *Prostate. Picture modified from: http://www.walktolive.ca/event/prostate-cancer/.*

Prostate cancer is classified according to the degree, which indicates the the status of the disease, the aggressiveness, and the stag. Depending on the stage in which the disease is, additional staging examinations such as CT (computed tomography) or MRI are carried out. To verify the presence of metastases to the skeleton is often used bone scintigraphy. The pathologist who analyzes the tissue taken by biopsy assigns to the so-called tumor Gleason score, that is a number between 1 and 5 which indicates how the appearance of the tumor glands is similar to or different from that of normal glands: are more similar, the lower the Gleason score. The tumors with Gleason score less than or equal to 6 are considered low-grade, intermediate grade of those with 7, while those between 8 and 10 high grade. The latter have a higher risk of progression and spread to other organs. Instead to define the stage at cancer typically uses the TNM system (T = tumor), where N indicates the status of the lymph nodes (N: 0 if not affected, if affected 1) M and the presence of metastasis (M: 0 if absent, 1 if present). For a complete characterization of the stage of disease these three parameters are also associated with the Gleason score and the PSA level. The correlation of these parameters (T, Gleason, PSA) can be attributed to the disease three different risk categories: low, intermediate and high risk. In general, in the case of a low-risk (ie, a disease that is difficult to spread and give rise to metastases) can also decide not to proceed with surgical removal of the gland but simply to monitor the evolution of the disorder.

In its early stages, prostate cancer is asymptomatic and is diagnosed after urological visit, which involves digital rectal examination or PSA control, with a blood test. When the tumor grows, it produces urinary symptoms: difficulty in urination (especially at start) or need to urinate often, pain when urinating, blood in the urine or semen, feeling of not being able to urinate in a comprehensive manner. Often urinary symptoms described above may be linked to prostate problems benign like hypertrophy: further tests are needed to classify : Low , intermediate and high risk patients. In the case of a low-risk (ie, a disease that is difficult to spread and give rise to metastases) surgical removal of the gland is avoided and the evolution of the disorder is simply monitored.

Prostate cancer is an androgen-dependent neoplasia, which, in the metastatic phase or locally in the advanced one, has been treated in the first instance pharmacologically with androgen deprivation via surgical or chemical castration. In the second instance to the patient surgical treatment (radical prostatectomy) and radiotherapy are planned. The drug therapy shows good results with tumor regression and decline in serum PSA, however within 2 years about 80% of castrated patients suffer a relapse of the disease, with tumor progression from an hormone-dependent stage to a hormone-independent one, extremely more aggressive.

The number of diagnoses of prostate cancer has increased progressively since, in the nineties, the test for the measurement of PSA has been approved by the Food and Drug Administration (FDA). Its real value in the diagnosis of a tumor, however, the debate is still open. The urinary symptoms of prostate cancer appear only in the later stages of the disease, however, may also indicate the presence of problems other than cancer. It is therefore very important that the diagnosis be performed by a physician who takes into account several factors before deciding how to proceed. In the evaluation of the prostate, the doctor may decide to perform the PSA test and digital rectal examination, which is performed in the physician's basic or urologist, and sometimes allows to identify the presence of any touch nodules at the level of the prostate. If this test gives rise to the suspicion of cancer, proceed normally with a biopsy of the prostate on ultrasound guidance. The only test that can identify with certainty the presence of cancer cells in the prostate tissue biopsy is performed under local anesthesia, which lasts a few minutes and is done in an outpatient setting. Through the guidance of the ultrasound probe inserted in the rectum are made with a special needle, at least 12 donations by trans-rectal or by trans-perineal (the area between the rectum and scrotum) which are then analyzed by the pathologist under a microscope to search of cancer cells.

Today there are many types of treatment for prostate cancer each of which has specific benefits and side effects. Only a careful analysis of patient characteristics (age, life expectancy, etc.) and the disease (low, intermediate or high risk) will allow the urologist to recommend the most suitable strategy and individualized therapy to agree also based on preferences of those who must submit to treatment. In some cases, especially for elderly or patients with other serious diseases, or in the case of tumors of small dimensions and with low risk (micro outbreak in biopsy), it may choose not to implement any kind of therapy and "wait" is to that the Anglo-Saxons called watchful waiting, a "watchful waiting," which does not require treatment, but only controls fairly frequent (PSA, rectal examination, biopsy) that allow you to control the evolution of the disease and check for any changes that merit intervention.

When it comes to active therapy, however, the choice often falls on radical surgery. Radical prostatectomy - removal of the entire prostate gland and lymph nodes in the region close to the tumor - is considered a curative intervention, where the disease is confined to the prostate. With the significant improvements in surgical instruments, now the surgery to remove the prostate can be done in a classic (retro pubic radical prostatectomy open), laparoscopically, or through the more modern system of robot-assisted laparoscopy. In Italy, the robot suitable for practicing the intervention are becoming increasingly popular all over the country. For the advanced stages of malignancies, the scalpel alone often fails to cure the disease and there is therefore the need to associate treatments such as radiation therapy or hormonal therapy. For the treatment of prostate cancer, in the treatments considered standard, it has been demonstrated that also the external beam radiotherapy is effective in tumors of low risk, with results similar to those of radical prostatectomy. Another radiotherapy technique that seems to offer similar to the previous results in diseases of low-risk brachytherapy, which involves inserting into the prostate small "seeds" that release radiation.

When prostate cancer is in a metastatic stage, unlike what happens in other tumors, chemotherapy is not the treatment of first choice and it is preferred to hormonal therapy. This is intended to reduce the level of testosterone - the male hormone that stimulates cell growth of prostate cancer - but it carries side effects such as reduction or cancellation of sexual desire, impotence, hot flashes, weight gain, osteoporosis, loss of muscle mass and fatigue. Among the local therapies still under evaluation are cryotherapy (elimination of tumor cells in the cold) and HIFU (focused ultrasound on the tumor). Are also being tested, in some cases already very advanced, even vaccines that lead the immune system to react against the tumor and destroy it, and anti-angiogenic drugs that block the formation of new blood vessels by preventing the cancer from receiving the nourishment need to evolve and develop further.

One of the main risk factors for prostate cancer is age the chances of getting sick are very slim before 40 years, but increases significantly after age 50 and about two out of three cancers are diagnosed in people over 65 years . Researchers have shown that a great many (between 70 and 90 per cent) men over the age of 80 have a tumor of the prostate, although in most cases the disease shows no sign and we notice of its presence only in case of autopsy after death. When it comes to prostate cancer, another significant factor is undoubtedly the familiar, the risk of illness is double for those who have a relative blood relative (father, brother, etc.) with the disease than someone who has no family in case . The presence of mutations in genes such as BRCA1 and BRCA2, are already involved in promoting the development of cancer of the breast and ovary, or gene HPC1, may increase the risk of developing prostate cancer. The probability of becoming ill could also be connected to high levels of hormones such as testosterone, which promotes the growth of prostate cells, and the hormone IGF1, similar to insulin, but which works on cell growth and not on the metabolism of sugars. No less important are the risk factors related to lifestyle: diet rich in saturated fats, obesity, lack of exercise are just some of the bad habits and becoming more widespread in the Western world that can promote the development and growth of the prostate cancer.

Among the more widespread cancers among the elderly population in the developed world and in Western countries, prostate cancer is one of the most common cancers and accounts for approximately 11% of cancer deaths in males. In Italy are estimated slightly more than 23,500 new cases each year, but the risk that the disease has a negative outcome could be lowered if ready diagnosed and cured. The incidence for prostate cancer, ie the number of new cases in a given period of time, is still growing, with a doubling in the last 10 years, due to the increase in the average age of the population and the introduction examination of PSA (prostate specific antigen). Measure through a simple blood levels of this molecule produced only by prostate cells allows, in many cases, to understand if the gland there is anything wrong, even if not necessarily the case of cancer, because the PSA increases even in the presence of simple inflammations, infections or benign thickenings of the gland itself.

2.6.2. MLT and prostate cancer

It was demonstrated an inverse relationship between MLT production and the incidence of prostate cancer in support of a potential preventive or in the very early stages function of development of prostate cancer (Pukkala et al., 2006).

In prostate cancer, MLT seems to act on the androgen mitogenic way. Activating the MT1 receptor, there is an upregulation of the gene p27 and an inhibition of proliferation through the mechanisms of activation of PKC and PKA (Tam et al., 2007). In a recent study, using a combination of pharmacological and genetic manipulations, Tam et al. (Tam and Shiu, 2011) demonstrated that MLT inhibits the proliferation of

LNCaP and VCaP prostate cancer cells via activation of the MT1 receptor-mediated antiproliferative signaling pathway, namely MT1/(Gas) PKA + (Gaq) PKC/p27Kip1, which they have previously identified in the cancerous 22Rv1 and the immortalized, nontumorigenic RWPE-1 prostate epithelial cell lines (Fig. 12).

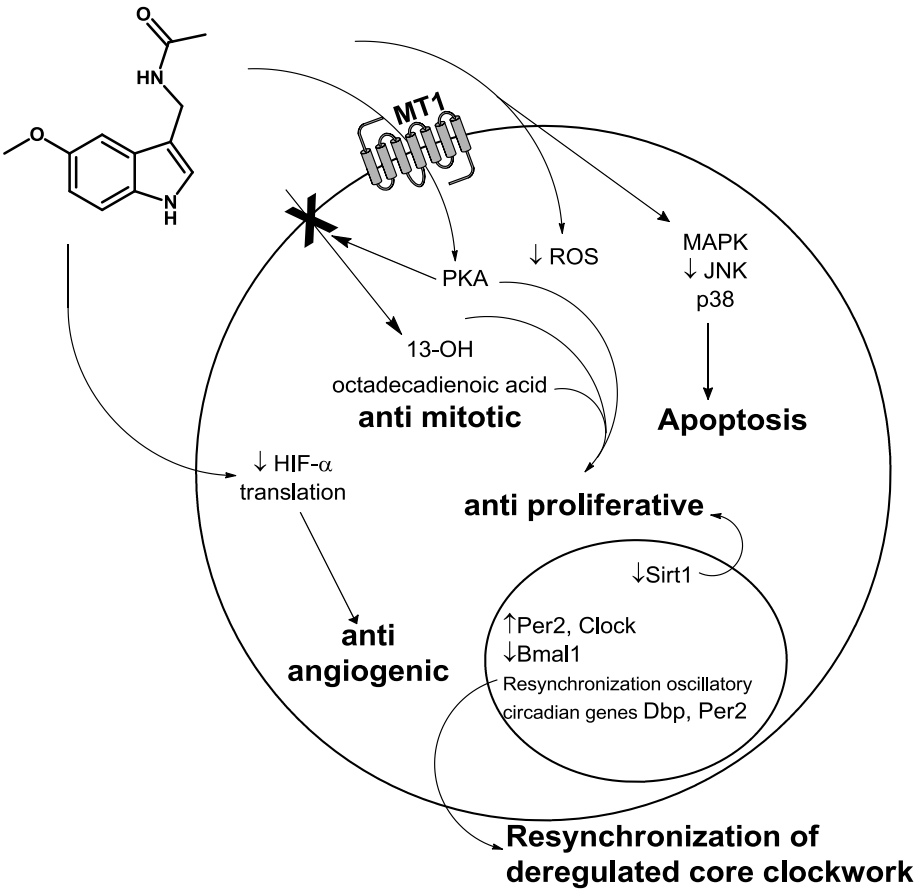

Fig. 12 - *MLT signaling in prostate cancer.*

The way MLT/MT1/PKA^{+} PKC/p27 characterized in prostate cancer appears to act independently from activation of the signal PI3K/AKT: the activation of PI3K/AKT may be the consequence of a mutation or loss of function of PTEN, as this event leads to a downstream inactivation of a transcription factor (FOXO) and the consequent decrease of p27 expression observed in prostate cancer (Shiu, 2007). Therefore MLT, acting independently, can counteract the mutations such as those of PTEN, promoting the synthesis of inhibitors of the mitotic cycle in cells in progression. Also it has been proved the relationship between MLT and androgens: the activation of the MLT / PKC induces downregulation of the transcriptional activity mediated by the binding of androgens with their AR receptor and induces exclusion of the AR from the nucleus (Shiu, 2007).

Contrary to the receptor hypothesis, the actions of MLT at the intracellular level for several research groups, are the key to explain the action that MLT has on cancer cells. At intracellular level, MLT can bind the MT3 receptor: the induction of this enzyme is associated with the decrease in tumor progression (Dietz et al., 2005). Some studies have also suggested how the binding of MLT to nuclear receptors RZR / RORα and RZRβ can alter the transcription of genes that play a role in cell proliferation as 5-LOX, p21, BSP (bone sialoprotein) (Carlberg, 2000). Another mechanism could involve the ability of MLT to modulate calcium and the activity of calmodulin, both important in cell cycle. MLT detriment of the calcium-calmodulin binding may have antiproliferative actions (Blask et al., 2002). The calmodulin is able to modulate some calcium receptors

(Li et al., 1999) so it may abolish the entry of calcium induced by sex hormones and the resulting mitogenic responses. This effect was observed on LNCaP cells (Xi et al., 2001). In the study, MLT-induced inhibition of LNCaP cell proliferation and attenuation of sex steroid-stimulated Ca^{2+} influx were associated with decreases in the levels of measurable PSA recovered from the culture fluids of LNCaP cells incubated with high physiological and pharmacological concentrations of MLT. These results are in line with the previously reported correlation between reduced production of PSA, as reflected by its level in the culture fluids, by LNCaP cells and decrease in cell proliferation induced by various pharmacological agents including calcium influx inhibitor. Since PSA is a serum marker available for monitoring the progression of prostate cancer and response to therapy, the data by Xi et al. suggest that it may be possible to use PSA for monitoring the response to therapy by MLT in future pre-clinical or clinical studies on prostate cancer.

In a study conducted by Jung-Hynes et al. (Jung-Hynes et al., 2011) , they recently demonstrated that Sirt1, a NAD+-dependent histone deacetylase, was overexpressed in prostate cancer (PCa) and its inhibition resulted in a significant antiproliferative response in human PCa cells. Studies have suggested a link between Sirt1 and circadian rhythms, the disruption of which has been linked to cancer. Interestingly, a decreased production of the pineal MLT has been shown to deregulate the circadian rhythm machinery and increase cancer risk. Furthermore, disruption in MLT production and circadian rhythmicity has been associated with aging. Jung-Hynes e al. challenged the hypothesis that MLT will impart antiproliferative response against PCa via inhibiting Sirt1. They demonstrated that MLT significantly inhibited Sirt1 protein and activity in vitro in multiple human PCa cell lines, and MLT-mediated Sirt1 inhibition was accompanied with a significant decrease in the proliferative potential of PCa cells, but not of normal cells. Forced overexpression of Sirt1 partially rescued the PCa cells from MLT's antiproliferative effects, suggesting that Sirt1 is a direct target of MLT. Employing transgenic adenocarcinoma of mouse prostate (TRAMP) mice, they also demonstrated that oral administration of MLT, at human-achievable doses, significantly inhibited PCa tumorigenesis as shown by decreases in (i) prostate and genitourinary weight, (ii) serum insulin-like growth factor-1 (IGF-1)/IGF-binding protein-3 (IGFBP3) ratio, (iii) mRNA and protein levels of the proliferation markers (PCNA, Ki-67). This anti-PCa response was accompanied with a significant decrease in Sirt1 in TRAMP prostate.

It is clear that the oncostatic action of MLT is not related to a unique function but rather to a sum of many factors which include the antioxidant properties, the activation of the immune system, the modulation of the endocrine system, the direct action on the tumor via specific membrane receptors. Most of these studies have been performed on cell lines "in vitro" or in animal models. The actual mechanism of action by which the molecule exerts its antitumor activity "in vivo" is not completely understood and deserves further preclinical and clinical research.

2.7. SOLID LIPID NANOPARTICLES (SLN)

In recent years it has become more and more evident that the development of new drugs alone is not sufficient to ensure progress in drug therapy. Exciting experimental obtained *in vivo* are very often followed by disappointing results *in vivo*. Main reasons for the therapy failure include:

- Insufficient drug concentration due to poor absorption, rapid metabolism and elimination (e.g. peptides, proteins). Drug distribution to other tissues combined with high drug toxicity (e.g. cancer drugs).
- Poor drug solubility which excludes intravenous (i.v.) injection of aqueous drug solution
- High fluctuation of plasma levels due to unpredictable bioavailability after oral administration, including the influence of food on plasma levels (e.g. cyclosporine)

A promising strategy to overcome these problems involves the development of suitable drug carrier systems. The *in vivo* fate of the drug is no longer mainly determined by the properties of the drug, but by the carrier system, which should permit a controlled and localized release of the active drug according to the specific needs. The size of the carrier depends on the desired route of administration and ranges from few nanometers (colloidal carriers), to the micrometer range (microparticles) and to several millimeters (implants). For parenteral administration, it is highly desirable to use biodegradable materials, which avoid surgery to remove the implant after complete drug release and which make the administration of micro- and nanoparticles feasible. The concept has been realized in several commercial products. Implants and microparticles based on biodegradable polyesters permit a controlled drug release over a period of weeks to months after subcutaneous (s.c.) or intramuscular (i.m.) implantation/injection. Commercially available systems have been developed for the treatment of prostate cancer and other GnRH-related diseases (Sandow et al., 1990). An example of the concept of localized drug release is the development of biodegradable implants for the treatment of gliomas, which ensure very high drug concentrations in the brain and minimize drug concentrations in other tissues, include bone marrow (Sipos et al., 1997).

Implants and microparticles are too large for drug targeting and i.v. administration. Therefore, colloidal carriers have attracted increasing attention during recent years. Investigated systems include nanoparticles, nanoemulsions, liposomes, nanosuspensions, micelles, soluble polymer-drug conjugates.

The existence of different colloidal carrier systems raises the question as to which of them might be the most suitable for the desired purpose. Of course, there is no simple answer to this question. Aspects to include:

- Drug loading capacity
- Possibility or drug targeting
- In vivo fate of the carrier (interaction with the biological surrounding, degradation rate, accumulation in organs)
- Acute and chronic toxicity
- Scaling up of production
- Physical and chemical storage stability
- Overall costs

Polymers from natural (Muller et al., 1996)and synthetic sources have been used. Polymer based systems in the submicron range size include water soluble polymer-drug conjugates, polymer nanocapsules

and nanospheres. A certain advantages of polymer systems is the wealth of possible chemical modifications, including the synthesis of block- and comb-polymers.

Problems of polymer based nanoparticles derive from residues from organic solvents used in the production process, polymer cytotoxicity and the scaling up of the production processes, the concentration of nanoparticles is low and does not exceed 2%. Polymer hydrolysis during storage has to be taken into account and lyophilization is often required to prevent polymer degradation.

Liposomes are spherical vescicles composed of one or more phospholipid bilayers. Lipophilic drugs can be incorporated into the lipid bilayers while hydrophilic drugs are solubilized in the inner aqueous core. Drug release, *in vivo* stability and biodistribution are determined by size, surface charge, surface hydrophobicity and membrane fluidity. Membrane permeability can be adapted by the selection of the phospholipids and the incorporation of additives (e.g. cholesterol). It is possible to prevent a rapid reticulendothelial uptake of the liposomes by the incorporation of natural compound or by use of chemical modified polyethylene glycols. The development of such sterically stabilized systems permits the practical realization of drug targeting strategies. Liposome based drug carriers also permit the intravenous injection of lipophilic drugs with very low water solubility. Chemical and physical stability problems might lead to liposome aggregation and drug degradation during storage.

Nanosuspensions are colloidal particles which are composed of the drug and the emulsifier only. Possible production procedures include ball milling or high pressure homogenization (Ford et al., 1999).

Lipid nanoemulsions are made of fatty vegetable oils or middle chain triglycerides, phospholipids and glycerol. These systems can be used for the purpose of nutrition and as drug carriers for lipophilic drugs and several formulations are commercialized.

The possibility of controlled drug release from nanoemulsions is limited due to the small size and the liquid state of the carrier. For most drugs, a rapid release has been seen. Advantages of nanoemulsions include toxicological safety and a high content of the lipid phase as well as the possibility of large scale production by high pressure homogenization.

The use of solid lipids instead of liquid oils is a very attractive idea to achieve drug release, because drug mobility in a solid lipid should be considerably lower compared with a liquid oil. Solid lipids have been used for several years in the forms of pellets in order to achieve a retarded drug release after oral administration. In the beginning of 80s, Speiser et al. developed solid lipid microparticles produced by dispersing of melted lipids with high speed mixers or ultrasound. The products contained relatively high amounts of microparticles. This might not be a serious problem for oral administration but it excludes an intravenous injection. Higher concentration of the emulsifier result in a reduction of the particle size, but also increase the risk of toxic side effects.

In the following years, it has been demonstrated that high pressure homogenization is a more effective method for the production of submicron sized dispersions of solid lipids compared to high shear mixing or ultrasound. Dispersions obtained in this way are called solid lipid nanoparticles (SLN). Most SLN dispersions produced by high pressure homogenization (HPH) are characterized by an average particle size below 500 nm and a low microparticle content. Other production procedures are based on the use of organic solvents (HPH/solvent evaporation) or on dilution of micro-emulsions.

SLN combine the advantages and avoid the disadvantages of other colloidal carriers. Advantages include:

- Possibility of controlled drug release and drug targeting
- Increased drug stability

- High drug payload
- Incorporation of lipophilic and hydrophilic drug feasible
- No biotoxicity of the carrier
- Avoidance of organic solvents
- No problems with respect to large scale production and sterilization

2.7.1. Ingredients

The ingredients of the SLN are solid lipid(s), emulsifying agent(s) to increase stability and water. The term lipid includes triglycerides (e.g. tristearine), partial glycerides (e.g. Imwitor), fatty acids (e.g. stearic acid), steroids (e.g. cholesterol) and waxes (e.g. cetyl palmitate). All classes of emulsifier (with respect to charge and molecular weight) have been used to stabilize the lipid dispersion. It has been found that the combination of emulsifiers might prevent particle agglomeration more effectively.

A clear advantages of SLN is the fact, that the lipid matrix is made from physiological lipids which decreases the danger of acute and chronic toxicity. The choice of the emulsifier depends on the administration route and is more limited for parental administrations.

2.7.2. Administration route

Numerous formulations SLN for different routes of administration (oral, parenteral, transdermal, ocular, pulmonary, rectal) have been developed and studied in vitro and *in vivo* for different drugs (Mehnert and Mader, 2001). Unloaded SLNs, administered i.v., can cross the blood brain barrier. In laboratory animals the stealth drug-loaded SLNs administered *via* the i.v. route have been demonstrated to cross the blood brain barrier to greter extent than commercial drug solutions (Gasco, 2007).The pharmacokinetic parameters are greatly improved compared to commercial forms, by increasing the amount of stealth agent (Zara et al., 2002).

