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# Arabinoxylan rice bran (MGN-3/Biobran) enhances natural killer cell—mediated cytotoxicity against neuroblastoma *in vitro* and *in vivo*

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## Abstract

*Background aims.* Natural killer cell (NK) cytotoxic activity plays a major role in natural immunologic defences against malignancies. NK cells are emerging as a tool for adoptive cancer immunotherapies. Arabinoxylan rice bran (MGN-3/Biobran) has been described as a biological response modifier that can enhance the cytotoxic activity of NK cells. This study evaluated the effect of MGN-3/Biobran on NK cell activation, expansion and cytotoxicity against neuroblastoma cells. *Methods.* NK cells were enriched with magnetic beads and stimulated with MGN-3/Biobran. NK cell activation was evaluated via analysis of their phenotype, and their expansion capability was tracked. The *in vitro* cytotoxic ability of the activated NK cells was tested against K562, Jurkat, A673, NB1691, A-204, RD and RH-30 cell lines and the *in vivo* cytotoxic ability against the NB1691 cell line. *Results.* MGN-3/Biobran stimulation of NK cells induced a higher expression of the activation-associated receptors CD25 and CD69 than in unstimulated cells ($P < 0.05$). The expression of NKG2D, DNAM, NCRs and TLRs remained unchanged. Overnight MGN-3/Biobran stimulation increased NK cell cytotoxic activity against all cell lines tested *in vitro* and decelerated neuroblastoma growth *in vivo*. The mechanism is not mediated by lipopolysaccharide contamination in MGN-3/Biobran. Furthermore, the addition of MGN-3/Biobran promoted NK cell expansion and decreased T cells *in vitro*. *Conclusions.* Our data show that MGN-3/Biobran upregulates NK cell activation markers, stimulates NK cell cytotoxic activity against neuroblastoma *in vitro* and *in vivo* and selectively augments the expansion of NK cells. These results may be useful for future NK cell therapeutic strategies of the treatment of neuroblastoma.

*Key Words:* arabinoxylan rice bran (MGN-3/Biobran), cytotoxic activity, natural killer (NK) cells, neuroblastoma

## Introduction

Natural killer cell (NK) cytotoxic activity plays a major role in our natural immunologic defences against the development of malignancies, as evidenced by the fact that decreased NK cell cytotoxic activity is associated with a higher risk of tumor development in healthy people [1]. Additionally, after hematopoietic stem cell transplantation, high NK cell cytotoxic activity is associated with a decreased risk of relapse in patients [2]. The cytotoxic activity of NK cells can be increased through healthy lifestyle practices [3—5], biological response modifiers [6,7], growth hormone [8] and cytokines [9—12]. Malignant cells can decrease NK cell cytotoxic activity through the release of suppressive cytokines and/or the reduction of activating receptors on NK cells [13,14]. NK cell activity can also be suppressed by antibodies [15,16] and chemotherapeutic drugs [17].

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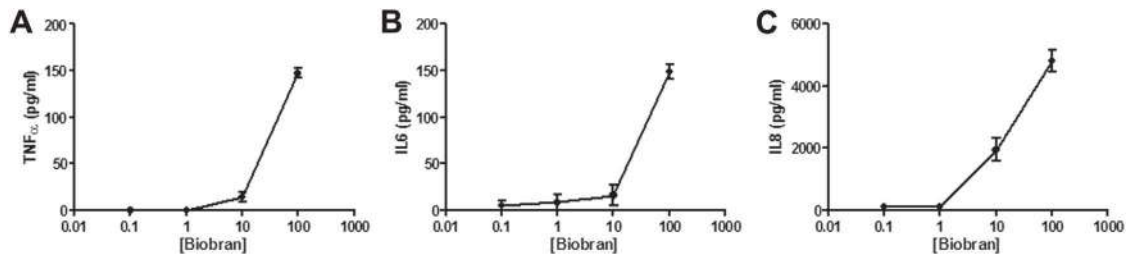


Figure 1. Cytokine levels in the culture supernatants were determined using the Cytometric Bead Array Flex Set (BD Biosciences) and analyzed by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences). (A) TNF-α levels, (B) IL-6 levels and (C) IL-8 levels.

Therefore, maintaining high NK cell cytotoxic activity should be targeted in both cancer patients and the healthy population.

MGN-3/Biobran is an arabinoxylan from rice bran that has been modified by carbohydrate hydrolysing enzymes from shiitake mushrooms [18]. This food supplement that has been reported to enhance NK cell cytotoxic activity against tumors in adult patients, *in vitro* and *in vivo* [19,20]. Furthermore, it has been described as having a synergistic anti-tumor effect with conventional treatment for some cancers, such as breast cancer and hepatocellular carcinoma [21−25]. These data have brought to light the possibility of using MGN-3/Biobran as a supplemental treatment for cancer in adult patients. However, no data have been reported for pediatric tumors. Our goal was to explore the role of MGN-3/Biobran as a NK cell stimulator against pediatric tumors *in vitro* and *in vivo* as well as the role of MGN-3/Biobran in NK cell expansion using various cytokine combinations and stimulator cell lines.