Moreover, unloaded SLNs administered duodenally, are targeted to lymph. The administration by the duodenal route of drug-loaded SLNs showed better pharmacokinetic parameters that the commercial i.v. solution forms. Tobramycin-SLN administered duodenally provides good absorption by the GI tract; the interest in this case is that tobramycin is still only administered by the pareneteral route (Cavalli et al., 2003).

2.7.2.1. Oral administration

Oral administration forms of SLN may include aqueous dispersions or SLN loaded traditional dosage forms, e.g. tablets, pellets or capsules. Increased bioavailability and prolonged plasma levels have been described after oral administration of cyclosporine containing lipid nanodispersions to animals. An increased uptake of SLN has been described by Bargoni et al. (Bargoni et al., 1998) after intraduodenal administration.

2.7.2.2. Parenteral administration

SLN (Fig. 13),has been administered intravenously to animals. In a recent study, conducted by Rezzani et al. (Rezzani et al., 2009), MLT and SLN-MLT with cyclosporine A (CsA) were injected in rats. CsA administration produced morphological and biochemical changes in the heart of rats, while MLT reversed the changes.

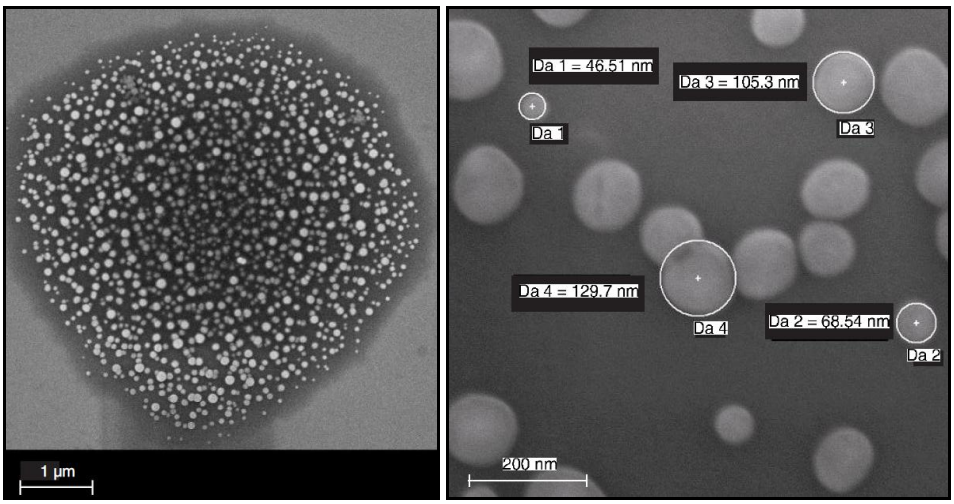

Fig. 13 *– Nanovector's SLN dried-size distribution and shape.*

In particular, since the antiapoptotic MLT's efficacy is mainly observed when it is loaded in SLN they suggest that MT1 /MT2 pathway is not sufficient for apoptosis antagonism and the additional intracellular effect may be required.

Pharmacokinetic studies on doxorubicin incorporated into SLN showed higher blood levels in comparison to a commercial drug solution after i.v. injections in rats. Concerning the body distribution, SLN were found to cause higher drug concentrations in lung, spleen and brain, while the solution led to a distribution more into liver and kidneys.

Yang et al. (Yang et al., 1999) reported on the pharmacokinetics and body distribution of campotethecin after i.v. injection in rats. In comparison to a drug solution, SLN were found to lead to much higher AUC/dose and mean residences times especially in brain, heart and reticulendothelial cells containing organs. The highest AUC ratio of SLN to drug solution among the tested organs was found in the brain.

2.7.2.3. Transdermal administration

The smallest particle sizes are observed for SLN dispersions with low lipid content (up to 5%). Both the low concentration of the dispersed liquid and the low viscosity are disadvantageous for dermal administration. In most cases, the incorporation of the SLN dispersion in a ointment or gel is necessary in order to achieve a formulation which can be administered to the skin.

In the study conducted by Priano et al. (Priano et al., 2007), MLT incorporated in SLN was administered by oral and trasdermal route in healthy subjects. This study demonstrated a significant absorption of SLN-MLT, with detectable plasma level achieved for several hours in particular after transdermal administration.

A dramatic increase of the elastic properties was observed with increasing lipid content. The rheological properties are comparable to typical dermal formulations. The results indicate that it is possible to produce high concentrated lipid dispersions in the submicron size range in a one-step production.

The cosmetic field offers interesting applications. It has been found in vitro that SLN have UV reflecting properties. The UV reflectance is related to the solid state of the lipid and was not evident in nanoemulsions of comparable composition. Those observations open the possibility of the development of SLN-based UV protective systems. The use of physiological components in SLN is a clear advantage over existing UV protective systems (UV blockers or TiO_2) with respect to skin penetration and potential of skin toxicity.

SLN have also been found to modulate drug release into the skin and to improve drug delivery to particular skin layers *in vitro*.

The loss of water after application on the skin causes changes of the lipid modification and SLN structure. Electron microscopy indicates that dense films are formed after drying (32°C) of SLN dispersions in contrast to spherical structures which have been proposed previously. The formation of the dense structure will favor occlusive effects on skin. It is interesting to note that the films made from melts of the lipid bulk do not form close films as dried SLN dispersions do. The surfactant plays a significant role in preventing pore formation.

2.8. CRYOPASS-THERAPY

Cryopass terapy is an applied physics technique that takes advantage of the action of photon kinetics of a laser beam to convey drug molecules frozen at -18°C through the skin to the target site. Penetration is atraumatic, and painless, regardless of the blood circulation and the density tissue (Fig. 14).

Fig. 14 *– Lasericemed-cryoRx- The instrument exists in two configurations, a fixed one, mainly used in medical studies and a portable one used for home purpose or directly in the sport field. The devices differ only for the appearance; the laser sources and the powers used are the same in both versions. There is also a portable version for veterinary purpose which is used primarily for horses treatment using a different configuration of laser capable of eliminating the problem of hair in the passage of the drug. Picture modified form: https://www.box.com/s/anr9knr2g9oyv2yb0fqj.*

At room temperature, if a photon strikes an electron, the energy applied to the electron make it jump at a higher energy level. This energy level is not stable so the electron come back to its originary level, giving back a photon (Fig. 15 –. If however, we freeze the substance and we apply again photonic energy, we observe that the process of electron decay to its originary level is slower than the process of electron excitation to a higher energy level (Fig. 15b). The low temperature causes that the energy absorbed by the drug molecules is hold in the upper energy level orbitals from low temperatures in the form of potential energy. In contact with the skin, this energy is released as kinetic energy, allowing the penetration of the drug through the skin barrier (Fig. 15c). The accelerated drug molecules cross through the skin thanks to the temporary depolarization of skin tight junctions.

This technique allows to use both polar and non-polar drugs, both soluble and non soluble substances in the form of dust suspended in the three-dimensional lattice, a particular blend of carboxymethylcellulose which in addition to the advantage to be neutral as water, prevents the dashing of the solute until formation of crystalline grid

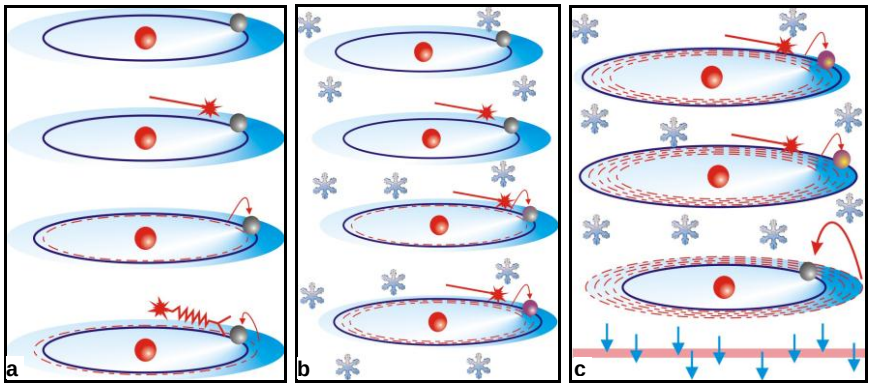

Fig. 15 *– (a) Energy orbitals at room temperature, (b) at -18°C, (c) and interface cryo applicator-skin. Picture modified form: https://www.box.com/s/anr9knr2g9oyv2yb0fqj.*

This technique allows to use both polar and non-polar drugs, both soluble and non soluble substances in the form of dust suspended in the three-dimensional lattice, a particular blend of carboxymethylcellulose which in addition to the advantage to be neutral as water, prevents the dashing of the solute until formation of crystalline grid.

A large variety has been used with cryoRx: anesthetics, antibiotics, anti-inflammatory corticosteroids, muscle relaxants, vasodilators, calcium antagonists, psoralenes, glucosamine sulfate, calcium chelators, dronats, controitin sulphate,poly deoxy ribonucleotids, vitamin complexes,hyaluronic acid, hyaluronidase,etc.

This technique demonstrated to be well tolerated by the patients, although some referred a slight intolerance to the direct application of ice on skin. This is attenuated by moving the cryo-applicator on the skin during the treatment. Skin lesions are avoided due to the low power laser used (source 635 nm, power 50 mW, laser safety class 3R), the lack of chemical allergenic or irritating carriers and the lack of electric current.

The active principles penetrate into the tissues to the target site in 15-20 min, depending to the site, with a maximum observed in the genital muscle of 6 cm (± 4 mm) (n= 6 patients) (Enrico e Emilio Bonizzoni, C.I.R.C.E. Srl, Magnago (MI), Itlaia, Criopass terapia- Nuove frontiere nella veicolazione del farmaco, Roma, 8 Giugno 2012).

Repeated serum withdrawals have shown a release of drug (aminophylline) from the tissue to the blood circle overlapping mesotherapy but with a less ripid and more prolonged release curve, thus suggesting a stronger link with membrane glycosaminoglycans and independence of capacity of penetration by microcirculation (Tab.1).

Tab.1						
Drug used	**Mesotherapy**		**Elastomesotherapy**		**Cryo-laser phoresis**	
Aminophylline (240 mg)	After 30 min	After 60 min	After 30 min	After 60 min	After 30 min	After 60 min
	8,4 mg/l	4,9 mg/l	7,9 mg/l	4,4 mg/l	6,3 mg/l	5,4 mg/l

Tab. 1 - *8 female patients (35 years±4)*

The major field in which cryoRx has been tested are: orthopedics and physiatry, dermatology, angiology, urology and ginecology, cosmetic medine, plastic surgery and sports medicine.

Cryotherapy (cryoRx), based on in situ freezing and devitalization of tissues, has been found more advantageous than surgical resection in cirrhotic patients because its focal application results in the loss of less hepatic parenchyma. Moreover, it is possible to treat several liver segments and the technique can be applied and controlled precisely to produce a predictable zone of necrosis. This technology has been used intensely in open surgical settings and, more recently, applied percutaneously to treat renal tumors and liver metastasis (Atwell et al., 2007).

Percutaneous management of solid renal tumors with radiofrequency ablation and cryoablation has been established as a technically feasible treatment in selected patients (Gupta et al., 2006). Allowing for relatively short-term follow-up, these percutaneous techniques are effective in tumor management The success rate is 90-100% for radiofrequency ablation (McDougal et al., 2005) and 92-100% for cryoablation (Silverman et al., 2005). Although the published findings on percutaneous cryoablation are limited, authors (Shingleton and Sewell, 2001) have tended to treat patients with smaller tumors, usually less than 5 cm, even though cryoablation technology allows simultaneous operation of several cryoprobes to generate large confluent ice balls for tumor treatment. Atwell et al. (Atwell et al., 2007) reviewed their experience in the percutaneous cryoablative management of renal tumors measuring 3 cm or more in diameter. They found that percutaneous cryoablation of selected renal masses 3 cm or larger was technically feasible, relatively safe, and, in the basis of short-term follow-up settings, appeared to be effective in local tumor control.

The clinical study conducted by Yang et al. (Yang et al., 2012) demonstrated that compared to sorafenib alone, the combined cryoRx and sorafenib therapy significantly improved time-to-progression and overall survival in HBV-related BCLC stage C hepatocellular carcinoma patients, with acceptable tolerance and similar safety profiles as previously reported, resulting in a improved clinical outcome.

Evolution of cryoRx as a minimally invasive treatment option for men with clinically localized prostate cancer is likely to result in modifications of the established surgical technique, including parenchyma-sparing modifications adjacent to the urethra and neovascular bundle. Bahn et al. (Bahn et al., 2012) reported the follow-up experiences with focal cryosurgery in 73 selected men with clinically unilateral low- to intermediate-risk prostate cancer. Primary focal cryoablation affords encouraging oncologic and functional outcomes over a median 3,7-years follow-up. Close surveillance with follow-up whole-gland biopsies is mandatory.

Mouraviev et al. (Mouraviev et al., 2012) reviewed current salvage cryoablation (SCA) outcomes in patients with locally recurrent prostate cancer following primary radiation therapy. SCA has proved to be feasible and efficacious treatment modality, especially using third-generation technology.

Spray cryoRx (SCT) uses a noncontact system to deliver liquid nitrogen (2 to 4 psi) through an endoscopic catheter. Rapid freezing and thawing of tissue causes cellular death and is also haemostatic. Finley et al. (Finley et al., 2012) reported the preliminary results from 6 institutions in which SCT was used for the treatment of malignant airway tumors. They found that SCT can be used in patients with highly vascular tumors, with reduced bleeding complications and a low overall complication rate. Caution is needed before SCT is used on a widespread basis, given the intra-operative complications.

2.9. Analytical methods for MLT determination

Due to the very low levels of endogenous MLT, the first important issue for its measurement is the adequate extraction from biological samples. From serum matrix, MLT can be extracted by simple liquid/liquid procedures, such as the addition of dichloromethane (1:1, v/v). Samples are then vigorously mixed and centrifuged to obtain aqueous and organic phases. With this procedure, MLT is retained in the dichloromethane phase that is collected and dried under nitrogen atmosphere to concentrate MLT. This yields a satisfactory recovery rate (generally more than 70%), and can be also applied to buffer-homogenized tissues. However, a low precision and accuracy with single liquid–liquid extractions of MLT for high performance liquid chromatography (HPLC) coupled to fluorescence detector have been reported (Rizzo et al., 2002). For multiple analyses of MLT and its precursors or metabolites, more profound liquid–liquid extractions have been described using a combination of different solvents.

In older investigations, chloroform was mostly used for MLT extraction and is still in use today. Although this method is effective, dichloromethane is preferred for reasons of lower toxicity. Generally, chlorinated methane should be of highest purity and protection from light and redox-active compounds is of utmost importance for avoiding formation of reactive intermediates which can destroy MLT.

Laganà (Lagana et al., 1995) described an extraction procedure for serum samples through an LC-18 cartridge plus a Carbograph cartridge with a recovery ranging from 86.3 to 91.7% for 10 to 200 pg MLT/ml. Briefly, 2 ml of serum sample is passed through an LC-18 cartridge, which is then washed with 2 ml of water and 2 ml of water–methanol (90:10, v/v). Thereafter, MLT can be eluted from the column with pure methanol, dried and resuspended in an appropriate solution for analysis or can be further purified by eluting with 2 ml of water–methanol (40:60, v/v) and loading onto a Carbograph cartridge. The cartridge is then washed with 10 ml of methanol and 3 ml of methanol–dichloromethane (80:20, v/v), and MLT is finally eluted with 1.5 ml of methanol–dichloromethane (10:90, v/v). The eluate is evaporated to dryness under N_2 atmosphere and resuspended in 100 µl of water–methanol (75:25, v/v) for analyses.

Sample preparation is also strictly dependent on the method used for analysis, since the presence of other compounds in the sample can interfere with the MLT signal. The extent of MLT pre-purification from biological samples can, in some cases, be fundamental for the sensitivity of the method used. The procedure described above allows MLT detection with high sensitivity and without interference from other components in the sample. It has been shown that homogenization in 10 vol of ice-cold 0.1 M perchloric acid can also represent an accurate means for MLT determination in tissues by HPLC coupled to electrochemical or fluorescence detection. In this case, the homogenate is centrifuged at 10,000×g for 20 min at 4°C and the resulting supernatant can be directly injected into the HPLC system. It has also been suggested that 90 µl of the supernatant fraction be mixed with 10 µl of 1 M sodium phosphate, pH 4.3, for better resolution of peaks.

Depending on the method used, further treatment of MLT extracts may be needed. Gas chromatography mass spectrometry (GC-MS) detection of MLT requires sample derivatization for MLT volatilization by, for example, the use of pentafluoropropionic anhydride or heptafluorobutyrylimidazole (Covaci et al., 1999). In another approach, human plasma samples have been directly injected into and evaluated in an HPLC system with fluorescence detection without prior extraction or purification, achieving a detection limit of 1 ng per ml of human plasma (4 pmol/ ml). Also, it has been reported that derivatization of MLT with sodium carbonate and hydrogen peroxide increases sensitivity almost 10-fold for measurement in HPLC systems coupled to fluorescence detectors (Tomita et al., 2003)

Rolčik (Rolcik et al., 2002) described a highly specific method for MLT isolation and purification from complex biological matrices by immune-affinity chromatography. Polyclonal antibodies highly specific against MLT were raised by Mannich synthesis and used for preparation of immune-affinity gel, with a 95% recovery rate for MLT extraction. In these samples, MLT concentration was determined by HPLC-mass spectrometry (HPLC-MS) with a detection limit of 10 fmol.

Regarding sample preparation for analysis by MS, the use of adequate isotopically labeled internal standards represents an important issue; this step improves quantification of the hormone and underestimation of actual levels of MLT due to losses which might have occurred in the samples during extraction.

Finally, the correct handling and maintenance of samples is also important. Samples of MLT should be kept constantly on ice and protected from light radiation, in order to avoid degradation. Despite its relative stability, MLT oxidation can occur over time, including reactions with singlet oxygen. The probability of this occurrence varies, and is dependent on oxygen availability and light incidence. For sample freezing, it is recommended that samples be dried and preferentially kept under vacuum or nitrogen atmosphere.

2.9.1. Immunoassay

For the monitoring of MLT in biological fluids, use of immunological methods is the most widespread method. Several commercial kits based on these methods are available for MLT determination. Some of these methods are highly sensitive and simple to use (lower limit of detection: 0.5 pg/ml) but may suffer from a potential risk of cross-reactivity to structurally similar compounds if MLT is not extracted.

The most crucial aspect of immunoassays is the preparation of the antiserum. Because MLT is too small to be capable of producing antisera on its own it must be coupled to an antigenic protein. In such a conjugate the small molecular weight substance is called a hapten. The resulting antiserum binds both the protein and the hapten plus a portion of the adjacent protein. The hapten has few antigenic determinants relative to the protein. Specificity studies of antisera produced by steroid–protein conjugates have shown that antisera are not able to discriminate structural differences in the hapten that are immediately at or close to the site of coupling.

The choice of the hapten and conjugation reaction should therefore be determined by the type of discrimination that is required. Indolealkylamines have in common a ring nitrogen (position 1) and an adjacent carbon (position 2). Thus for MLT, coupling via the position 1 or position 2 should allow resulting antisera to discriminate different indoles that are commonly found in tissues.

Studies of antisera resulting from Mannich coupling of MLT to bovine serum albumin (BSA) have revealed that this approach leads to a highly specific MLT antiserum as shown by cross-reactivity studies in radioimmunoassay (RIA) (Yang et al., 2006). To determine the locus of attachment of MLT to protein, model reactions have been conducted and the resulting products analyzed by nuclear magnetic resonance and infrared spectroscopy. The results of the study indicated that coupling was likely at position 2. Further studies were done of cross-reactivity of intermediate reaction products revealing that the highest cross-reactivity occurred with C-2 substituted MLT derivatives. Thus it was concluded that the methylene bridge conjugating MLT to BSA occurred at the number 2 position of the indole molecule. This approach has been used widely for MLT immunoassays. Recently, two different groups have used this approach for generating monoclonal antisera against MLT (Soukhtanloo et al., 2008).

Coupling at the ring nitrogen using 1-(p-carboxybenzyl)-MLT coupled to BSA as antigen results in antisera that bind MLT specifically. MLT-1- propionic acid coupled to BSA also stimulates production of highly specific antisera. A similar approach by coupling 1-(4-carboybutyl)-MLT to protein resulted in a highly specific RIA. The MLT derivative, 3-(3-(2-acetamidomethyl)-5-methoxyindol-l-yl) propionic acid coupled to bovine gamma globulin produces a specific antiserum that has been used widely in RIA. Yet another derivative N-(3-(2-aminoethyl)-5-methoxyindole) hemi succinamide has been used to generate antiserum as the basis for a specific RIA. Thus MLT coupled at the N position gives rise to antisera that are highly specific for MLT as compared to other indoles.

Coupling at the side chain has also successfully produced useable MLT antiserum. The methods used include N-acetyl-5-methoxytryptophan coupled using carbodiimide, succinyl-5-methoxy-tryptamine coupled to protein and indomethacin coupled to protein. MLT coupled via a diazo linkage has also been reported to produce a reasonably specific antiserum, however the sensitivity of the resulting assay was found to be low.

Coupling of NAS using formaldehyde generates antiserum that binds MLT and NAS equally; cross-reactivity studies and model reactions have shown that coupling occurs at the 4^{th} position of the molecule. The resulting antiserum has been used as the basis of an RIA that required prior extraction and column chromatography to eliminate the cross-reacting indole.

The chief metabolite of MLT in urine, aMT6s has also been measured by immunologic means. The antiserum typically used for this assay is generated by use of the Mannich reaction and is highly specific. Antisera produced using these approaches have been used extensively not only for RIA, but also for immunohistochemistry and for enzyme-linked immunoassays (ELISA).

2.9.2. Radioimmunoassay

The principle of RIA method for MLT measurement is that a known amount of radioactive MLT (2-I^{125}-iodoMLT or ^{3}H-MLT) is mixed with a fixed amount of antibody raised against MLT. Increasing concentrations of unlabeled MLT are added to the mixture, which will compete with labeled MLT causing its displacement from the antibody. Free labeled MLT is then separated from remaining antibody-bound radioactive MLT and radioactivity is measured. As the concentration of unlabeled MLT increases in the mixture, competition for the antibodies also increases and bound labeled MLT decreases. A calibration curve constructed from known amounts of labeled and unlabeled MLT allows the determination of unknown MLT concentrations in biological samples.

Fraser (Fraser et al., 1983) described a protocol for MLT measurement by RIA in plasma that has been adopted by several researchers, some with slight modifications. Briefly, 200 µl of 1000-fold diluted antibody is added to 500 µl of solutions containing different amount of MLT standard (2.5 to 250.0 pg). The solution is vortexed and kept at room temperature for 30 min. ^{3}H-MLT is added to the tubes(100 µl, 4,000 cpm), mixed, and kept at 4°C for 18 h. Then, 0.5 ml of Dextran-coated charcoal solution (0.1 g of dextran 75 plus 10 g of charcoal per 500 ml of buffer) is added and the solution is centrifuged for 15 min at 1500×g and 4°C, in order to separate the antibody-bound MLT from the free fraction. The supernatant fraction is finally decanted into 10 ml of scintillation fluid and radioactivity is counted on a beta scintillator counter.

Several variations in RIA methods have been described, by using different antibodies (as noted above), by changing ^{3}H-MLT to 2-I^{125}-iodoMLT, or by altering the separation procedure. In general, because of its higher specific activity 2-I^{125}-iodoMLT allows a lower detection limit thus allowing the use of a smaller

amount of sample. The concentration of MLT during daylight can be as low as 0.2 to 0.3 fM (Rolcik et al., 2002). This could be especially important if measurements are not preceded by MLT purification. However, ^{125}I is more prone to nonspecific binding so that some determinations can be faulty.

Sieghart (Sieghart et al., 1987) reported that prior MLT purification from plasma using reversed-phase column chromatography greatly reduces the problems of cross-reactivity. Moreover, Rolčik (Rolcik et al., 2002) used immunoaffinity chromatography employing specific antisera to process samples prior to HPLC-MS analysis. Nonetheless, it should be recognized that even a weak cross-reactivity can be a problem if the cross-reacting molecule is present in large quantities. Thus independent validation of the procedure is essential when a different matrix is assayed.

One example of such a different matrix is saliva for which several RIAs have been described. To obtain saliva, different methods have been used, from chewing gum, chewing on cotton swabs, or using commercial apparatus. Again, extraction is usually essential especially since levels in saliva are about 40% of those in plasma. Saliva is particularly useful if repeated sampling is required: for example to characterize the full 24 h rhythm of MLT or to determine the dim light MLT onset (DLMO), a measure that has been shown to be very useful in studies on circadian rhythmicity in sleep disorders (Pandi-Perumal et al., 2007).

Several variants of the time-consuming charcoal separation procedure have been developed and successfully applied. In the so-called scintillation proximity assay, the MLT antibody is bound to a secondary antibody (e.g. antisheep) attached to scintillator-containing microbeads ("fluomicrospheres"). This relatively convenient procedure depends, however, usually on the commercial availability of suitable fluomicrospheres, since preparation and standardization of such beads is too time-consuming for the average laboratory. In the proximity assay, bound radioactivity is detected directly by the scintillator system of the microspheres. For physical and geometrical reasons, such a system has to have a lower scintillation efficiency than a homogeneous scintillation cocktail. However, this procedure has other advantages. Apart from being more rapid, the system is less affected by nonspecific binding (values close to background) such as occur in the charcoal procedure, has a better reproducibility and shows a much lower assay drift upon repetitive measurements (proximity assay: about 10% change within 84 h; charcoal method: about 25% over the same period). Other variants include separation using a double antibody procedure and ammonium sulfate precipitation.

Considerable interest has also been shown in the major urinary metabolite of MLT, aMT6s. The 24-h pattern of excretion of the metabolite accurately reflects the pattern of MLT in blood. RIAs for this substance are available and have been useful in assessing pineal function in various conditions(Fideleff et al., 2006).

2.9.3. Enzyme-linked immunoassay

A variety of ELISAs for MLT have also been reported that employ antisera identical to those used in the RIA described above. One such immunoassay employed MLT– hemi succinate–human serum albumin absorbed on polystyrene spheres, with the MLT competing for a fixed amount of peroxidase labeled IgG antibody to MLT. This method had a detection limit of 22 fmol per tube and therefore required extraction. A competitive solid phase ELISA for human and rat serum and rat pineal gland has been described and validated using microtiter plates that has a much lower detection limit (1.0 fmol per well) as well as precision comparable with other methods and that can be applied without extraction to rat serum. An improved version of this assay with a shorter incubation time was subsequently reported. A comparative study of an RIA and a commercial ELISA reported that the ELISA required a purification step to be valid when applied to human

serum, a step that was not part of the procedure recommended by the manufacturer. With the extraction step, the assay had distinct advantages, Enzyme assays have major advantages in that the enzyme conjugate is stable, is more convenient than ^{3}H or ^{125}I and present no problem with disposal of radioactive waste. Furthermore if microtiter plates are used centrifugation is not necessary. Although not an enzyme immunoassay, it is of interest that a time resolved fluoroimmunassay has also been described (Yamada et al., 2002). An enzyme immunoassay for aMT6s has been reported, and commercial kits are available.

2.9.4. HPLC coupled to electrochemical and fluorescence detection

In many studies, RIA methodology has been replaced by HPLC with electrochemical and fluorescence detection for MLT evaluation, due to its great sensitivity and specificity. However, this procedure is more adequate for MLT alone, and not for mixtures of several indoles, such as serotonin and tryptamine among others, that can cause disruptions in the assay. For example, serotonin/ MLT ratio is higher than 100 in rat pineal. This high ratio can cause disturbances in chromatographic separations that can make MLT detection difficult, and thus requires a good procedure for MLT extraction. However, the avoidance of partial co-elution with other indoles is mostly a matter of the art of chromatography. In the work conducted by de Almeida et al. (de Almeida et al., 2011), they have been able to detect MLT with great accuracy in blood plasma after simple dichloromethane extraction as described above, and using an HPLC system connected to electrochemical detection. Good peak separation was achieved by using a LC-18 column and 50 mM sodium acetate-100 mM acetic acid (pH 4.3), 0.1 mM Na_2-EDTA, and acetonitrile (75:25, v/v) as mobile phase pumped isocratically at 1 ml/min.

Harumi (Harumi et al., 1996) also successfully determined MLT by HPLC with electrochemical detection, with very clear peak separation for different indoleamines among MLT. However, the sensitivity of this procedure depends on the model of electrochemical cell. Amperometric-based electrochemical cells are generally less sensitive than coulometric cells, so that the adequate potential should be previously optimized by the construction of hydrodynamic voltammograms. With the coulometric electrochemical system, the best MLT signal is obtained at 600 mV. Sensitivity can be also greater with coulometric electrochemical detectors such as the ESA coulochem III model (ESA, Bedford, MA, USA), which uses porous electrochemical cells that allow greater accuracy in MLT peak resolution. Harumi et al. reported the use of a higher potential, 900 mV, for good MLT signal with their graphite carbon working electrode, and even so they detected MLT at very low levels. Rizzo (Rizzo et al., 2002) also used 900 mV for MLT detection with an amperometric electrochemical detector.

With respect of fluorescence detection, some high sensitive methodologies have been reported for MLT detection at the femtomole level (Yang-2002). MLT can be separated on a C18 column by using 75 mM sodium acetate pH 5.0 and acetonitrile (72:28, v/v) as the mobile phase pumped isocratically at 1.0 ml/min, and directly detected by setting up the fluorescence detector at an excitation wavelength of 275 nm and an emission wavelength of 345 nm (Rizzo et al., 2002. Nevertheless, in some cases in which MLT concentration is very low, derivatization is recommended to enhance the MLT signal. An oxidation procedure that can enhance MLT fluorescence by 6.8 times (allowing its determination at attomole levels) has been described using biological samplesMLT was oxidized to a new fluorescent compound with sodium carbonate and hydrogen peroxide. However, precautions should be taken when using this kind of approach, because other components in the biological sample may lead to the generation of fluorophores, which in turn could interfere with the determination of the correct level, thus preventing method specificity (Tomita et al., 2003).

In any case, care with sample preparation can improve the MLT signal. Pre-purification of MLT as described before will decrease chromatogram noise and avoid the co-elution of MLT with other compounds that can interfere with MLT peaks. Generally, the use of fluorescence techniques are affected not only by co-elution with other fluorescent compounds in the sample, but also by the presence of quenchers. This should not be underrated since the majority of aromates absorb around the excitation maximum of MLT. Therefore, samples should be tested in advance for quenching by adding known amounts of MLT.

2.9.5. Mass spectrometry

The GC-MS technique is very sensitive and offers more specificity than HPLC with electrochemical or fluorescence detectors; however, a difficulty with this technique is the need of derivatization, and thus it has been gradually substituted by liquid chromatography-mass spectrometry procedures. Thus, alternative HPLC-MS methods appropriate for use in biological issues have been developed (Motoyama et al., 2004). However, this approach is limited by the need of adequate internal standards. Yang et al. described their methodology which used N-acetyltryptamine as the internal standard (Yang-2002); however, several factors make this approach less than ideal. It is appropriate to use a labeled internal standard whose structure is the same of the analyte except for the mass difference. The addition of an isotopically labeled internal standard prior to the analysis improves the method's confidence level.

Another analytical method has been developed which uses column-switching semi-microcolumn liquid chromatography/mass spectrometry and selected ion monitoring (Anisimov et al., 2006) for detecting endogenous MLT in human saliva. In the relevant study MLT was monitored based on its fragment ion at m/z 174 by in-source dissociation and using deuterated MLT as the internal standard, and a detection limit of 10 fmol was obtained (Rolcik et al., 2002). The main limitation of this methodology is the use of the SIM mode to detect the ions generated in the probe, which does not imply an absolute specificity. Yet, Eriksson (Eriksson et al., 2003) reported a method for the determination of MLT in human saliva by HPLC-MS/MS, using 7-D-MLT as internal standard. The limit of detection was 1.05 pg/ml and the limit of quantification was 3.0 pg/ml. It has been reported the development of a new HPLC-MS/MS assay with electrospray ionization (Regodon et al.) to quantitatively determine MLT and also its degradation product AFMK with high sensitivity and specificity. A stable isotopic internal standard MLT-D_3 (deuterated MLT) was easily synthesized by the reaction of 5-methoxytryptamine with deuterated acetyl chloride (CD_3COCl).

The predominant ion [M+H]+ in the full scan mass spectra of MLT, and MLT-D_3 were located. The fragments generated in collision-induced dissociation chamber revealed a predominant fragment at m/z=174 for MLT and MLT-D_3 (loss of the N-acetyl group). The m/z transitions from 233 to 174 (MLT) and from 236 to 174 (MLT-D_3) were therefore chosen for the Multiple Reaction Monitoring (MRM) detection experiments, which ensured a higher specificity and an accurate quantification of MLT in human plasma.

2.9.6. Other techniques

Some laboratories have taken and developed capillary electrophoresis (CE) for the separation and determination of MLT in blood plasma (Musijowski et al., 2006) and in pineal gland (Chen et al., 2001)Detection of analyte was performed with a UV and fluorescence (Pobozy et al., 2005) or electrochemical detector (Wu et al., 2006). The detection limit of MLT with CE is comparable with the data obtained by HPLC methods reported previously. Recently, for the separation of MLT from related compounds, CE with micellar electrokinetic chromatography was applied (Hevia et al., 2010). This technique

permitted the effective separation of MLT and its precursors or metabolites. Sodium dodecyl sulfate is used to produce a pseudo-stationary phase.

3.0. AIMS

3.1. MAIN OBJECT

The number of signaling pathways which modulation, at different cellular hierarchy, has been attributed to MLT molecule, is exceptional. The practical applicability of MLT as a therapeutic agent, however, remains unconfirmed inasmuch as most of the effects described have not been validated in a clinically relevant setting with the use of a well -defined pharmaceutical formulation and therapeutic scheme.

The main object of my PhD thesis is to confirm the pleiotropy of MLT molecule by testing its activity in two of the most promising clinical applications: the cure of prostate cancer and the regulation of the sleep/wake rhythm as adjuvant in the sedative therapy in critically ill patients. The attaining of these objectives is necessarily linked to a comprehensive study to assess whether alternative and novel strategies to deliver the drug may be competitive (both in animal and in humans) with the routine methods used till now. Providing that MLT appears active in fighting prostate cancer and/or in ameliorating critically ill patients hospitalization, I will try to elucidate the molecular mechanisms underlying the observed activities, with particular attention to the hypoxia signaling pathway and to the antioxidant and scavenger activity. To elucidate the molecular events underpinning MLT clinical effects is pivotal to plan effective strategies for further research.

3.2. SPECIFIC AIMS

3.2.1. MLT as antitumoral molecule

1. To evaluate the oncostatic effect of MLT administered intraperitoneally (i.p.) by saline solution on human prostate tumor. To this purpose I will select an in-vivo experimental model of nude mice (athymic), xenografted subcutaneously with tumor cells of a human prostatic line (LNCaP), an approach much closer to the clinical situation than *in vitro* cultured cells and that looks therefore adequate to investigate the underlying molecular mechanisms *in vivo*. Tumor growth will be monitored over time (4-6 weeks) in relation to a repeated treatment with MLT administered i.p. . The efficacy will be stated in terms of inhibition on tumor growth, good animal compliance and low toxicity. This part of the research will provide the evaluation of amount of MLT distributed systemically and accumulated in the tumor mass.
2. Using the same animal model and the same administration route (i.p.) and treatment schedule of MLT administered in saline, to investigate the efficacy of a novel and promising pharmaceutical formulation: MLT included in a solid lipid nanoparticles system (SLN-MLT). The nanoparticles systems should be able to enhance the role of MLT as an inhibitor agent of tumor growth, to reduce the first pass effect through the liver, increase the systemic distribution, avoid the destruction in the gastro-intestinal tract reducing the inter-individual variation in bioavailability of the drug. This study is intended to find that nanocarrier (SLN) are able to promote the systemic absorption of MLT in respect to MLT dispersed in a buffered solution, used as reference. The pharmacokinetics (distribution and metabolism of MLT) will be investigated in the mouse model. The specific tropism for cancer tissue and for all the target organs (prostate, brain, cerebellum, liver, kidney) will be studied.
3. Using the same mouse model of human prostate cancer, to test whether MLT can be administered efficiently using alternative ways that are more sustainable for prolonged treatments than i.p. MLT, e.g., transdermal delivery through the skin barrier directly onto the tumor *via* a novel and patented technique

named cryoRx. CryoRx is a system for the delivery of drugs based on the topical application of drugs frozen in suitable cryo-applicators connected to a laser source. To assess whether transdermal MLT application negatively affects tumor growth in a concentration- and time-dependent manner, so to be considered for further experimentation, and the advantages in terms of lower toxicity and greater efficacy.

4. To focus on the underlying action mechanism of MLT at the tumor cellular micro-environment and the possible influence on such a mechanism of the lipid nanocarrier employed. To this purpose the cell signaling mechanisms involved in the reduction of tumor growth will be investigated and in particular, interactions with other regulatory factors such as "hypoxia inducible factor" (HIF-1α), a positive regulatory factor induced by hypoxia. Moreover, to complete the understanding of MLT loaded-nanocarrier effects at the tissue microenvironment in physiological or pathological conditions, the antioxidant properties of such systems will be evaluated by studying oxidative stress, damage from free radicals and possible residual antioxidant defenses in tissues and blood.

   Attaining this aim will also help addressing the relevance of these signaling paths during cancer development.

The results achieved from the proposed aims will be integrated together and will add knowledge both in understanding the crucial pathways for targeting in prostate cancer and in the development of new pharmacological devices for the cure and prevention in clinical patients

3.2.2. MLT in ICU patients as sleep/wake regulator

1. To evaluate in a cohort of critically ill patients admitted to a high-risk Intensive Care Unit (ICU), if the circadian rhythm of MLT secretion is disrupted and to which extent MLT administration by different routes and different drug formulations (MLT as a tablet administered orally, MLT encapsulated in SLN administered orally as a suspension, MLT encapsulated in SLN applied transdermally as a suspension with the aid of a patch) is feasible in terms of efficiency of absorption and adequacy in achieving and maintaining nocturnal peak plasma hormone even in the early phase of hospitalization in the ICU. To study the differences in the pharmacokinetics profile (absorption peak, plasma half-life, mean concentration) of MLT as a function of administration by different routes and drug formulations and to understand the pathophysiological characteristics that may affect. To test whether some of these administration ways are competitive and more likely sustainable in a clinical setting. The nano-encapsulation should allow for a more effective therapeutic action related to the bioavailability and the intracellular hormone concentration. The SLN in fact act as a reservoir of the hormone allowing a constant and prolonged effect at the cellular site of action, so it could be more evident a clinical effect thanks to the achievement of the intracellular environment regardless of the specific receptors–MT1 and MT2. Transdermal administration may have several advantages in critical patient, in particular: ease of administration (the application of a patch on the skin is possible in almost all clinical settings situations excluded from hyperhidrosis, large burns, allergic skin manifestations, cutaneous vasoconstriction etc..) and reduced hepatic first-pass effect (reduction of the administered dose with lower peak plasma maintaining a constant plasma concentration).
2. To evaluate if the restoration of the melatoninemia by the different ways of drug delivery in critically ill patients may be useful to restore the pleiotropic function of this hormone: facilitate the resolution of

sleep-wake cycle disorders, improve the quality of sleep, reduce the number of episodes of anxiety, confusion and agitation, and reduce the amount of sedatives used, especially at night. As a first approach, the total antioxidant defenses of the patients after the different MLT treatment will be evaluated, in order to understand the underlying mechanism of the observed clinical activity.

4.0. MATERIALS AND METHODS - MLT AS ANTITUMORAL MOLECULE

4.1. MATERIALS

4.1.1. LNCaP cells

LNCaP cells are androgen-sensitive human prostate adenocarcinoma cells derived from the left supraclavicular lymph node metastasis from a 50-year-old caucasian male in 1977. They are adherent epithelial cells growing in aggregates and as single cells (Fig. 16). The LNCaP cell line was established from a metastatic lesion of human prostatic adenocarcinoma. The LNCaP cells grow readily in vitro (up to 8 x 10^5 cells/sq cm; doubling time, 60 h). The malignant properties of LNCaP cells are maintained. Athymic nude mice develop tumors at the injection site (volume-doubling time, 86 h). Functional differentiation is preserved; both cultures and tumor produce acid phosphatase. High-affinity specific ARs are present in the cytosol and nuclear fractions of cells in culture and in tumors. The cell line does express PSA and MLT receptor (MT1). In vivo, the frequency of tumor development and the mean time of tumor appearance are significantly different for either sex. Male mice develop tumors earlier and at a greater frequency than do females. Hormonal manipulations show that, regardless of sex, the frequency of tumor development correlates with serum androgen levels. The rate of the tumor growth, however, is independent of the gender or hormonal status of the host.

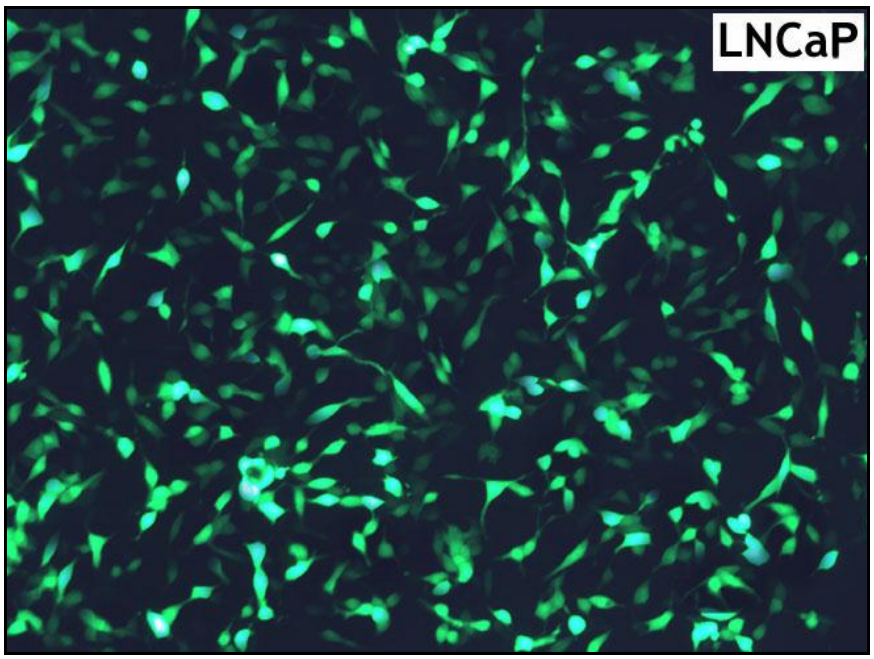

Fig. 16 *- LNCaP cells by fluorescence microscope .Picture modified from: http://signagen.com/index.php?main_page=product_info&products_id=747.*

4.1.2. Cultures

The androgen-dependent human prostate cancer cell line LNCaP from American Type Culture Collection (purchased from Rockville, MD, USA) was grown as monolayer in RPMI-1640 medium (Euroclone, Westherby West Yorkshire, UK) supplemented with 10% fetal bovine serum (Euroclone), 100

U/ml penicillin (Invitrogen, San Giuliano Milanese, Italy), 100 µg/ml streptomycin (Invitrogen), and was maintained at 37°C in 5% CO_2 in air. The medium was changed at intervals of 2-3 days.

4.1.3. Animals

Animals were cared in accordance to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). Seven-week-old male Foxn $1^{nu/nu}$ mice (purchased from Harlan Bioproducts for Science, San Pietro al Natisone, Italy), weighing 27-30 g, were housed in a pathogen-free environment inside a laminar-flow hood and fed with food sterilized by ^{60}Co-γ-irradiation. Water and bedding were heat-sterilized. Mice had free access to water and diet until 24 h before sacrifice. A 12/12 h light/dark cycle was maintained. Sterile gloves and masks were used whenever the animals were handled.

4.1.4. Matrigel

The following characteristics of Matrigel Basement membranes are thin extracellular matrices underlying cells in vivo. Matrigel Basement Membrane Matrix is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins. Its major component is laminin, followed by collagen IV, heparin sulfate proteoglycans, entactin and nidogen. It also contains TGF-β fibroblast growth factor, tissue plasminogen activator and other growth factors which occur naturally in the EHS tumor. Matrigel Basement Membrane Matrix is effective for the attachment and differentiation of both normal and transformed anchorage dependent epithelioid and other cell types. Basement membranes are highly specialized, continuous sheets of extracellular matrices which underlie epithelial and endothelial cells and surround muscle, fat and the entire nervous system. The basement membrane plays a key role in diverse biological processes such as providing mechanical support for cell layers and formation of barriers between tissue compartments that impede the transmigration of cells and passively regulate the exchange of macromolecules. The basement membrane also serves as an interactive surface for cells by providing adhesion, cell shape and migratory signals, as well as communicating information for regeneration and/or differentiation.

Matrigel gels rapidly at 22°C to 35°C. Therefore it was thawed at 4°C overnight on ice and kept on ice before use. The product was handled using sterile area and gently pipetted using a pre-cooled pipette to ensure homogeneity.

4.1.5. LNCaP xenograft

LNCaP cells were grown to 90% confluence, washed in serum-free RPMI, harvested by quick tripsinization, recovered in Falcon tube pooling same cells from different flasks, spinned down (1000 rpm for 5 min) and finally counted by the Trypan blue test. After centrifugation (800 rpm for 5 min), the cells were resuspended in a 50% (v/v) mixture of ice-cold Matrigel (BD Bioscience, Buccinasco, Italy) and serum-free RPMI-1640 medium in order to get a suspension containing 3.0 x 10^6 cells/0.1ml.

Since Matrigel Matrix forms a gel above 10°C, Matrigel Matrix solution was kept at low temperatures and all related equipment (syringes, needles, etc.) and reagents were chilled in an ice bath prior to injection. After mixing Matrigel Matrix with cells, the mixture was injected into each flank of the mice subcutaneously. An appropriate needle size (26G) was selected to prevent the destruction of cells. To increase the contact

area of the injected mixture into tissue, a wide subcutaneous pocket was formed by swaying the needle right and left after a routine insertion. The mixture (0.1 ml) was then slowly injected into the pocket.

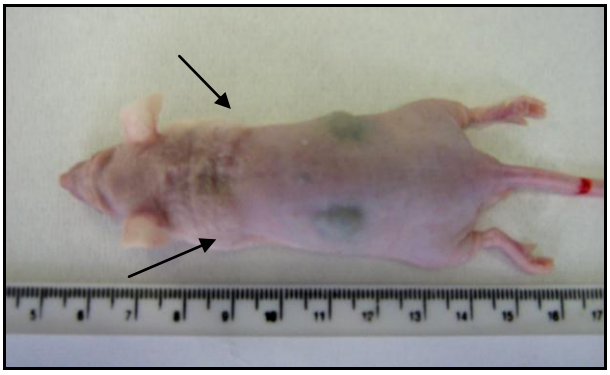

Fig. 17 *- Mice xenografted with human LNCaP cells.*

4.2. EXPERIMENTAL PROTOCOL

For the evaluation of the antitumoral activity of MLT we have studied 2 different administration routes: intraperitoneal (i.p.) and topical by cryoRx.

4.2.1. Time Schedule

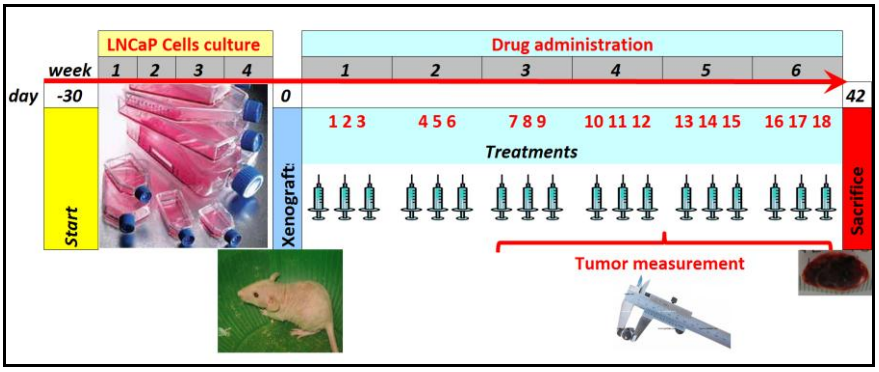

Fig. 18 *- Treatment schedule*.

For all the experiments we have used the same treatment schedule as shown in (Fig. 18 - Treatment schedule which provides that the MLT administration (i.p., or by topical laser administration) has been carried out 3 times a week starting from the day after the xenograft for 6 weeks (18 treatments), the time required because the tumor to be developed. At the end (42^{th} day) the animals were sacrificed and along the treatment period the mice weight was recorded as well as the tumor volume was measured by a caliper as soon as it became apparent.

4.2.2. Dosage and MLT preparation for i.p. administration

The MT i.p. dose delivered at each of the 18 treatments (see above) was 1 mg/Kg both in saline solution and enclosed into SLN. As the mice weight rank about from 26-32 g, we planned to administer for each i.p. treatment a dose of 0.030 mg/mouse in a 100 µL volume. For each experiment, MT or SLN-MT in the suitable quantity for the programmed number of animals, was dissolved in sterile saline solution at the final concentration 0.3 mg/mL. This solution was sterilized by filtration through 0.2 µm filter under sterile laminar flow hood, capped and stored at 4°C along the treatment period.

Saline solutions of the drug have been prepared fresh in our laboratory, while SLN loaded with MLT has been provided by Nanovector Srl, Turin, Italy (Gasco and Gasco, 2007) as lyophilized powder in vials containing 3 mg SLN-MLT and 41 mg excipients (stearic acid, Epikuron 200, sodium glycocholate, UP water and trehalose).

4.2.2.1. Treatment Groups

Control group (Ctrl): mice treated with injection of saline alone i.p. This group allows the assessment of tumor growth without any anticancer treatment.

Group treated with MLT (MLT): mice treated according to the scheme described above with injection of MLT dissolved in saline. This group allows to evaluate the effect of MLT has on tumor growth in relation to the positive control.

Group treated with MLT incorporated in SLN (SLN-MLT): mice treated with i.p. injections of MLT incorporated in SLN. This treatment allows to evaluate the effect MLT incorporated in SLN has on tumor growth in relation to positive control and evaluating differences compared to MLT injected without the formulation SLN.

4.2.3 Dosage and MLT preparation for topical administration

For this set of experiments, mice xenografted with human LNCaP cells as described above, received MLT topically by the treatment schedule described above. MLT has been prepared fresh each week by emulsifying 0,048 mg MLT/ml of 0,8-1% hydroxyethyl cellulose for 7 min at full speed with a Politron ultra-turrax, keeping the drug in ice and in the dark. Then, 15 ml of the suspension (0,72 mg MLT) have been transferred into the cryo-applicator and frozen at -20°C overnight. Each stick have been used for the treatment of 6 animals (Figure X). The final dose administered topically is approximately 0,120 mg MLT / mouse / treatment, ie 4 mg/kg. In the first stage, the frozen stick connected to a laser source giving the energy to penetrate the cutaneous barrier and deliver the active principle to the target area, was rubbed on the back of the animal (Fig. 19 –), where the tumors where xenografted, for 2,5 min (≈ 2.5 min per mouse corresponded to the melting of ≈2.5 mL). This treatment time was studied to avoid that the animals of small size such the nude mice used here, can go into hypothermia. Longer treatment times are not recommended for healthy animals. When necessary, the mice have been placed on a hot plate at 37°C during the treatment.

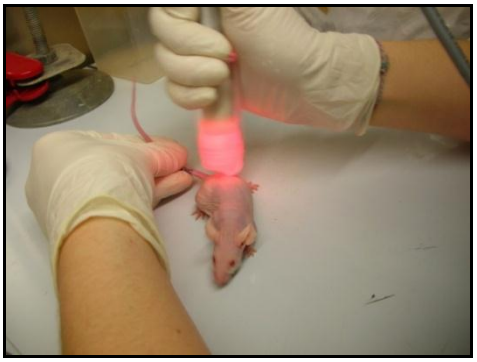

Fig. 19 – *Frozen stick connected to a laser source rubbed on the back of the animal.*

The concentration of MLT has been studied to administer the required amount topically in 2,5 min. After this first phase, in which the drug was pushed under the skin, mice have been placed in special constrictors, immobilized and a high-power laser scanning was applied on the area of drug application for 15 min to optimize the adsorption through the tissues and facilitate the drug to reach the desired site of activity (Fig. 20). At this stage the use of a laser source with a pendulum movement allows the positioning of drug molecules to the desired depth.

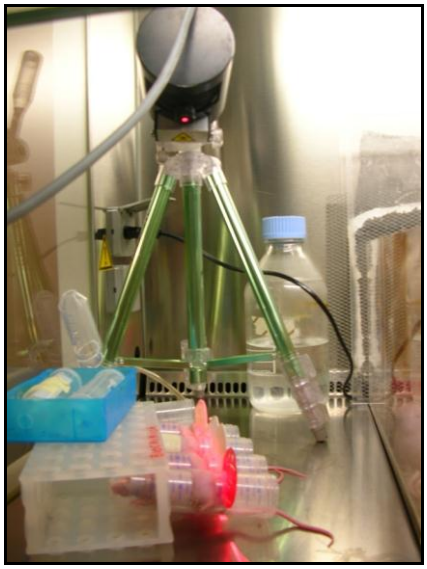

Fig. 20 – *Portable configuration of Lasericemed-cryoRx that we used for our experiments.*

4.2.3.1. Treatment Groups

Control group (Ctrl): the tumor are xenografted to athymic mice and, without any anticancer treatment, the growth in mice, he tumor mass and mortality have been recorded for all the experiment period. It shows the tumor growth for the period of the experiment and the growth of mice and/or mortality in the absence of other variables.

Control group treated with cryoRx (laser): the tumors are xenografted to athymic mice and then the mice have been treated with frozen gel excipient containing MLT, 3 times a week for 42 days after xenograft. This group has the responsibility for determining whether the laser itself can have an influence on tumor growth, growth and mortality of mice.

Tumor group treated with cryoRx and MLT (MLT- laser): the tumors are xenografted to athymic mice and they have been treated with frozen gel excipient containing MLT,3 times a week for 2 days after xenograft. For comparison with the control groups, one can assess the possible increase of the preventive effect and / or antitumor activity of MLT is associated with the laser treatment.

4.2.4. Sacrifice

At the end of the observation period, animals were anesthetized by i.p. Na-thiopental (10 mg/100 g body weight) plus heparin (500 units) and weighted. After euthanasia by cervical dislocation, animals were taken out of the chamber to be thoracotomized, blood was withdrawn into a heparinized syringe from the left

ventricle, tumors were quickly dissected away from surrounding skin and fascia and weighed, internal organs were removed, isolated, and harvested. The tumors and the organs were immediately frozen in liquid nitrogen and stored at -80°C for biochemical analyses and for histological study. No precaution was taken for N mice.

4.3. MEASUREMENTS

4.3.1. In vivo measurements

Body weight and tumor volume were measured three times a week. No precaution was taken for N mice. The linear dimensions of the tumor were measured with electronic caliper and the tumor volume (in mm^3) was calculated by the formula :

$$V = \frac{1}{2}\left(\frac{4\pi}{3}\right)\left(\frac{length}{2}\right)\left(\frac{width}{2}\right)(height) = 0.5236 \times length \times width \times height$$

This formula was derived from a formula for calculating the volume of a hemi-ellipsoid, the geometric figure most nearly approximating the shape of tumors (Williams et al., 2007). Tumor growth had to be followed through at least four separate measurements to be admitted to statistical analysis.

4.3.2. Trypan Blue test

The number of viable cells was determined using Kova Glasstic Slide 10 with grids (Hycor Biomedical GmbH, Kassel, Germany) under an inverted microscope (Motic AE 31, Motic Incorporation LTD, Hong Kong). The cells were trypsinized, centrifuged (1,000 rpm for 5 min) and resuspended in 2 ml RPMI. Cell viability was determined by the Trypan Blue exclusion assay, by mixing 10 µL cell suspension, 10 µL 0.4% Trypan blue solution and 80 µL PBS. Dead cells were counted as blue cells and the live cells were counted as cells that did not absorb dye.

4.3.3 Protein extraction and Western blot

Separate extracts were prepared for each biopsy at 4°C. To obtain the cytosolic extract, frozen tissue (50-80 mg) was homogenized in a glass potter at 4°C with 1:3 (w:v) solution containing 10 mM HEPES, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF, 10 mM KCl and 10% Protease Inhibitor Cocktail (Complete Protease Inhibitor Cocktail Tables, EDTA-free, Roche Diagnostics GmbH, Mannheim, Germany), pH 7,9. The homogenate was kept in ice for 20 min and centrifuged for 20 min at 14000 rpm at 4°C. The pellet was resuspended in the same solution, kept in ice for 10 min and centrifuged for 10 min at 14,000 rpm at 4°C. The cytosolic extract was obtained pooling the supernatant fractions from both centrifugations. The pellet was resuspended in the solution containing 20 mM Hepes, 1.5 $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMFS, 25% glycerol, 10% Protease Inhibitor Cocktail (Roche) pH 7,9, kept in ice for 20 min and centrifuged for 20 min at 14000 rpm at 4°C to obtain the nuclear extract. The proteins concentration was measured by the Coomassie Plus Protein Assay reagent Kit (Pierce, Rockford, IL).

Either the nuclear or the cytosolic extract were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE. Both extract were separated by 8% acrylamide gels and 50-70 µg protein was loaded per each lane. After separation, proteins were blotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) and blocked with 5% nonfat dry milk

in TRIS-buffered saline containing 0.1% Tween (1 h, room temperature). Membranes incubation overnight at 4°C with the primary antibody, was followed by incubation with horse-radish peroxidase-conjugated secondary antibody (1 h, room temperature). The following primary antibodies and dilutions were used: rabbit polyclonal anti-HIF-1α (Santa Cruz Biotechnology, 1:2000), rabbit polyclonal anti-VEGF165 (Calbiochem, 1:1000), mouse monoclonal anti-actin (Sigma Aldrich, St Louis, Mi 1:4000), rabbit polyclonal anti phospho-Akt-Ser^{473} (Cell Signaling Technology, 1:1000), rabbit polyclonal anti Akt (Cell Signaling Technology, 1:1000), rabbit polyclonal anti α-tubulin (Santa Cruz Biotechnology, 1:500). The secondary antibodies included horseradish peroxidase-conjugated anti-mouse IgG (Jackson Immuno Research, West Grove, PA, 1:10000) or anti-rabbit IgG (Jackson Immuno Research, West Grove, PA, 1:10000). Chemiluminescence was detected by incubating the membrane with LiteAblot Chemiluminescent substrate (Lite Ablot, EuroClone, EMPO10004) followed by x-ray film exposure (Kodak X-Omat Blue XB-1 Film, Eastman Kodak Company, Rochester, NY). The resulting image was acquired and quantified by Gel Doc (Bio-Rad quantitation software Quantity One).

4.3.4. Hemoglobin

Hemoglobin (Bartsch et al.) concentration/content was measured in blood, tumor mass and a reference organ (left kidney). Blood Hb concentration was measured by diluting 10 μl of well-stirred blood to 1 ml of Drabkin reagent, followed by incubation for 30 min at room temperature and absorbance reading at λ=540 nm. The concentration was calculated assuming ε=11.05 cm^{-1} mM^{-1}.

To measure the tissue Hb content, 10 μl extract was diluted in 1 ml Drabkin reagent, (Bartsch et al.) was measured and expressed as μg Hb/mg tissue and was calculated by the formula:

$$\frac{\mu gHb}{mgTissue} = \frac{Abs \times 101}{11.05} \times 16000 \times \frac{Extraction\,Volume_{ml}}{BiopsyWeight_{mg}}$$

4.3.5. Immunohistochemistry

Biopsies from the frozen organs were included into embedding medium (OCT, optimal cutting temperature-compound, Leica Instruments, Nussloch, Germany) and serial 5-μm thick sections were obtained in a cryomicrotome (Leica CM1510, Nussloch, Germany) and placed on SuperFrost Plus glass slides (Menzel-GmbH & CoKG, Braun Schweig, Germany). The sections were dried at room temperature for 3 min, fixed in 4% buffered formalin for 45 min at 4°C, rinsed two times for 5 min in PBS, post-fixed with ethanol–acetic acid 2:1 (v/v) at -20°C for 5 min, rinsed twice for 5 min in PBS, boiled in 10 mM citrate buffer, pH 6.0 for 10 min, washed one time in distilled water and two in PBS, and finally used for either HIF-1α immunoperoxidase, HIF-1α immunofluorescence or DNA fragmentation staining.

4.3.5.1. HIF-1α immunoperoxidase staining

Immunoperoxidase staining reveals HIF-1α -linked peroxidase activity as brown spots in the nucleus or in the cytosol. The sections were exposed to 3% H_2O_2 (5 min) to block endogenous peroxidase, treated for 1h with 10% normal goat serum under gentle agitation, incubated overnight at 4°C with a rabbit anti-HIF-1α polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:200 in 1.5% normal goat serum in PBS), washed three times for 5 min in PBS, incubated at room temperature for 45 min with goat anti rabbit

IgG peroxidase conjugated secondary antibody (Sigma, St. Louis, Missouri, diluted 1:800 in 1.5% normal goat serum), rinsed four times with PBS, incubated at room temperature with 1 mg/ml diaminobenzidine (Dako, Carpenteria, CA), counterstained with Gill's hematoxylin, and mounted in 9:1 glycerol/ PBS medium, pH 7.4. A negative control was prepared for each biopsy by substituting the primary antibody with 1.5% normal goat serum. The slides were examined at 10X and 40X magnification in a microscope (Axiolab E, Carl Zeiss, Göttingen, Germany) equipped with a CCD camera (Nikon DS 5M, Tokyo, Japan) and the images were stored in a PC.

4.3.5.2. HIF-1α quantitative immunofluorescence

To give a quantitative estimation of HIF-1α, we employed an immunofluorescence technique by which the sections were incubated with anti-HIF-1α antibody, then with fluorescein-labeled secondary antibody (directed against the primary antibody) which yields a green signal measured by a semi-automatic method. After treatment with 10% normal goat serum for 1 h under gentle agitation, the sections were incubated overnight at 4°C with a rabbit anti-HIF-1α polyclonal antibody (Santa Cruz Biotechnology, diluted 1:200 in 1.5% normal goat serum), washed in PBS, treated at room temperature for 45 min with a goat anti-rabbit IgG fluorescein-conjugated secondary antibody (Santa Cruz Biotechnology, diluted 1:150 in 1.5% normal goat serum), rinsed with PBS four times, and mounted in a 9:1 glycerol/PBS medium, pH 8.5, containing 0.1% p-phenylenediamine as anti quenching agent. A negative control was prepared for each biopsy by substituting the anti-HIF-1α antibody with 1.5% normal goat serum. The slides were examined at 40X magnification in an inverted fluorescence microscope (Axiovert 25 CFL, Carl Zeiss, Göttingen, Germany), equipped with a filter for detection of fluorescein (filter set 09, excitation band-pass 450–490 nm, emission low-pass 515 nm), randomly chosen images were acquired by a CCD camera (Nikon DS 5M, Tokyo, Japan) and stored in a PC. For HIF-1α quantification, we used an algorithm that allows to perform quantitative immunohistochemistry by calculating the cumulative signal strength, or energy, of the digital file representing the image. The algorithm involves subtracting the energy of the digital file encoding the control image (i.e., not exposed to antibody) from that of the experimental image (i.e., antibody-treated). In this manner, the absolute amount of antibody-specific chromogen per pixel could be determined for any cellular region or structure. The images were analyzed by IPlab Software (Scanalytics, Inc., MA) and split into RGB channels. The green channel was used to calculate the color intensity as the sum of the pixel intensity values. Five random fields were selected for each slide, the green color intensity was averaged and subtracted of the signal detected in the negative controls. HIF-1α abundance in the image is expressed as the sum of green pixel intensity*10^5/0.037 mm^2.

4.3.5.3. Apoptosis

The degree of apoptosis was assessed by the TUNEL method, using the *In Situ* Cell Death Detection Kit, Tetramethylrhodamine (TMR) red (Roche Diagnostics GmbH, Germany). The test is based on labeling DNA strand breaks by Terminal deoxynucleotidyl transferase (TdT), which catalyzes polymerization of TMR red-labeled nucleotides to free-3'-OH DNA ends in a template-independent manner. The sections were incubated in a solution containing TMR red-nucleotides and TdT for 1 h at 37°C in the dark, then washed three times in PBS. A negative control was established for each biopsy by substituting TdT with the label solution (TMR red-nucleotides without TdT enzyme). The nuclei were then counterstained with 150 ng/ml bisbenzimides (Hoechst 33258, Sigma Aldrich, St. Louis, MI), for 3 min in the dark at room

temperature. Then the sections were rinsed with PBS four times, and mounted in a 9:1 glycerol/PBS medium, pH 8.5. Randomly chosen images were acquired as described for HIF-1α immunofluorescence, but using 2 different filters: the rhodamine detection filter (filter set 15, excitation band-pass 546 ± 12 nm, emission low-pass 590 nm) or a filter for Hoechst staining (Filter set 02, excitation bandpass 365, emission 420). Then the 2 images acquired with the 2 filters were merged. Two operators counted the number of TdT-labeled and total nuclei by examining at least 5 random fields in a blinded procedure. Results are expressed as number of TdT-labeled nuclei/total nuclei/0.037mm^2.

4.3.5.4. CD68 quantitative immunofluorescence

To give a quantitative estimation of CD68, we employed an immunofluorescence technique by which the sections were incubated with anti-CD68 antibody, then with fluorescein-labeled secondary antibody (directed against the primary antibody) which yields a green signal measured by a semi-automatic method. After treatment with 10% normal goat serum for 1 h under gentle agitation, the sections were incubated overnight at 4°C with a rabbit anti-CD68 polyclonal antibody (Santa Cruz Biotechnology, diluted 1:100 in 1.5% normal goat serum), washed in PBS, treated at room temperature for 45 min with a goat anti-rabbit IgG fluorescein-conjugated secondary antibody (Santa Cruz Biotechnology, diluted 1:150 in 1.5% normal goat serum), rinsed with PBS four times, and mounted in a 9:1 glycerol/PBS medium, pH 8.5, containing 0.1% p-phenylenediamine as anti quenching agent. A negative control was prepared for each biopsy by substituting the anti-CD68 antibody with 1.5% normal goat serum. Randomly chosen images were acquired as described for HIF-1α immunofluorescence.

4.3.5.5. Estimation of the tumor and infiltrate sizes

In order to get a semi-quantitative estimation of the size of the tumor and infiltrate, the digital image of the tumor obtained by immunoperoxidase staining was used to delimit by hand the border of the infiltrate with IPlab software (Scanalytics, Inc., MA) on a high-resolution monitor. Then the area within the border limit (expressed as Pixel) was measured by IPlab software and used to calculate the tumor/inflammatory area. This operation was performed in 5 different fields from 3 tumors belonging to N and CH groups. Then the ratios were averaged.

4.3.6 MLT quantification by ELISA test

To measure MLT plasma levels, blood samples were withdrawn by intracardiac puncture. Serum samples were immediately separated by centrifugation and stored at -20°C until assayed. MLT plasma and tumoral levels were determined by a competitive enzyme immunoassay kit (Immuno Biological Laboratories, Hamburg, Germany) according to manufacturer's instruction. To evaluate tumoral levels of MLT, the tumors were weighed (5-20 mg), homogenized in an appropriate volume of PBS using a Politron ultra-turrax for 1 min and then centrifuged at 12000 rpm for 5 min. Tumor homogenate supernatants were used for MLT determination. This procedure was adjusted from one used for tissue sample by Sanchez-Hidalgo et al. (Sanchez-Hidalgo et al., 2009). MLT from 500 µL of the samples, standards and control was extracted using C18 reversed phase columns (IBL-Hamburg, Germany) and methanol elution. The dried extracts (after evaporating methanol) were stored at -20°C for up to 48 hours. MLT levels were measured in duplicate using 96 well microtiter plate coated with captured antibody goat anti-rabbit Ig. Each microtiter plate was filled either with 50 µL blank reagent, extracted calibrators, extracted samples or extracted standard solution

(containing 0, 3, 7, 33, 110 or 250 pg/ml of MLT). Then, 50 µL of MLT biotin and 50 µL of rabbit-antiserum were added into each well, shaken carefully, sealed with adhesive foil and incubated overnight (14-20 hours) at 2-8°C. After washing three times with 250 µL diluted washing buffer, 150 µL of ant-biotin conjugate to alkaline phosphatase was added into each well and incubated 2 hours at room temperature. The reaction was developed using p-nitrophenyl phosphate and optical densities were determined at 405 nm in an automatic microplate reader. The sensitivity of the MLT assay was 1.6 pg/ml. Both intra- and inter-assay coefficients of variation (Gregory et al.) were less than 10%.

4.4. STATISTICS

Data are expressed as mean±SEM. Significance level was P=0.05 (two-tailed). To detect differences among two group we performed the Student unpaired t test. To detect differences among three groups, we performed one-way ANOVA. If this test resulted significant, the differences between selected pairs of data were tested using the Bonferroni procedure (InStat, Windows version 3.01, GraphPad Software, San Diego, California, USA). To assess the tumor growth rate, we fitted by the least square method the volume of each tumor on the equation $y=y_0*e^{kt}$, e.g., a first-order exponential growth where t represents time and k the rate constant, then averaged the k values for each group.

5.0. MATERIALS AND METHODS - MLT IN ICU PATIENTS AS SLEEP-WAKE REGULATOR

5.1. STUDY POPULATION

The present study (randomized, perspective, pharmacokinetics, monocentric, single blind) named "Pharmacokinetics of exogenous MLT in high-risk patients", was conducted in the Intensive Care Unit (ICU) of H. S. Paolo (Milan, Italy), in the period between January and October 2011. For this study, 21 high-risk patients have been enrolled, subdivided into three different groups, based on the different administration and pharmacological formulation of MLT. The criteria adopted for the enrolment to the treatment was the minimization in order to reduce the differences between the major prognostic factors, maintaining the groups homogeneous; this allows to attribute to each treatment the eventual differences observed in the outcome. For each group we studied seven subjects: this abundance allows enough data collection for a pharmacokinetic study.

5.1.1 Inclusion and exclusion criteria

The inclusion criteria were the following: patients over 18 years old, high-risk patients (SAPS II > 32 and expected mechanical ventilation > 48 hours).

Exclusion criteria were: underage, hepatic failure (Child-Pugh class C), dialytic treatment, gut impracticability, pregnancy and lactation, home mechanical ventilation, neuropsychiatric disorders, DNR orders (Heyland-2003). As soon as their clinical and neurological conditions improved, patients were duly informed of the study and their consent was obtained.

5.2. MLT DOSAGE AND FORMULATION

The drug formulations used in the study were:1st GROUP : 1 cp Tranquillus (Ingredients per tablet: MLT (3 mg), additives, microcrystalline cellulose, calcium phosphate, inulin, talc, magnesium stearate) 400 mg tablets manufactured in Florence-via D. Veneziano, 13 for Functional Point Srl-via Pietro Paleocapa 19 24122 Bergamo-ITALY)2nd GROUP: MLT incorporated into SLN has been provided by Nanovector Srl, Turin, Italy (Gasco-2007) as lyophilized powder in vials containing 3 mg SLN-MLT and 41 mg excipients (stearic acid, Epikuron 200, sodium glycocholate, UP water and trehalose. A hot microemulsion was prepared with stearic acid as lipid matrix, phospholipids and taurocholate. The average diameters of the SLN-MLT vary depending on the amount of melatonin incorporated. The SLN-MLT had e an average diameter of 120 nm.
3rd GROUP: MLT incorporated into SLN , 3 mg SLN-MLT and 41 mg excipients were dissolved in BD water (0.5 mL) and the solution was applied on an area of 9 cm^2, using a patch.

After a period of clinical stabilization after ICU admission (2 days), the pharmacokinetics study began on ICU day 3, time at which MLT was administered to the patients.

1st GROUP (**MLT os**) received standard MLT by nasogastric tube: 1 Tranquillus tablet (400 mg) containing 3 mg of MLT.

2nd GROUP (**SLN-MLT os**) received SLN-MLT by nasogastric tube: 3 mg SLN-MLT.

3rd GROUP (**SLN-MLT td**) received SLN-MLT transdermally: 3 mg SLN-MLT, duration of application 12 h.

5.3. TIMING OF BLOOD SAMPLES

1st day ICU stay: recruitment and stabilization; collection informed consent.

2nd day ICU stay: blood sampling for basal endogenous MLT levels at 20:00-24.00-03.00-06.00-14.00 h.

3rd day of ICU stay: last blood sampling for basal endogenous MLT levels at h 20:00.

Immediately after this blood sampling, administration of MLT (MLT os/ SLN-MLT os/ SLN-MLT td). Blood sampling for exogenous MLT levels at 20:05-20:10-20:20-20:30-20:45-21:30-22:15-23:00-24:00-03:00-06:00-07:00-14:00-20:00 h.

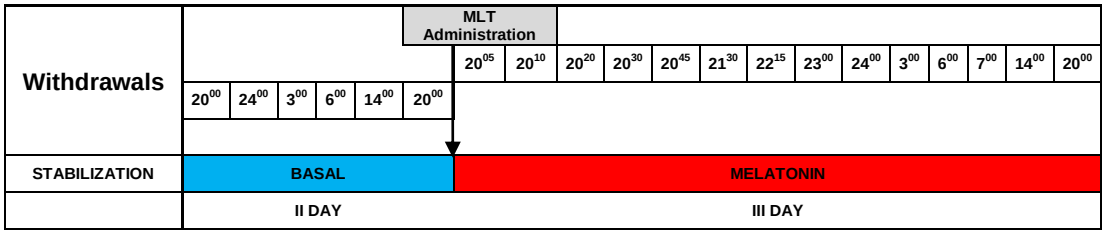

Fig. 21 *– Withdrawals timing.*

Extreme care has been made to maintain darkness at night when the blood samples were taken via central venous catheters. Patients' sleepiness was monitored by nurses, assuming that observing a calm patient with closed eyes meant that the patient was sleeping. All patients received standard intensive treatment including ventilator, cardiovascular, nutritional support (continuous enteral nutrition or a combination of enteral and parenteral nutrition) and sedatives based on their clinical needs.

5.4. MEASUREMENTS

To measure MLT plasma levels, blood samples were collected, as previously described, from central venous catheters, placed in the internal jugular vein before the beginning of the study. Samples were collected in plastic tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Serum samples were immediately separated by centrifugation at 2000 rpm room temperature, separated from RBC and stored at -20°C until assayed. MLT plasma levels were determined by a competitive enzyme immunoassay kit (Immuno Biological Laboratories, Hamburg, Germany) according to manufacturer's instruction (see cap 4.4.6).

5.5. STATISTICS

The basic characteristics and patient outcomes were analyzed using one-way ANOVA for continuous variables equally distributed, Kruskal Wallis test for continuous variables not equally distributed; for categorical variables Chi square test was used instead. The pharmacokinetic analysis was obtained by means of an integral of the trapezoidal melatoninemia. The analysis of the relationship between the pharmacokinetic variables and clinical characteristics of patients was conducted by non-linear quantile regression and Spearman correlation. All analyzes were performed using the statistical program "Stata 12" (Stata Corporation, College Station TX, USA).

6.0. Results – MLT as antitumoral molecule

6.1. MLT and SLN-MLT intraperitoneal (i.p.)

6.1.1. Effect of intraperitoneal MLT and SLN-MLT on animal homeostasis

This set of experiments was aimed at assessing whether MLT administered by this quite traditional route results into an oncostatic situation, and whether administration of the same amount of SLN-MLT is oncostatic as well. The athymic mice were implanted with LNCaP cells and not-treated (n=6) or treated with MLT 1 mg/kg (n=13) and SLN-MLT 1 mg/kg (n=13). The xenograft rate of success was over 65% and the tumors could be measured after 15 days. The (Fig. 22) evidences that animals of two MLT treated groups grew better than the control animals all along the treatment period. As far as the final body weight, the mice treated with MLT and SLN-MLT showed a significantly higher ($p<0,001$ vs control for both groups) body weight resulting in a better animal compliance. The treatment with MLT in solution and in SLN has not been shown to have different effects, compared to the group control (not-treated), as regard as the changes in blood Hb concentration (data not shown). The treatment with MLT and SLN-MLT at concentrations previously reported showed no obvious acute toxic effects and mortality was found to be 0% in all three references groups.

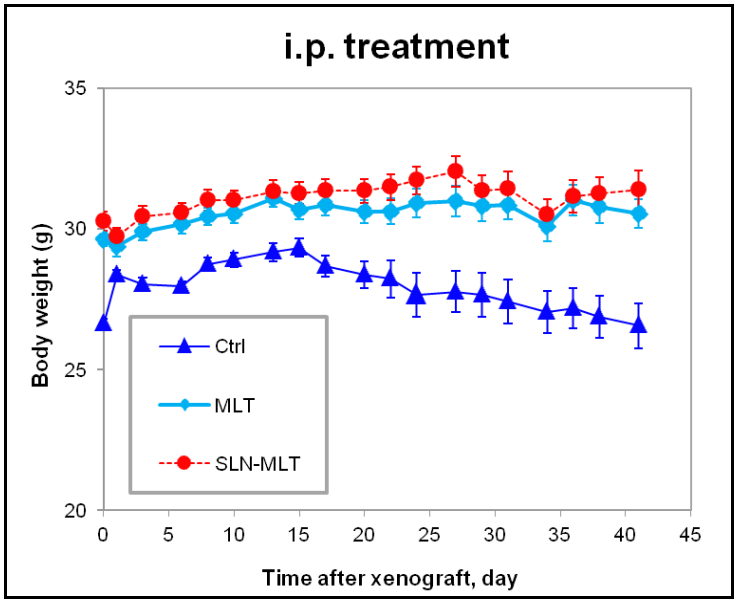

Fig. 22 – *Body weight trend for 3 groups (Ctrl, SLN-MLT and MLT) during all the treatment.*

6.1.2. Effect of intraperitoneal MLT and SLN-MLT on tumor growth

The effect on tumor growth was evaluated in the three treatment groups with measurements of tumor volume over time as described in materials and methods. In the control group (not treated) athymic mice implanted with human LNCaP cells developed 12 of 12 tumors (100%). In the group treated with ip administration of MLT have been developed 20 of 26 tumors (77%) and in the group treated with SLN-MLT have been developed 17 over 26 tumors (65 %).

The statistical analysis shows that starting from day 34 treatment (Fig. 23) with MLT significantly inhibited tumor growth than in control ($p<0,01$ for SLN-MLT and $p<0,001$ for MLT). The day 41 (last

measured point) showed a highly significant difference ($p<0,05$) for the two groups treated with MLT vs control group (Fig. 24).

Data confirmed that ip treatment with MLT and SLN-MLT has important oncostatic potential. Of interest, MLT does not delayed appreciably the time of appearance of the tumors after the xenograft, but rather it decreased the growth rate. SLN-MLT seemed to have more or less the same oncostatic potential of MLT in solution.

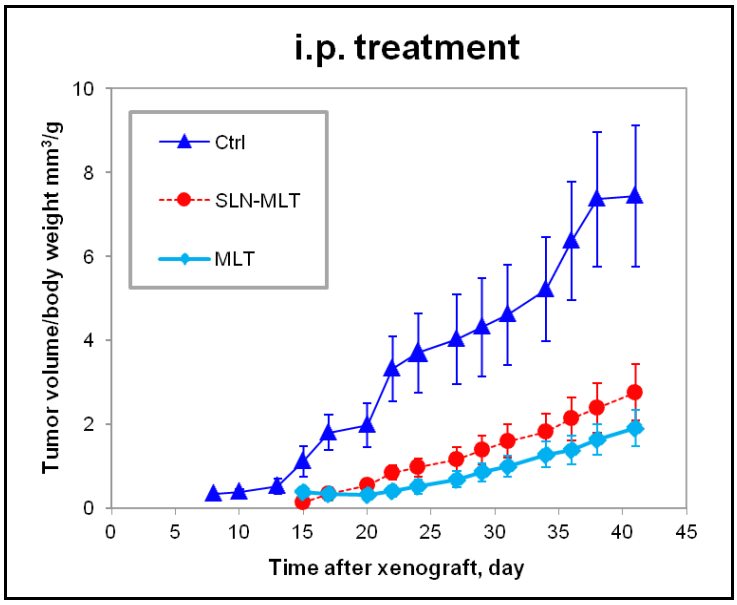

Fig. 23 *– Kinetic of tumor volume/body weight for 3 groups (Ctrl, SLN-MLT and MLT) during all the treatment.*

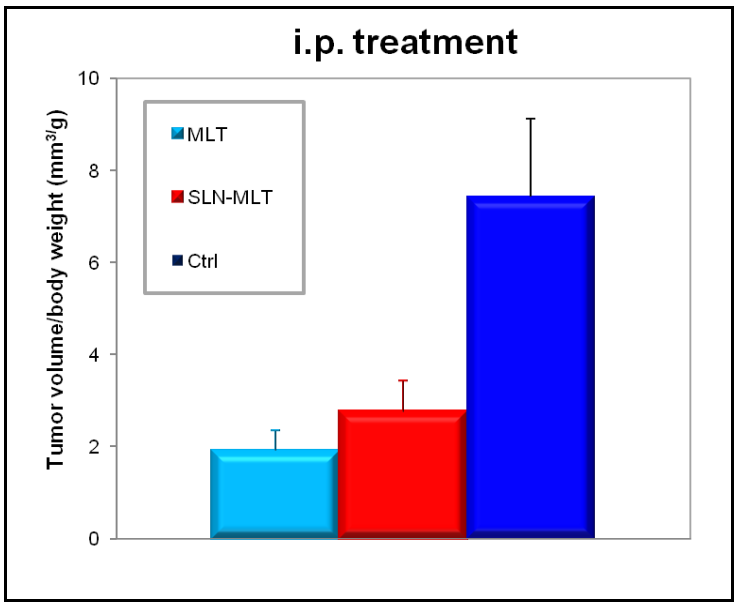

Fig. 24 *– Final (at day 42nd day)tumor volume/body weight for 3 groups (Ctrl, SLN-MLT and MLT).*

6.1.3. Effect of intraperitoneal MLT and SLN-MLT on MLT levels in plasma and tumors at sacrifice

We have evaluated the MLT levels in plasma and tumors at sacrifice by ELISA assay as described under material and methods. This method resulted immediately suitable for MLT determination in mouse plasma following the producer's instructions, however we needed to conduct different experiments testing

different dilutions of the plasma samples, before extracting them using the C18 columns. The diverse dilutions were aimed at dosing samples with a MLT concentration within the linearity range of the ELISA kit, and were chosen on the base of the putative concentration in relation to the treatment received.

The determination of MLT at tumor tissue levels was particularly challenging, especially concerning the MLT extraction step, since few papers reports experiences on this issue. We have conducted different experiments using different methodologies to extract MLT from the tumor samples. We have then adjusted the methodology reported by Sanchez-Hidalgo et al. (Sanchez-Hidalgo-2009); the frozen tumors were cut on dry ice, weighed (5-30 mg), and immediately homogenized in cold PBS (500 µl) with Politron ultra-turrax for 1 min keeping the tube in ice during the operation. and then centrifuged at 12000 g for 5 min in a mini Spin centrifuge. The homogenate supernatants were then used for MLT determination.

Results showed that MLT levels in plasma at the time of the sacrifice were significantly higher ($p<0,05$) both in MLT and SLN-MLT groups, *vs* controls, as expected. No significant differences were found between the two methods of MLT delivery (Fig. 25). A similar trend was observed for the MLT levels in tumors of treated animals ($p<0,05$ vs control) (Fig. 25). Although the MLT concentration in plasma and tumors of SLN-treated animals resulted always slightly lower than in the group treated with MLT dissolved in saline, this difference never resulted significant (Fig. 25).

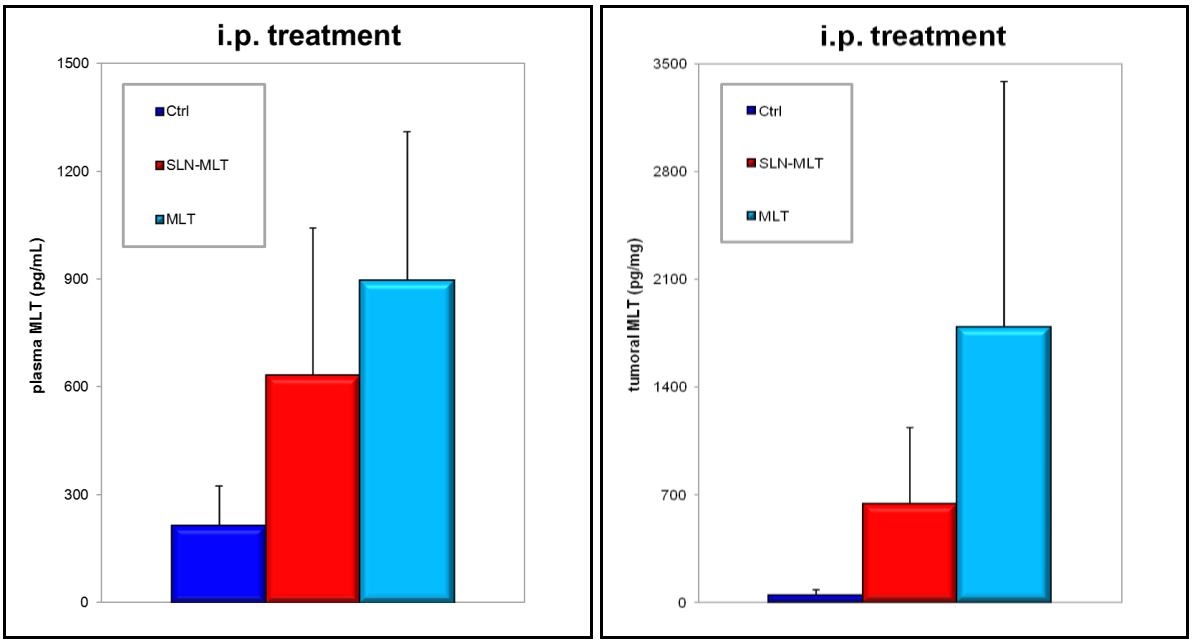

***Fig. 25** –Plasma MLT levels (at day 42nd day) for Ctrl (n=5),SLN-MLT (n=12) and MLT (n=13) and tumoral MLT levels (at day 42nd day) Ctrl (n=12),SLN-MLT (n=5) and MLT (n=7).*

6.1.4. Effect of intraperitoneal MLT and SLN-MLT on HIF-1α expression

In Fig. XX are reported the values of HIF-1α expression in the tumor tissue evaluated by immunofluorescence as described previously. In both treated groups HIF-1α expression appeared increased in respect to controls, although not reaching the significance. Saline SLN, as previously seen for other parameters, seem to shift more efficiently HIF-1α expression (Fig. 26).

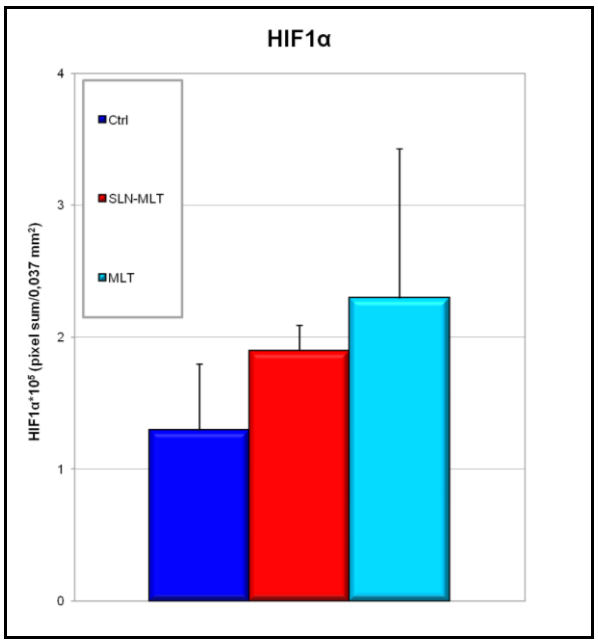

Fig. 26 *– HIF-1α expression by immunofluorescence for 3 groups (Ctrl, SLN-MLT and MLT).*

6.1.5. Effect of intraperitoneal MLT and SLN-MLT on tumor mass morphology

For the morphological analysis, the tumor samples, which had different composition and density, were treated in such a way to make a homogenous mass. The samples were fixed in formalin, dehydrated trough the passage in alcohol and clarified in xylene. After being immersed in paraffin, they were cut into sections. These sections were then collected on microscope slides. To staining hematoxylin-eosin was used, which allows to display effectively the morphological structure.

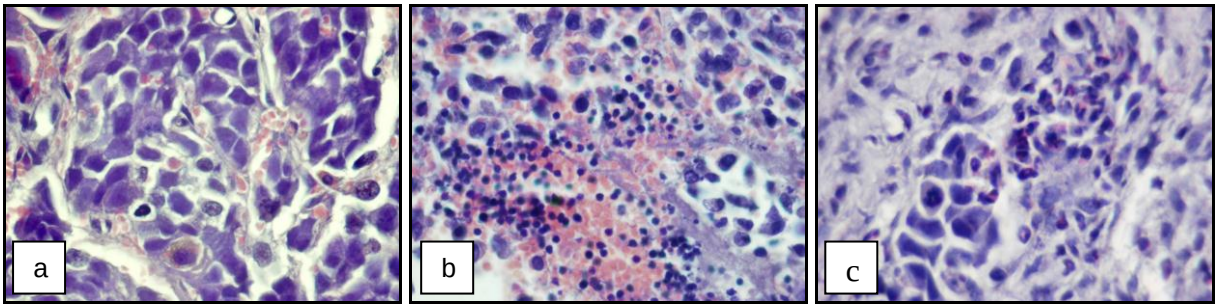

Fig. 27 *– Morphological imagines for 3 groups: Ctrl (a), SLN-MLT (b) and MLT (c).*

In all groups, the tumor mass has a lobular organization, with tumor cells interspaced by parenchymal cells and characterized by a high nucleus/cytoplasm ratio as well as by nuclear alterations and areas of necrosis.

Untreated tumors exhibit moderate neo-vascularization and lack of significant inflammatory response (Fig. 27a). MLT ip tumors were characterized by nests of tumor cells circumscribed by an important inflammatory reaction and for the massive presence of lymphocytes and some granulocytes surrounding and infiltrating tumor cells (Fig. 27b). Tumors of the SLN-MLT group show characteristics similar to those of the MLT group with regard to angiogenesis and the inflammatory response. In this case, the tumor appears circumscribed by a high lymphocytes response and by the presence of fibroblast-like cells (Fig. 27c).

Immunofluorescence analysis carried on the same tumors essentially confirmed the differences seen above with control tumor tissue characterized by homogeneous and compact structure without disaggregated areas, whereas after MLT i.p. treatment we evidenced small to medium regions of cells highly disorganized in all the three dimensions of the space. SLN-MLT treatment seems to impair more strongly the tissue architecture, showing some areas still organized with some incipient fragmentation and a strong leukocyte infiltration, and some other tumor areas with a much more disorganized structure with overlapping tumor cellular layers highly fragmented (Fig. 28).

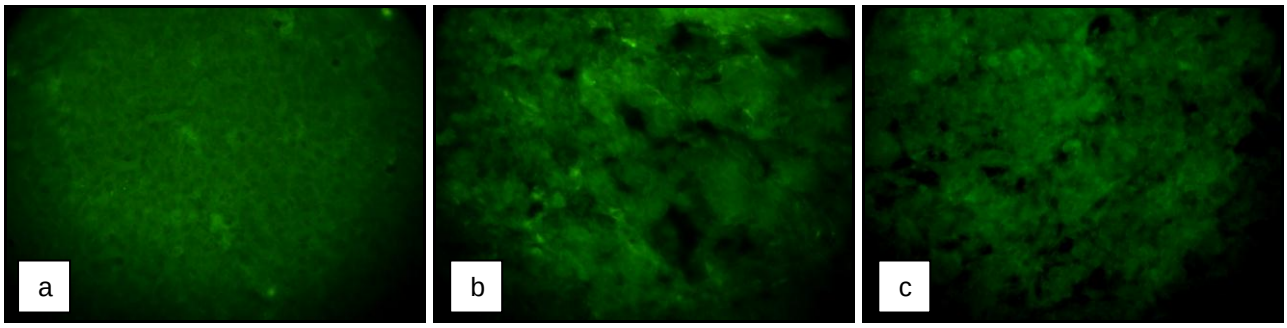

Fig. 28 *-Immunofluorescence imagines for 3 groups: Ctrl (a), SLN-MLT (b) and MLT (c).*

6.1.6. Effect of intraperitoneal MLT and SLN-MLT on CD4 and CD8 expression

The CD4 and CD8 expression was evaluated by quantitative analysis immunohistochemistry.

The number of CD4 were not significantly different in the two treated groups *vs* control (not treated) (Fig. 29). The number of cells positive for the marker of CD8 showed a trend to increase in the MLT and in the SLN-MLT groups, *vs* control, although not significantly (Fig. 29). The $CD4^+/CD8^+$ ratio resulted significantly lower in both treated groups, in respect to controls (Fig. 29).

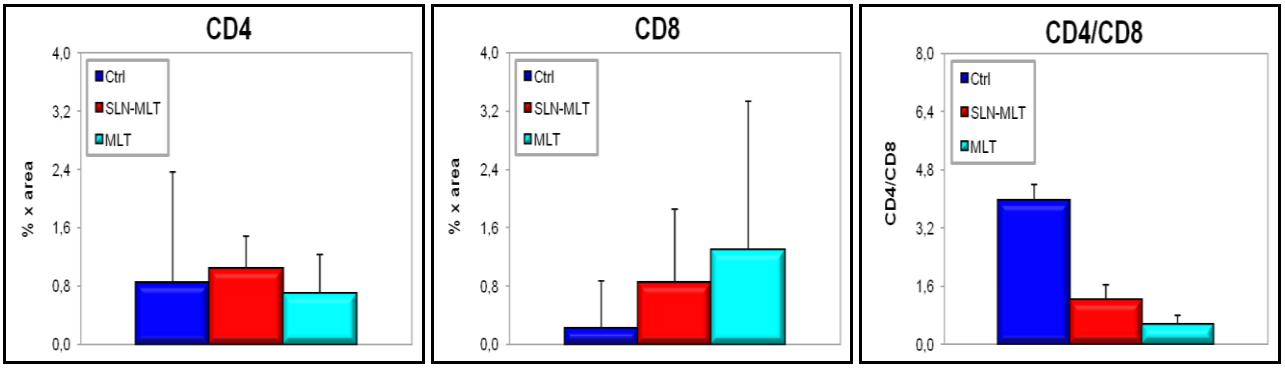

Fig. 29 *– CD4, CD8, CD4/CD8 expression for 3 groups (Ctrl, SLN-MLT and MLT).*

6.2. MLT transdermal by cryolaser

6.2.1. Effect of transdermal MLT by cryolaser on animal homeostasis

This set of experiments was aimed at assessing whether a novel system of drugs administration (Cryopass therapy) is efficient for MLT and comparing this system with traditional administration route (i.p.). The athymic mice were implanted with LNCaP cells and not-treated (n=6) or treated with cryoRx (laser) (n=10) or with cryoRx and MLT (MLT- laser): 4mg/kg (n=14). The xenograft rate of success was over 65% and the tumors could be measured after 15 days. Both the treatment with MLT-laser and with laser alone

seemed to be well tolerated by the animals, with a good compliance and a body weight higher in respect to not treated animals all along the treatment period. The final body weight of treated groups was significantly higher ($p<0,001$ vs control for both groups).

The treatment with MLT-laser and with laser alone has not been shown to have different effects, compared to the group control (not-treated), as regard as the changes in Hb concentration. The treatment with laser alone at concentrations previously reported showed no obvious acute toxic effects, and mortality was found to be 0% in all three references groups.

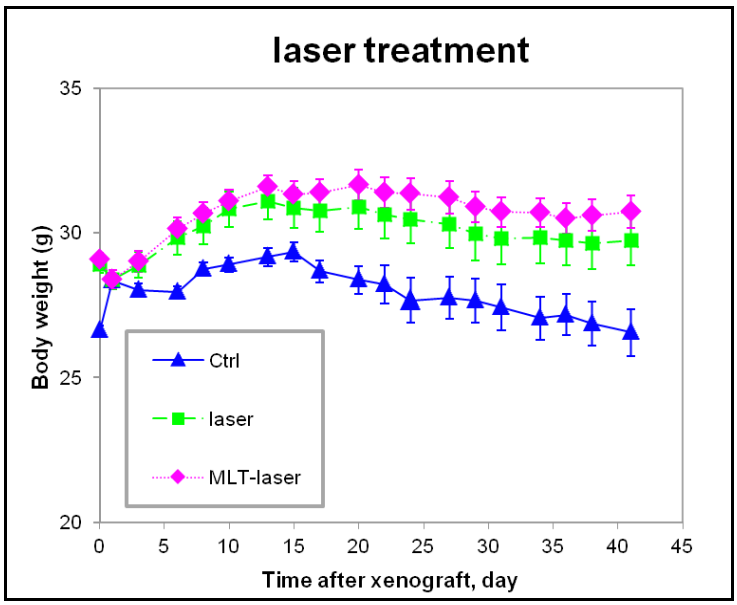

Fig. 30 *- Body weight trend for 3 groups (Ctrl, laser and MLT-laser) during all the treatment.*

6.2.2. Effect of transdermal MLT by cryolaser on tumor growth

The effect on tumor growth was evaluated in the three treatment groups with measurements of tumor volume over time. In the control group (not treated) athymic mice implanted with human LNCaP cells developed 12 of 12 tumors (100%). In the group treated with laser alone have been developed 15 of 20 tumors (75%) and in the group treated with topical MLT have been developed 21 over 28 tumors (75%).

The statistical analysis shows that starting from day 34 treatment, transdermal MLT-laser significantly inhibit tumor growth than in controls ($p<0,05$) (Fig. 31). The day 41 (last measured point) shows a highly significant difference ($p<0,01$) for the group treated with MLT-laser vs control group (Fig. 31.

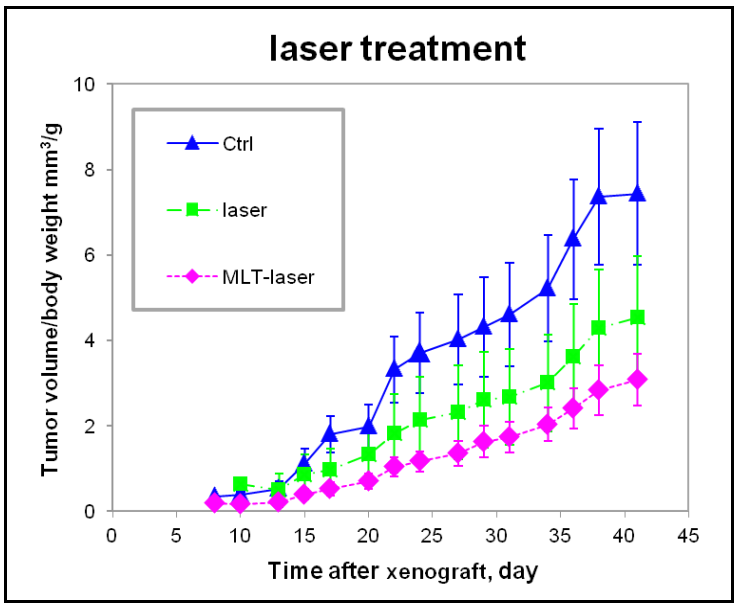

Fig. 31 *– Kinetic of tumor volume/body weight for 3 groups (Ctrl, laser and MLT-laser)during all the treatment.*

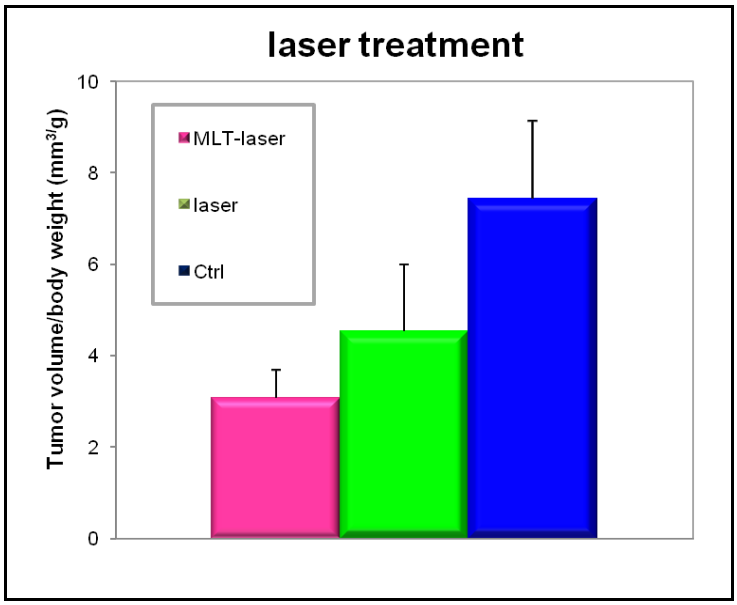

Fig. 32 *- Final (at day 42nd day)tumor volume/body weight for 3 groups (Ctrl, laser and MLT-laser).*

6.2.3. Effect of transdermal MLT by cryolaser on MLT levels in plasma and tumors at sacrifice

We have evaluated the MLT levels in plasma and tumors at sacrifice by ELISA assay as described under material and methods and following the same strategy followed for the i.p. treatment.

Results showed that MLT levels in plasma at the time of the sacrifice were significantly higher ($p<0,05$ in MLT-laser group *vs* controls, as expected. A similar trend was observed for the MLT levels in tumors of MLT-laser treated animals ($p<0,05$ *vs* control) (Fig. 33). The group treated with laser alone did not show any difference in both tumor and plasma MLT levels respect to controls.

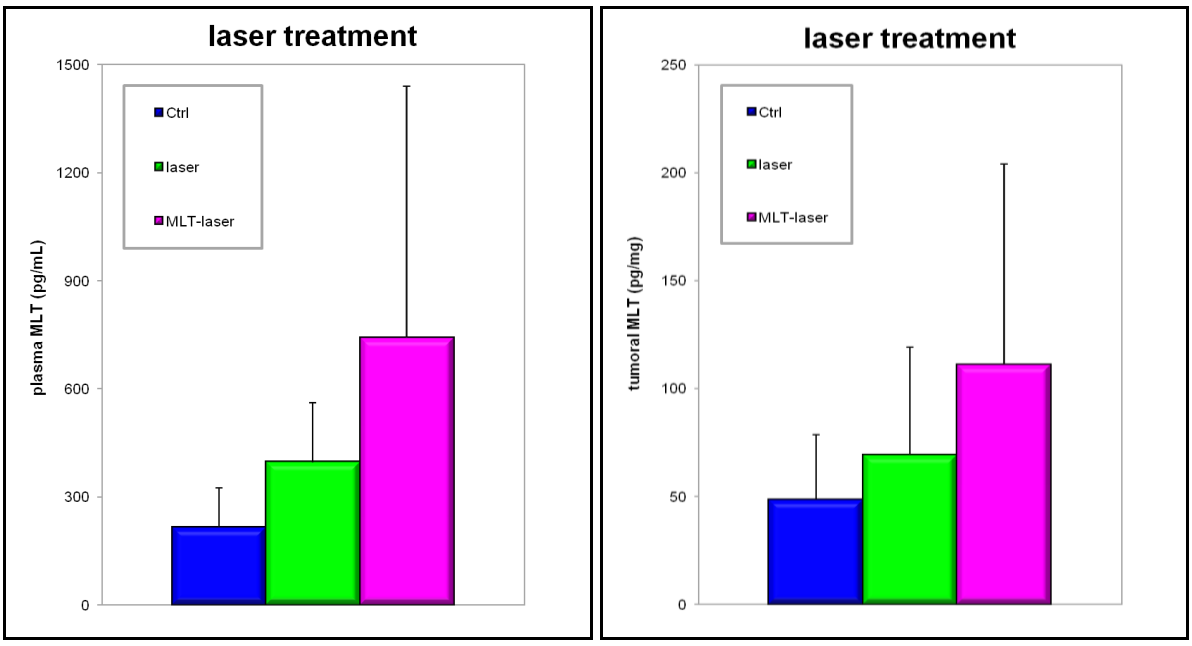

Fig. 33 *- Plasma MLT levels (at day 42ndday) for Ctrl (n=5),laser (n=9) and MLT-laser (n=13) and tumoral MLT levels (at day 42ndday) Ctrl (n=12), laser (n=15) and MLT-laser (n=21).*

6.2.4. Effect of transdemal MLT by cryolaser on HIF-1α expression

HIF-1α expression was measured as described above using immunofluorescence. The results are similar to those observed for the MLT ip experiments, in fact the group treated with MLT-laser showed an increased HIF-1α expression ($p<0,05$) in respect to controls. This result has been confirmed with Western blot and still deserves further investigation on the mechanisms underlying MLT action. The group laser alone showed more or less the same HIF-1α expression of the control using both methods.

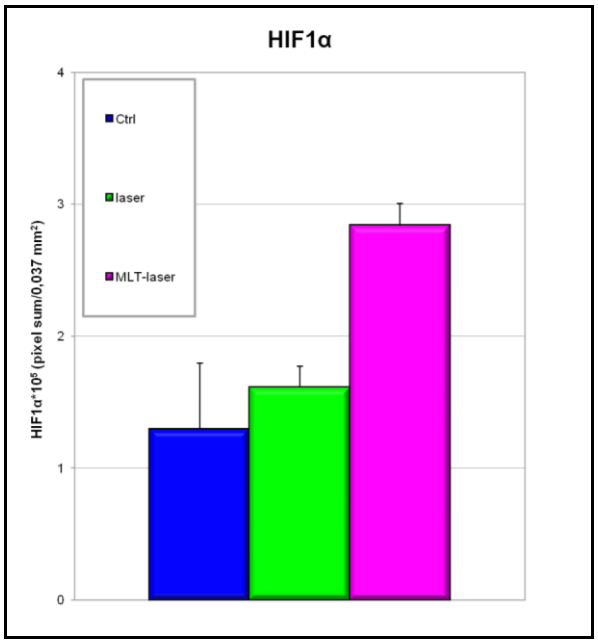

Fig. 34 *- HIF-1α expression by immunofluorescence for 3 groups (Ctrl, laser and MLT-laser).*

6.2.5. Effect of transdermal MLT by cryolaser on tumor mass morphology

For the morphological analysis, the tumors samples were treated as described above In all groups the tumor has lobular organization, with tumor cells that appear interspaced by some parenchymal cells and characterized by a high ratio nucleus / cytoplasm as well as by nuclear well evident alterations. It can also be observed areas of necrosis within the tissue. The tumors in the control group exhibited a moderate neovascularization and the absence of a significant inflammatory response.

The tumors in the group treated with MLT-laser were characterized by nests of tumor cells circumscribed by a major inflammatory reaction, mainly characterized by a chronic response represented by the massive presence of lymphocytes and some granulocytes, which seem to surround and infiltrate the tumor cells. It 's also possible to note an elevated development of blood vessels, which grow indefinitely forming cavity rich in erythrocytes. The tumor appears limited not only by high lymphocyte response, but also by the presence of fibroblast-like cells. (Fig. 35).

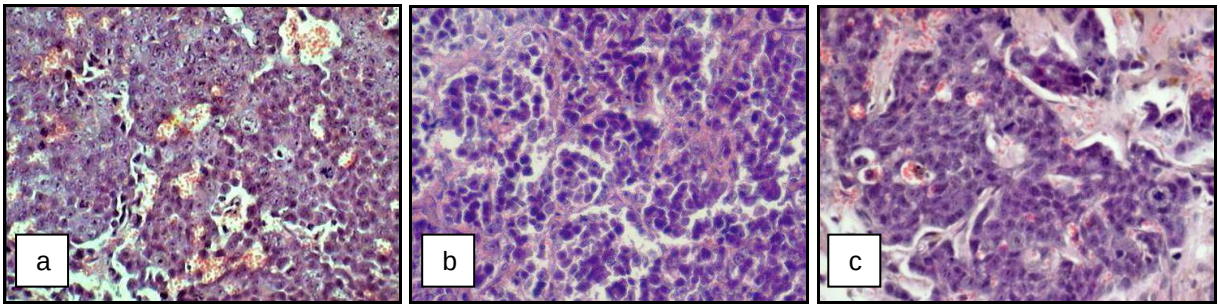

Fig. 35 *- Morphological imagines for 3 groups: Ctrl (a), laser (b) and MLT-laser (c).*

Immunofluorescence analysis carried on the same tumors essentially confirmed the differences seen above with a control tumor tissue with a homogeneous and compact structure not showing disaggregated areas. The treatment with laser alone still conserve a structure with compact cellular blocks but reveals the tendency to form a network of small cords disorganized. When MLT was used as an adjuvant to laser (MLT-laser group), the tumor tissue tends to be much more disaggregated, showing a mixed structure with compact cords and areas most highly disorganized. The luminescence showed in Fig. 36 is due to white blood cell infiltrates

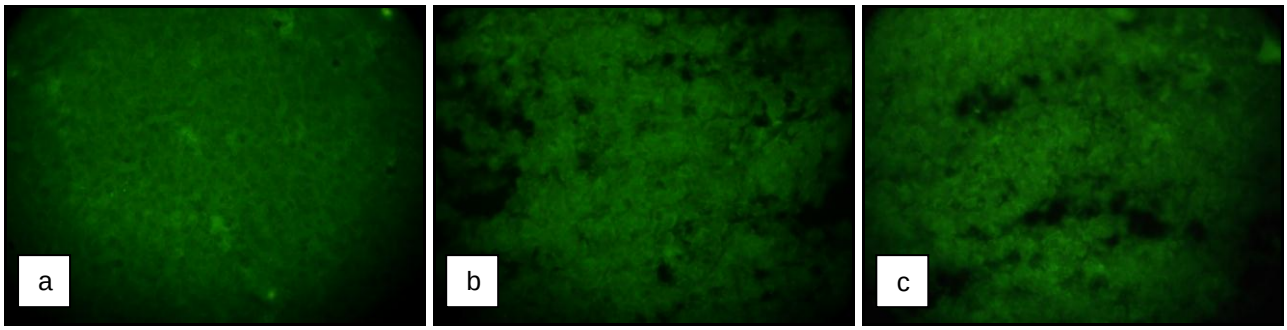

Fig. 36 *- Immunofluorescence imagines for 3 groups: Ctrl (a), laser (b) and MLT-laser (c).*

6.2.6. Effect of transdermal MLT and SLN-MLT on CD4 and CD8 expression

The CD4 and CD8 expression was evaluated by quantitative analysis immunohistochemistry. The number cells positive for $CD4^+$ and for $CD8^+$ are significantly higher ($p<0,05$) in the MLT-laser group *vs* control (Fig. 37). Not significant difference vs control was observed both for CD4 and CD8 expression for the group treated with laser. The $CD4^+/CD8^+$ ratio resulted therefore not significantly different in the MLT-treated group respect to control.

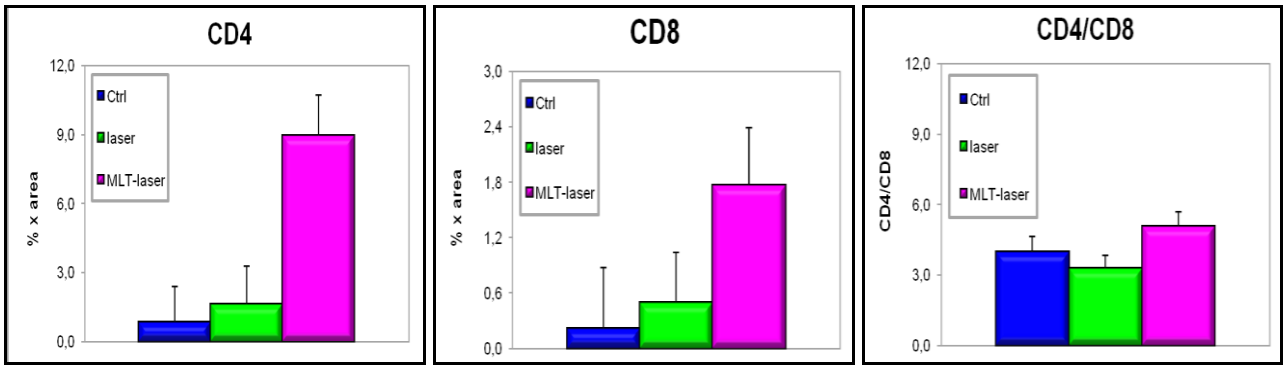

Fig. 37 *- CD4, CD8, CD4/CD8 expression for 3 groups (Ctrl, laser and MLT-laser).*

7.0. Results - MLT in ICU patients as sleep-wake regulator

The study involved 21 patients admitted to the Intensive Care Unit of the Hospital San Paolo, Milan. Of these patients, 11 were hospitalized as a result of medical conditions and 10 after emergency surgery or complications within the 7th postoperative day, the diagnosis of entry is pneumonia for 5 patients, 3 for anastomosis dehiscence colic, 2 for fasciitis, 2 for aortic aneurysm rupture, 2 for hemorrhagic shock, as well as individual cases of cardiogenic shock, mediastinitis, infection, vascular flap, cholecystitis, diabetes insipidus, pulmonary thromboembolism, bowel obstruction. The clinical characteristics of the patient enrolled in the study are reported in Table 2.

Tab. 2 Characteristics of patients

	MLT os (n=7)	**SLN-MLT os (n=7)**	**SLN-MLT td (n=7)**	**p value**
Age (years)	69±13	71±12	73±5	0,78
Men n (%)	6 (86)	5 (71)	5 (71)	0,77
Weight (kg)	76±10	81±15	71±14	0,44
Height (Patel et al.)	169±6	170±7	167±9	0,72
Entry SAPS II (points)	59±16	52±10	53±14	0,57
Entry SOFA score (points)	9±3	6±1	8±4	0,28
Medical admission n (%)	5 (71)	2 (29)	4 (57)	0,26

Table 3-5 show the patients clinical values collected during the day in which was performed the blood sampling for detection of baseline MLT levels (day 1), the day of MLT administration (day 2), and the last day of the study in which blood samples were obtained to analyze the pharmacokinetics of exogenous MLT (day 3).

Tab. 3 Blood test analysis of patients					
	day	**MLT os (n=7)**	**SLN-MLT os (n=7)**	**SLN-MLT td (n=7)**	**p value**
WBC/mm³	1	10684±5137	9729±2963	18680±13561	0.127
	2	10140±3254	10414±3002	16595±10108	0.153
	3	9300±2754	9403±3320	13859±7752	0.214
Platelets/mm³	1	120429±62801	172143±68307	207000±145777	0.288
	2	87500±26987	154857±67800	142500±69229	0.129
	3	88333±30540	138857±80549	179429±93643	0.124
Creatinine (mg/dL)	1	2.1±1.4	0.9±0.3	1.4±0.9	0.145
	2	1.9±1.3	0.7±0.2	1.3±0.9	0.078
	3	1.9±1.5	0.7±0.2	1.1±0.9	0.097
Urea (mg/dL)	1	93±75	41±24	64±36	0.255
	2	93±65	41±17	69±27	0.101
	3	97±67	42±20	51±35	0.094
AST (units/L)	1	90±125	33±27	35±14	0.454
ALT (units/L)	1	30±14	63±98	28±12	0.534
Total bilirubin (mg/dL)	1	1±0.4	2.6±3.8	1.3±1.4	0.490
Procalcitonin (ng/mL)	1	50.65±45.74	6.5±11.78	11.65±10.19	0.073
	3	5.4±3.11	3.5±3.99	4.28±5.56	0.913

Tab. 4 -Severity indicators of patients					
	day	**MLT os (n=7)**	**SLN-MLT os (n=7)**	**SLN-MLT td (n=7)**	**p value**
SOFA score (points)	1 2 3	6±3 7±3 6±3	5±2 5±2 3±2	6±3 6±3 5±3	0.892 0.593 0.195
Shock (numbers)	1 2 3	3 3 2	2 2 0	3 4 3	0.817 0.558 0.159
SIRS/severe sepsis/septic shock (numbers)	1 2 3	2/3/2 2/4/1 2/4/1	3/2/2 3/2/2 3/4/0	2/2/3 2/1/4 2/2/3	0.860 0.414 0.366
Lactates (mmol/L)	1 2 3	1.6±0.6 1.4±0.5 1.1±0.3	1.5±0.5 1.2±0.3 1.4±0.5	1.5±0.9 1.2±0.5 1.1±0.5	0.987 0.763 0.384
Perfusion state: bad/poor/good	1 2 3	1/2/4 0/3/4 0/2/5	1/3/3 3/2/2 2/0/5	2/2/3 1/3/3 1/3/3	0.919 0.353 0.247
Systemic vasoconstrictors Yes/No	1 2 3	1/6 1/6 1/6	3/4 2/5 0/7	3/4 4/3 4/3	0.424 0.223 0.033
O_2/C-PAP/PSV	1 2 3	0/0/7 0/0/7 1/0/6	0/0/7 1/1/5 2/1/4	1/1/5 1/1/5 1/1/5	0.350 0.504 0.754
Gastric retention Yes/No	1 2 3	0/7 1/6 0/7	2/5 1/6 1/6	1/6 2/5 1/6	0.311 0.734 0.575
Prokinetic Yes/No	1 2 3	2/5 2/5 2/5	4/3 4/3 2/5	2/5 2/5 3/4	0.446 0.446 0.807

Tab. 5 Clinical parameters of patients					
	day	**MLT os (n=7)**	**SLN-MLT os (n=7)**	**SLN-MLT td (n=7)**	**p value**
Hourly diuresis (mL/h)	1	122±63	96±49	67±35	0.150
	2	94±46	84±68	78±28	0.840
	3	91±62	130±101	95±39	0.543
Axillary temperature (˚C)	1	37.4±0.8	37.7±0.9	37.5±1	0.807
	2	38±0.6	37.4±0.9	37.7±0.8	0.340
	3	37.9±0.4	37.5±0.6	37.4±0.6	0.225
Heart rate (bpm)	1	95±20	92±9	101±41	0.823
	2	92±21	95±16	92±14	0.935
	3	94±18	96±12	90±20	0.774
systolic BP (mmHg)	1	131±20	122±17	119±19	0.482
	2	125±14	123±11	112±18	0.221
	3	122±15	112±11	118±10	0.320
diastolic BP (mmHg)	1	57±10	59±12	53±8	0.535
	2	62±15	66±14	53±9	0.193
	3	55±10	56±12	63±13	0.407
Respiratory rate (breaths/min)	1	21±3	17±7	16±5	0.170
	2	23±6	21±4	19±6	0.366
	3	23±7	22±4	23±5	0.869
spO_2 (%)	1	98±2	99±1	98±2	0.732
	2	98±1	99±1	99±2	0.384
	3	99±1	99±1	98±1	0.729
pH	1	7.44±0.04	7.43±0.03	7.42±0.06	0.479
	2	7.46±0.03	7.43±0.03	7.43±0.04	0.241
	3	7.43±0.05	7.45±0.01	7.45±0.03	0.522
pCO_2 (mmHg)	1	48±5	47±8	47±8	0.986
	2	48±7	47±9	48±6	0.962
	3	51±9	49±9	46±6	0.534
RASS ≤-1/0/≥1	1	2/4/1	3/3/1	3/4/0	0.755
	2	3/4/0	2/4/1	2/5/0	0.329
	3	3/3/1	1/6/0	2/5/0	0.202
Sedation: nothing/enteral/mixed	1	2/5/0	2/5/0	1/5/1	0.460
	2	3/4/0	2/5/0	1/6/0	0.497
	3	4/3/0	6/1/0	2/5/0	0.097

The MLT baseline levels resulted below the nocturnal physiological levels in 71% of patients enrolled in the study. In a recent study conducted by Khaleghipour et al. (Khaleghipour-2012) the nocturnal MLT plasmatic levels in healthy subjects were found to be: 67.42 ± 16,17 pg/ml. In our study, the MLT baseline levels were between 34.1 (14.8-50.3) and 10,2 (8,5-16,4) pg/ml for the MLT os group; between 44.9 (19.3-68.9) and 7.4 (6.6-10.1) for SLN-MLT os group and between 58.0 (42.9-110.5) and 16.1 (14.0-22.4) for the SLN-MLT td group. Administration of exogenous MLT led all patients to reach pharmacological MTL levels (Fig. 38).

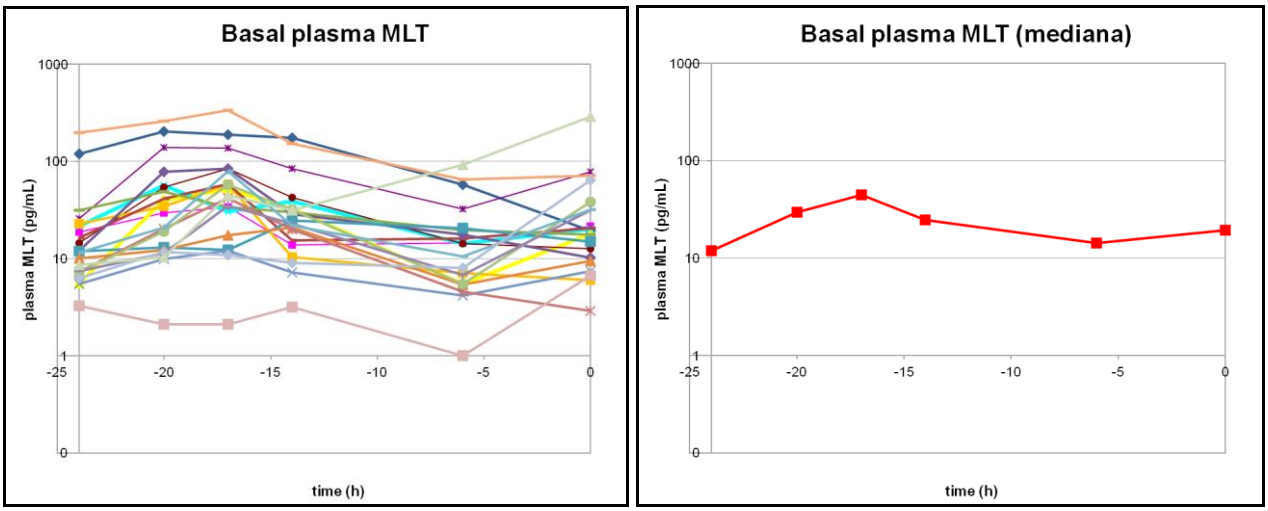

Fig. 38 *– Basal values trend and median basal value recorede in the 2nd day of ICU stay in all patients enrolled for the study.*

In patients who have received formulations *per os* (MLT os and SLN-MLT os groups), absorption was rapid: the peak plasma concentration has a median of 30 minutes and after just 5 minutes MLT plasmatic levels were greater than those physiological in all patients; the group who received transdermal MLT (SLN-MLT td group) instead, presented a delayed peak plasma concentration (median value 4 h), exceeding, however, the higher endogenous MLT concentration recorded in the 24 h prior the application.

The maximum concentration and the area under the curve (AUC) values showed statistically significant differences among the three groups ($p < 0.01$); they were significantly higher in the group that received SLN-MLT per os (SLN-MLT os). This group had a peak plasma 2 times higher than that received melatonin in the standard formulation (MLT os) (median 61226 pg/mL vs 26793 pg/mL) and 220 times higher than the group who were given SLN –MLT td (303 pg/mL). In patients who received enteral nanocapsules AUC was 3.8 times higher than those who received MLT per os (148578 pg / mL * h vs. 39126 pg / mL * h), and the SLN-MLT td group presents an area under the curve 19 times lower compared to the group which received the MLT per os (2050 pg/mL* h vs. 39126 pg/mL* h). The elimination fraction and plasma half-life did not differ significantly between the groups ($p = 0.56$) (Table 6).

Tab. 6 MLT pharmacokinetic characteristics

	MLT os (n=7)	**SLN-MLT os (n=7)**	**SLN-MLT td (n=7)**	**p value**
Maximum concentration (Cmax) (pg/mL)	26793 [16344-36673]	61226 [40408-75353]	303 [101-1548]	0.0003
Time to reach Cmax (h)	0.5[0.16-0.5]	0.5[0.5-1.5]	4[3-7]	0.0011
Halflife (h)	3.9[2.6-4.7]	3.1[1.7-3.9]	3.9[2.7-8.0]	0.56
AUC (pg/mL*h)	39126 [21706-86150]	148578 [55635-195758]	2050 [860-12784]	0.0009

Values are reported as median [interquantile range].

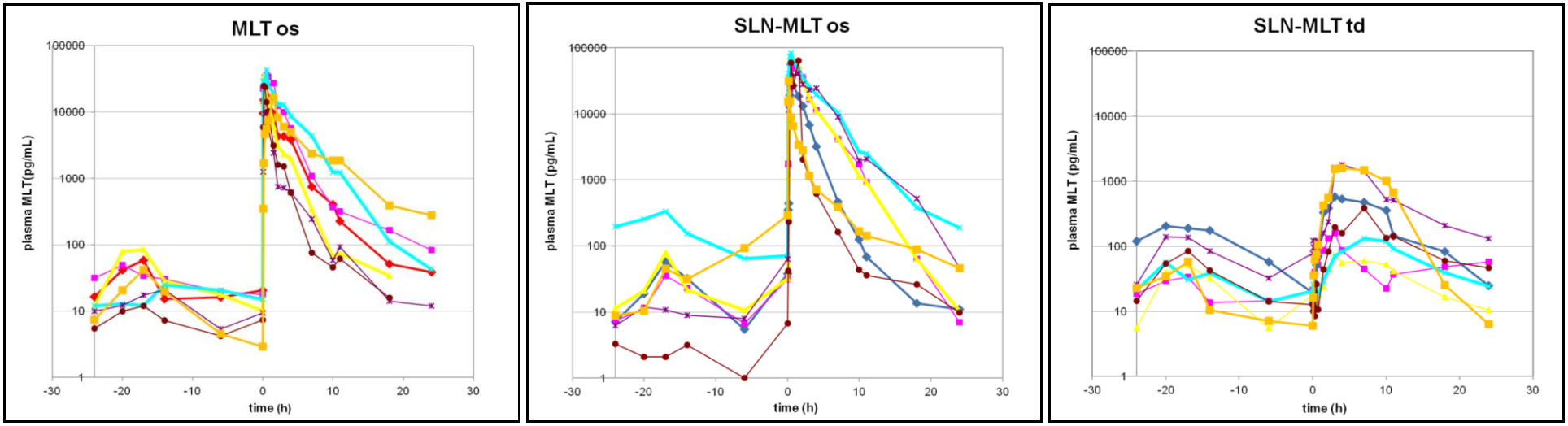

Fig. 39 *– Pharmacokinetics MLT plasma levels in the three groups: MLT os (n=7), SLN-MLT os (n=7) and SLN-MLT td (n=7).*

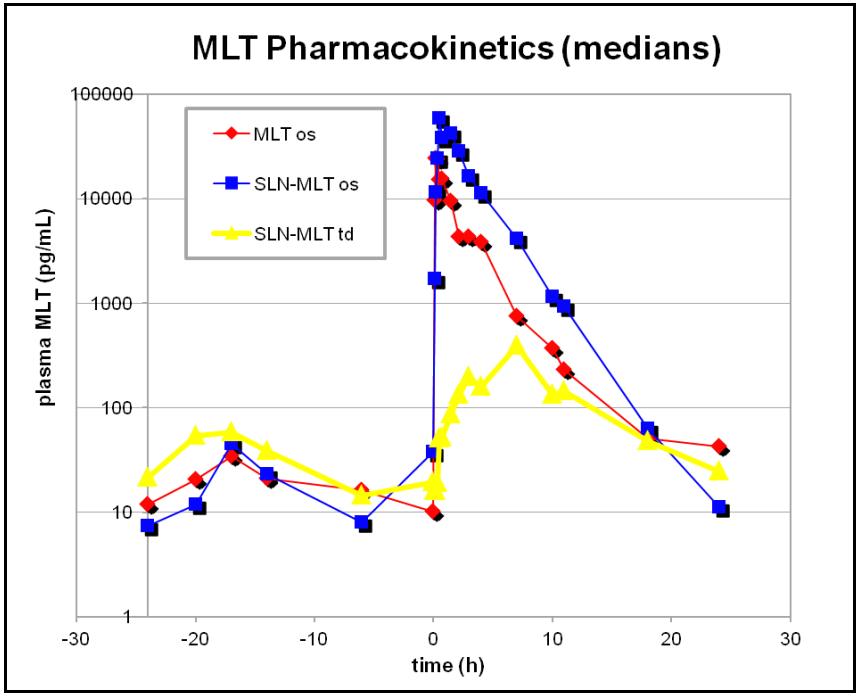

Fig. 40 *– Median plasma MLT levels for 3 groups: MLT os, SLN-MLT os and SLN-MLT td.*

Table 7 shows that the patients outcome did not vary in relation to the formulation or route of administration used (Table 7).

Tab.7 Outocome of the patients				
	MLT os (n=7)	**SLN-MLT os (n=7)**	**SLN-MLT td (n=7)**	**p value**
LOS	13 [13-24]	10 [7-11]	12 [8-17]	0,12
C-LOS	13 [10-24]	6 [5-9]	9 [6-14]	0,14
Ventilation (days)	13 [10-24]	5 [4-9]	9 [5-14]	0,13
ICU dead n(%)	3 (42,9)	1 (14,3)	1 (14,3)	0,35

Table 8 reports the results obtained from the correlation analysis to find a relationship between the clinical variables of the patients and the pharmacokinetics parameters.

Tab.8. Relationship between clinical variables and pharmacokinetics parameters

		MLT os		SLN-MLT os		SLN-MLT td		Overall	
	day	**Coef.**	**p**	**Coef.**	**p**	**Coef.**	**p**	**Coef.**	**p**
C_{max}									
	1	14114	0.515	1839	0.06	**384**	**0.035**	**8438**	**0.012**
Bilirubin	**2**	-11745	.	68850	0.148	948	0.143	-22728	0.523
	3	-23489	.	o.ins	o.ins	5474	.	**11567**	**0.023**
	1	-0.717*	0.069*	0.347*	0.446*	**-0.775***	**0.041***	0.061*	0.792*
Perfusion*	**2**	-0.722*	0.069*	0.416*	0.354*	**-0.81***	**0.027***	0.247*	0.28*
	3	0.474*	0.282*	-0.158*	0.735*	**-0.81***	**0.027***	-0.006*	0.979*
AUC									
	1	67178	0.205	14824	0.131	**3022**	**0.050**	**21519**	**<0.001**
Bilirubin	**2**	-22538	.	452776	0.065	7406	0.191	-10607	0.656
	3	-45076	.	o.ins	o.ins	51960	.	**26405**	**0.025**
	1	*391260	*0.169	8139	0.512	-2163	0.671	**113455**	**0.015**
Gastric retention	**2**	20363	0.583	-29940	0.728	-2163	0.803	24366	0.654
	3	39126	0.169	-29940	0.728	-1531	0.774	**113455**	**0.019**
T_{max}									
	1							-1	0.583
Shock	**2**							-1	0587
	3	0	1	0.5	0.081	-1	0.685	**2.5**	**0.002**
	1	0	1	-1	0.074	-1	0.685	-1	0.583
Vasoactive	**2**	0	1	-0.18	0.78	-4	0.182	**2.5**	**0.003**
	3	0	1	0.5	0.081	-4	0.182	**2.5**	**0.002**
	1	*0.5	*0.114	0	1	-1	0.512	-1	0.5
Gastric retention	**2**	**1**	**<0.001**	0	1	-1	0.77	1	0.595
	3	0.5	0.114	0	1	**4**	**0.023**	0	1
Half-life									
Bilirubina	**1**	**2.43**	**0.046**	*3.0426	*0.127	0.4488	0.951		

The analyzes were carried out using nonlinear regression quantile;

** analysis using Spearman correlation.*

8.0. DISCUSSION

8.1. MLT AS ANTITUMORAL MOLECULE

The pleiotropy of MLT molecule is an unique feature that has been proved by the exceptional number of papers (17829) appeared on the scientific scenario since 1958 (http: // www. ncbi. nlm. nih. gov. pros.lib.unimi.it/ pubmed). In a recent review Carpentieri et al. (Carpentieri et al., 2012) confirm the multifaceted activity of this molecule, focusing with a special emphasis on the clinical aspects and potential uses of MLT in the sleep-wake rhythms, in the immune function, in cancer therapy, in neuroprotection against oxidative damage and antioxidant activities in different tissues. Combined effects of MLT with other drugs are also discussed.

Today, prostate cancer is the leading cancer type (29% of new cases) in the US and accounts for 9% of the estimated male cancer deaths (Siegel et al., 2013). Planning research on chemoprevention of prostate cancer and strategies to improve the quality of life of survivors is thus mandatory. MT-based therapies appear promising because in 2007 the WHO International Agency for Research on Cancer (IARC) remarked that shift work, e.g., individuals exposed to light during night work that involves circadian rhythm disruption, increases the general risk for cancer (Straif et al., 2007), especially prostate cancer (Dumont et al., 2012). A recent meta-analysis found an aggregated risk of breast cancer of 1.40 associated with prolonged exposure to night work (Viswanathan and Schernhammer, 2009). Other studies also suggest that increased cancer risk related to night shiftwork may also extend to endometrial cancer (Viswanathan et al., 2007), prostate cancer (Kubo et al., 2006), and colorectal cancer (Schernhammer et al., 2003). In 2012 at least three important papers appeared on this issue (Davis et al., 2012) (Bonde et al., 2012) (Dumont, 2012 #1494) relating the decreased pineal MLT secretion in night workers exposed to light with increased cancer risk. MLT reduces the initiation and progression of tumor growth by multiple mechanisms chronodisruption, sleep deprivation and immunosuppression (Blask, 2009) so a decreased production would deprive night workers from MLT's protective oncostatic effects.

For some years there has been a growing interest of the scientific community and the public on the potential therapeutic effects of MLT in the treatment of certain diseases including prostate cancer, for which current treatment options are not yet fully satisfactory. It is not surprising then if research groups have begun to study in vitro and in vivo the effect of MLT on the proliferation of tumor cell lines benign (Gilad et al., 1997) or malignant (Hill and Blask, 1988).From the above it is clear that the oncostatic action of MLT is not related to a single function, but rather to a sum of several factors which include the activation of the immune system (Maestroni et al., 1987) the modulation of the endocrine system and direct action on the tumor. The actual mechanism of action by which the molecule exerts its antitumor activity is not so completely understood and deserves further research. Furthermore, while many evidence supports a possible direct effect of MLT on different types of cancer cells through its antiproliferative action (Shiu et al., 1999) others show controversial results (Papazisis et al., 1998). Regarding the in vitro proliferation of cells of prostate cancer, has been reported inhibition of MLT against PC-3 (androgen independent) (Gilad et al., 1999) LNCaP (androgen-dependent) (Moretti et al., 2000) and DU 145 (androgen independent). *In vivo* at contrary, has been reported an absence of inhibition in the growth of PC-3 cells due to a possible lack of MLT receptors in these cells (Xi et al., 2001). Other in vitro studies show an effect independent receptor (Moretti et al., 2000), using for example inhibitors of the receptor of MLT (Sainz et al., 2005). Conflicting data and different results must also

be evaluated in view of the different experimental design and MLT concentrations used, but in any case, the effect of MLT on prostatic carcinoma cells must be confirmed and deepened.

In the light of these data the first aim of this study was to investigate on the MLT potential as oncostatic molecule, testing also different pharmaceutical formulations and different and innovative delivery routes.

At this purpose we decided to use an in-vivo experimental model of nude mice (athymic), xenografted subcutaneously with androgen-sensitive human prostate adenocarcinoma (LNCaP) cell , an approach much closer to the clinical situation than in vitro cultured cells and that looks therefore adequate to investigate the underlying molecular mechanisms in vivo. This protocol has been set up and is running in our laboratory, with a success rate of 70% and <5% mortality.

In the first set of experiments MLT dissolved in saline solution (1mg/Kg) was administered repeatedly (18 treatments) i.p. to the animals for a period of 6 weeks. induced. The results obtained from this set of experiments confirmed that treatment with MLT is able to strongly inhibit the prostate tumor growth, inducing a significant decrease in the rate of growth of prostate cancer androgen-dependent (LNCaP) in athymic mice compared to an untreated control group evident from the 30^{th} day of treatment, and a significant decrease in the volume and weight of the tumor to 41 days by xenotransplantation, reaching the highest statistical significance in respect to not-treated animals, after 18 treatments just before the sacrifice (Fig. 23). In addition to this quite challenging behavior, a second interesting observation from this first set of experiments was the complete absence of toxicity (no animal died) and that the MLT treated animals showed all a better compliance to the xenograft in respect o not-treated (Fig. 22).

The clear oncostatic activity of MLT that we observed using this animal model, prompted us to investigate about eventual differences in activity and/or toxicity when MLT is loaded into SLN and administered by the same way (i.p.) and with the same treatment schedule. These experiments were intended to find that nanocarrier (SLN) systems are able to promote the systemic absorption of MLT and to enhance its oncostatic activity in respect to MLT dispersed in a buffered solution, used as reference. Numerous SLN formulations for various routes of administration (parenteral, oral, transdermal, ocular, pulmonary, rectal) have been developed and studied in vitro and in vivo for different drugs. The SLN-MLT system should present advantages so to affect the kinetics and the ability to concentrate the drug at the intracellular level.

Results showed that treatment with the SLN-MLT is safe and well tolerated by the animals that grew along the 42 days treatment as the ones treated with saline solution as carrier (Fig. 22). The oncostatic activity of SLN-MLT revealed slightly lower in respect to MLT in saline solution, when evaluated as tumor volumes over time, although the mean tumor size and weight (at sacrifice) in the two treatment groups was never different.

The evaluation of amount of MLT distributed systemically and accumulated into the tumor mass in both treatment groups revealed that at the end of the IP treatment with MLT in physiological or entrapped in SLN we got plasma MLT levels significantly higher than in controls, with a lower, but not significant, level in SLN-treated animals (Fig. 24). This behaviour inversely mirrored the tumor volumes recorded at the end of the treatment (Fig. 23). The MLT tumor uptake showed the same trend observed in plasma but in this case

we have found an overload of MLT in the tumor of IP saline solution treated animals in respect to SLN, suggesting a different disposition of the drug or a different metabolic fate (Fig. 25).

Despite the low average life of MLT plasma reported (Yeleswaram et al., 1997), the protocol of intraperitoneal injections of MLT has been shown to induce functional responses in our as in other studies Xi et al., 2001). The microscopic examination of LNCaP tumors showed changes in histology of the tumor cells between treated and not treated with MLT, according to some studies (Siu et al., 2002) and to what has been observed in an in vitro study where MLT change morphologically LNCaP cells (Sainz et al., 2005) with changes similar to those described in the literature in the terminal differentiation of LNCaP cells (Burchardt et al., 1999). This same work shows that the differences in the action of MLT, are dose dependent.

Observing the histology of the group treated with MLT compared to the control (Fig. 27) the same necrotic centers and similar extension of the vasculature are shown, suggesting that inhibition of the vascularization is not the only mechanism by which MLT acts to decrease the speed of growth of LNCaP tumor in vivo. The differences that were detected by microscopic analysis relate to the presence of a predominantly lymphocytic infiltrate in the group treated with MLT. This shows an attempt by the body to reject the tumor in mice lacking thymus. This data is not reported in other studies carried out in vivo on LNCaP cells (Siu et al., 2002) and have been also thoroughly confirmed by immunofluorescent analysis (Fig. 28). The morphological evaluation of the tumors treated with SLN-MLT has shown a development of the inflammatory response as in the group of MLT, showing an interaction with the immune system although probably slightly different. The infiltrate present in the group treated with SLN-MLT in fact presents in addition to lymphocytes, also other types of immune cells. Again this picture was confirmed by the immunofluorescence analysis. Furthermore it detects an attempt to isolate the tumor through fibrous capsules, effect not observed in the treatment with MLT, and in other works using MLT *in vivo* (Siu et al., 2002). There seem to be an important effect in the tumor microenvironment of SLN-MLT that shows an attempt to tumor rejection and isolation by the immune system. This type of cancer may have less invasive capacity compared to cancer shown in the control group and in the one treated with MLT. The volume and the tumor mass in the SLN-MLT treated group may also be distorted by the large percentage of connective tissue present in the tumor mass. With regard to the vasculature and the morphology of the tumor cells there were no significant differences between MLT-saline treated group and the control group.

The CD4 and CD8 expression evaluated by quantitative analysis immunohistochemistry showed a $CD4^+/CD8^+$ ratio significantly lower in both MLT treated groups, in respect to controls, although again in MLT-saline this ratio was lower than in SLN-MLT. Diederichsen (Diederichsen et al., 2003) has reported that a lower $CD4^+/CD8^+$ ratio is associated with a better clinical course, in patients with colorectal cancer. Diederichsen also found that there was no association between the $CD4^+/CD8^+$ ratio and tumor localization or growth pattern, while the $CD4^+/CD8^+$ ratio was inversely associated with tumor differentiation and a better prognosis of tumor.

Hypoxia (low O_2 supply with respect to needs) is predicted to play a pivotal role in the development of most solid cancers thereby triggering mechanisms leading to inducing greater resistance of tumor cells in a hostile environment (Vaupel, 2004), which translates into repression of apoptosis and autophagy, faster turnover and growth (Marignol et al., 2008). When the hypoxia severity in the tumor cell environment *in vivo* is increased, the growth rate in prostate LNCaP cancer near doubles (Terraneo et al., 2010). Many *in vitro* studies indeed point at the paradigm that most of the hypoxia-downstream adaptive patterns are mediated

by the hypoxia-inducible factor-1α (HIF-1α), an O_2 sensor that activates hundreds downstream genes in response to hypoxia. But this pattern was not confirmed by the *in vivo* study (Terraneo et al., 2010), where HIF-1α was not over-expressed in tumors despite faster growth.

Recently, it has been reported that, in LNCaP cells exposed to mimetic hypoxia, MLT may down-regulate HIF-1α (Park et al., 2009). Furthermore, MT destabilizes HIF-1α protein in HCT116 human colon cancer cell line, secondary to its antioxidant activity, possibly by a mechanism involving the dephosphorylation of p70S6K and its target RPS6 (Park et al., 2010). As a matter of facts, however, such evidence derives essentially from *in vitro* experiments, and the interaction of MT with the hypoxia-signaling path *in vivo* is still to be proved. Many clinical trials have started making use of HIF-1α inhibitors, yet with non-encouraging results (Onnis et al., 2009), thereby questioning the paradigm of HIF-1α as a central player in cancer growth. This controversial issue reflects in apparently contradictory results. For example, whereas in one study HIF-1α appears instrumental to determine tumor growth because the drug acriflavine inhibits its activity (Lee et al., 2009), in another the drug digoxin inhibits HIF-1α but does not affect tumor growth (Gayed et al., 2012).

So, to clearly the underlying action mechanism of MLT and the role of HIF-1α at the tumor cellular micro-environment, we have evaluated the HIF-1α expression in the tumor tissues by immunofluorescence.

In both MLT treated groups HIF-1α expression appeared unexpectedly increased in respect to controls, although not reaching the significance of the difference. Saline SLN, as previously seen for other parameters, seem to shift less efficiently HIF-1α expression (Fig. 26). This preliminary observation if confirmed by additional experiments and using also other techniques (western-blot analysis), is in contrast with the finding of Park et al (Park et al., 2009). in *in vitro* experiments suggesting that MLT could play a pivotal role in tumor suppression via inhibition of HIF-1-α mediated angiogenesis and opens a different scenario to explain the MLT oncostatic activity and its role in the angiogenic signaling pathway during cancer development.

After the demonstration that i.p. MLT (both in saline solution and entrapped into SLN carrier) is able to efficiently reduce the prostate tumor growth in our “in vivo” animal model, the second objective, using the same mouse model of human prostate cancer , was to test whether MLT can be administered efficiently using alternative ways that are more sustainable for prolonged treatments than i.p. MLT, e.g., transdermal delivery through the skin barrier directly onto the tumor via a novel and patented technique named cryoRx. Using this technique we delivered the frozen MLT by topical application with a suitable cryo-applicators connected to a laser source and we applied the same schedule (18 treatments for 6 weeks) used for i.p. study. By this administration route we chose to use a dosage 4-fold higher than i.p. in order to reach about the same MLT concentrations at systemic level. The topical laser treatment was perfectly tolerated by the animals and when MLT was added to the treatment this produced the same beneficial effects on mice growth seen for i.p. treatment. Curiously, the laser itself seem to give a better compliance to the xenograft compared to controls not treated (Fig. 30). The MLT-laser treatment impaired tumor growth all along the 42 days and produced tumors with a significant lower weight in respect to control at the sacrifice. Again we noticed that the laser itself is able to reduce the tumor growth, although less efficiently than with MLT as adjuvant. The topical treatment with MLT significantly increased the level in the systemic circulation and in the tumor tissue, with levels comparable to those found after i.p. treatment. HIF-1α expression, as observed with i.p. treatment, again resulted significantly overexpressed by the presence of MLT, and this correlates

with the concentration of MLT in the tissue thus confirming that additional study is needed o clarify the role of MLT in Hypoxia signaling pathways in prostate cancer.

The effect of transdermal MLT by cryolaser on tumor mass morphology are quite similar to those reported after i.p. treatment. MLT is able to elicit a immunological response into the tumor characterized by

nests of tumor cells circumscribed by a major inflammatory reaction, represented by the massive presence of lymphocytes and some granulocytes, which seem to surround and infiltrate the tumor cells. It was also possible to note an elevated development of blood vessels, which grow indefinitely forming cavity rich in erythrocytes and this may be in line with the high expression of HIF-α found in the tissue. The tumors appears limited not only by high lymphocyte response, but also by the presence of fibroblast-like cells (Fig. 35). Immunofluorescent analysis confirmed that when MLT is used as an adjuvant to laser scan tends to disaggregate the tumor tissue even more than with laser alone, and a mixed structure with compact cord and areas most highly disorganized is evidenced (Fig. 36). The luminescence evidenced in the figure is due to white blood cell infiltrates.

The results obtained by transdermal application of MLT by cryoRx are extremely promising as this application proved to negatively affects tumor growth in a time-dependent manner, so to be considered for further experimentation, with the advantages in terms of lower toxicity and greater efficacy.

CryoRx is a very interesting technique routinely employed for a lot of different applications spanning from orthopaedics and physiatry, dermatology, angiology, urology and gynaecology, aesthetic medicine plastic surgery, sports medicine. The use of cryoRx has been tested to treat patients with burn scars and to carry metilprednisolone in acute spinal cord injury in rats. Moreover has been used to deliver ganglioside GM1 for treatment of peripheral nerve damage in rats showed more regenerating nerve fibers in the distal segment compared to the untreated groups (Prof. Ciro Silva, Md,PhD, University of San Paulo Medical School unpublished observations). As far as we know, the this is the first experience in which cryoRx is employed for prostate tumor treatment in association with MLT. The results demonstrate that the technique is safe and feasible and that is able to vehicle the MLT molecule at the systemic level (plasma) efficiently almost as i.p. administration. The tumor growth was inhibited almost at the same level after topical administration by cryoRx, although the tumor tissue uptake seems to be lower than after i.p. (Fig. 25 and Fig. 34). On the other hand, the histological analysis and the immunofluorescence confirm the strong affect of MLT-laser treatment in affecting of the tumor tissue architecture and in elicit a significant immunological response Additional investigation are needed on this route of administration in order to understand if the uptake at cellular level of the drugs is also linked to a different metabolic fate of the molecule.

The results achieved from this part of the thesis will be integrated together and will add knowledge both in understanding the crucial pathways for targeting in prostate cancer.

8.2. MLT in ICU patients as sleep-wake regulator

The encouraging results obtained with transdermal application of MLT in mice for the cure of prostate cancer, prompted us to be involved also in a clinical study, testing MLT transdermal delivery in *in a* cohort of critically ill patients admitted to a high-risk Intensive Care Unit (ICU). In this case the endpoint was totally different, as MLT was administered, through different modalities (enteral administration and

transdermal through solid lipid nanoparticles, SLN), to test its activity as potential adjuvant in the therapy sedative.

The potential of MLT are not limited to its sleep inducer effects but include a wide range of activities, including the immunomodulatory and the antioxidant effect (Leon-Blanco et al., 2003). Critically ill patients admitted to the ICU present frequently an altered circadian secretion of the pineal hormone (Mundigler et al., 2002). It has recently been hypothesized that the restoration of the melatoninemia in critically ill patients may be useful (Bourne and Mills, 2006): to this end, numerous formulations and different doses of the hormone have been tested. In particular, a study of 2010 has demonstrated the practicability of enteral administration of MLT in terms of effectiveness of absorption, adequacy in the rate of establishment of the plasma peak and in the maintenance of plasma concentrations pharmacological hormone (Mistraletti et al., 2010) also in the early phase of hospitalization in the ICU. Other studies have demonstrated the effectiveness of transdermal administration (Aeschbach et al., 2009).

The research here presented includes the transdermal administration because it may have different advantages in critically ill patients, in particular: ease of administration (the application of a patch on the skin is possible in almost all clinical contexts excluding situations of hyperhidrosis, large burns, allergic skin manifestations, cutaneous vasoconstriction, etc.) and reduced hepatic first-pass effect (reduction of the administered dose with lower peak plasma maintaining a constant plasma concentration). Furthermore, the possibility of incorporating the MLT in SLN should allows to obtain a greater therapeutic efficacy connected intracellular action of the hormone itself. The SLN in fact act as a reservoir of the hormone effect allowing a constant and prolonged the site of action, so it could more evident clinical effect thanks to the achievement of the intracellular environment regardless of specific receptors MT1 and MT2 (Priano et al., 2007).

The design of this clinical study was aimed at evaluate, at first, if the circadian rhythm of MLT secretion is disrupted in ICU patients by studying the pharmackinetics of basal secretion in the 2nd day after ICU admission. The results show a very high variability among MLT basal secretion in patients enrolled in this study, with some subjects characterized by MLT levels well below the control values, and some others with values in the normal range, if not even higher than normal. It can be conceivable that all patients could take advantage from exogenous administration of MLT.

The second aim of this study was to test to which extent MLT administration by different routes and different drug formulations (MLT as a tablet administered orally, MLT encapsulated in SLN administered orally as a suspension, MLT encapsulated in SLN applied transdermally as a suspension with the aid of a patch) is feasible in terms of efficiency of absorption and adequacy in achieving and maintaining nocturnal peak plasma hormone even in the early phase of hospitalization in the ICU.

All patients enrolled in the study presented very serious medical conditions requiring invasive treatment and intensive high during hospitalization, the Simplified Acute Physiology Score II (SAPS II) indicates that the expected mortality for the study population is 50% and the Sequential Organ Failure Assaesment (SOFA) shows the need for a high level of intensive care (mean score ≥ 6), all patients required ventilatory support.

From the analysis of the different pharmacokinetics profile (absorption peak, plasma half-life, mean concentration) obtained as a function of MLT administration by different routes and drug formulations emerged that:

- the time in which the two oral formulations reach the maximum concentration is similar despite of the different vehicle used to delivery the drug. In some patients it was significantly delayed because of the presence of gastric stagnation or shock requiring the use of vasoconstrictors
- The AUC of SLN-MLT administered enterally was significantly higher than when MLT was administered by saline solution. This figure is probably determined by the intrinsic properties of the formulation that avoids the hepatic first-pass effect
- Administration of SLN-MLT by transdermal route, although showed a lower bioavailability compared to the enteral administration, however, ensured the restoration of suitable MLT plasma levels that should be suitable enough to restore the pleiotropic function of this hormone: facilitate the resolution of sleep-wake cycle disorders, improve the quality of sleep, reduce the number of episodes of anxiety, confusion and agitation, and reduce the amount of sedatives used, especially at night.
- The peak plasma concentration, maximum in the case of administration by SLN, was increased in disease states where there are higher levels of bilirubin;
- The peak plasma level was decreased, only for the transdermal formulation, in conditions of peripheral hypoperfusion, condition that may affect the adsorption of drugs by this administration route.
- The plasma half-lives of MLT elimination were not different in the three administration routes, indicating that the clearance of the active principle follows the same pathway.
- Patients with impairment of the excretory organs, such as those with high bilirubin levels, presented a higher MLT bioavailability.
- The outcome of patients do not vary with the formulation used.

Patients characterized by serious medical conditions have reduced levels of endogenous MLT. It is well known that supplementation of the hormone in these patients armies favorable effects. The objective for this study was to evaluate the existence of differences in the pharmacokinetic profiles of melatonin, depending on the formulation and route of administration used in a group of patients treated in intensive care. MLT in SLN showed the best bioavailability. Transdermal administration guaranteed the achievement of drug levels and constitute a valuable alternative in cases of impracticability of the gastrointestinal tract.
Additionally studies are required to understand the underlying mechanism of the observed clinical activity, for instance by evaluating the total antioxidant defenses of the patients after the different MLT administration

9.0. CONCLUSIONS AND PERSPECTIVES

The main object of this PhD thesis, has been successfully reached. The pleiotropy of MLT molecule was demonstrated by getting positive results in two of the most promising clinical applications: the cure of prostate cancer and the regulation of the sleep/wake rhythm as adjuvant in the sedative therapy in critically ill patients. In addition, during this PhD program a comprehensive study to assess the capability of alternative and novel strategies to deliver the drug (both in animal and in humans) has been performed demonstrating that the transdermal way of administration for MLT is safe and feasible and is able to provide systemic concentrations of the drug adequate to sustain the pharmacological activity. During this PhD program the cryoRX system for drug delivery was used on animals displaying an excellent performance. In future this system merits more attention especially for its use in humans, both as an adjuvant for prostate cancer managing and for the treatment of other kind of tumors i.e. melanoma and/or other skin disease. The results obtained with MLT in an animal model of prostate tumor provide a rational basis for possible future use in humans of a non-toxic substance of natural origin and for treatment in cases of suspected prostate cancer (PSA borderline). The cryoRX system may also be shifted to treatment with other drugs which have high toxicity and low therapeutic ranges, with the aim of obtaining advanced mode of administration, specifically "targetted" to the area of interest and characterized by lower dosages and reduced adverse reactions.

Providing that MLT appeared active in fighting prostate cancer and/or in ameliorating critically ill patients hospitalization, an initial approach to elucidate the molecular mechanisms underlying the observed activities has been done, with particular attention to the hypoxia signaling pathway and the immunological response.

This part which gave some interesting preliminary evidence on the molecular mechanism underpinning MLT cellular activity (MLT seems to increase HIF-1α), deserves to be more deeply investigated. MLT oncostatic activity has been recently related in addition to hypoxia, also to sphingolipids intracellular signalling pathways. However, the underlying mechanisms of MT antiproliferative activity remain unclear, the interplay between HIF and SPHK1 in “in vivo” experiments is lacking and whether HIF is tumor promoting or inhibitory is still under debate, being, in some tumor types, the pro- or antitumorigeneic effects of HIF-α, isoforms specific. It is pivotal to plan effective strategies for further experiments in this field. Finally, recent literature give a lot of attention to the MLT metabolite N-acetyl-N-formyl-5-methoxykynuramine (AFMK) rather than its commonly measured urinary excretory product 6-hydroxymelatonin sulfate. Via the AFMK pathway, a single MLT molecule is reported to scavenge up to 10 ROS/RNS. That the free radical scavenging capacity of MLT extends to its secondary, tertiary and quaternary metabolites and explains how it differs from other conventional antioxidants. It has been proposed that the ratio of AFMK to another SLN metabolite, cyclic 3-hydroxymelatonin, may serve as an indicator of the level of oxidative stress in organisms. From all the above considerations, appears that a method for MLT quantification characterized by high precision and specificity so as to be regarded as the definitive method for the measurement of different molecules in biological matrices (MLT and its metabolites as well) (i.e. liquid chromatography mass spectrometry, LC-MS-MS), is pivotal to explain and confirm the results till now obtained.

10.0. REFERENCES

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