## Methods

### Cell preparation

Our local institutional ethics committee approved this study. peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation from blood samples taken from healthy volunteers. Blood was gently layered onto an equal volume of Ficoll-Paque Plus (GE Healthcare) and centrifuged at 400 $g$ for 20 min at room temperature. The PBMCs were harvested from the interface and washed twice with phosphate-buffered isotonic saline (PBS) and centrifuged at 400 $g$ for 10 min. NK cells were then enriched by magnetic bead selection (NK cell isolation KIT or CD56 microbeads; Miltenyi Biotec) (see online supplementary Figure 1 for reviewers). Whole blood was layered on top of a Ficoll cushion and centrifuged at 1800 rpm for 30 min at room temperature. The lymphocyte/monocytic fraction was isolated, washed with PBS and subjected to red blood cell lysis (ammonium chloride solution; Stem Cell Technologies) for 5 min at room temperature, and following an additional wash with PBS, monocytes were cultured under adherent conditions in RPMI 1640 medium (Gibco-BRL, Life Technologies Ltd) supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% $CO_2$ at 37°C. Adherent monocytes were cultured for 7−10 days to allow for differentiation into macrophages. Macrophages were used as biosensors to identify the optimal dose of MGN-3/Biobran to stimulate NK cells without stimulate macrophages.

### Reagents

Anti-human monoclonal antibodies (mAbs) used in the study were CD3PE-Cy7, CD45-FITC, CD69-FITC and CD314 (NKG2D)-APC (all from Becton Dickinson); CD56-APC, CD25-PE, CD336

Table I. Biobran and IL-15 overnight stimulation effect on activating NK receptors.

| | Resting | | Biobran | | IL-15 | | Biobran/resting | IL-15/resting |
|---|---|---|---|---|---|---|---|---|
| | MFI | SD | MFI | SD | MFI | SD | Ratio | Ratio |
| CD69 | 508.3 | 889.2 | 1591.7 | 741.1 | 18032.6 | 14136.6 | **3.13** | **35.48** |
| CD25 | 481 | 448.9 | 1537 | 520.3 | 1864 | 2843.8 | **3.2** | **3.88** |
| NKG2D | 4634.2 | 5762 | 5074.3 | 4761 | 9914.9 | 11491 | 1.09 | **2.14** |
| DNAM | 1960.6 | 2529 | 2501.1 | 1174 | 3344.8 | 5391 | 1.28 | 1.71 |
| NKp44 | 1017.7 | 1473 | 1808 | 2780 | 886.3 | 2110 | 1.78 | 0.87 |
| NKp30 | 1300.7 | 1990 | 1519.8 | 2508 | 4971.5 | 1567 | 1.17 | **3.82** |
| NKp46 | 1134.4 | 1044 | 1159.4 | 1695 | 1568.8 | 1187 | 1.02 | 1.38 |
| TLR4 | 2654 | 2858 | 1348 | 2533 | 1963 | 2150 | 0.51 | 0.74 |
| TLR9 | 5854 | 6284 | 5200 | 6448 | 8779 | 6231 | 0.89 | 1.50 |

Data express MFI, SD, and ratios from 3 healthy controls. Bold indicates statistical significance.

(NKp44)-PE and CD335 (NKp46)-PE (all from Beckman Coulter); CD337 (NKp30)-PE (Miltenyi Biotec). Fluorochrome-labeled mAbs against TLR-4 and TLR-9 were obtained from Enzo Life Sciences AG.

Interleukin (IL)-15 was obtained from CellGenix. IL-2 (Proleukin) was obtained from Novartis. MGN-3/Biobran was provided by Daiwa Pharmaceuticals Co Ltd. Lipopolysaccharide (LPS; Sigma 0127:B8) was used as toll-like receptor-4 (TLR-4) ligand, and polymyxin B (InvivoGen) was used as an inhibitor of LPS-induced activation of TLR-4.

*Cell lines*

K562 erythroleukaemia, Jurkat T lymphoid leukaemia, A673 Ewing sarcoma (all from ATCC), NB1691 neuroblastoma cell line (kindly provided by Dr. A. Davidoff of St. Jude's Children's Research Hospital), A-204 embryonic rhabdomyosarcoma, RD embryonic rhabdomyosarcoma and RH-30 alveolar rhabdomyosarcoma (all from DSZM) cell lines were used as targets for NK cell natural cytotoxicity assays *in vitro*. The luciferase-transduced neuroblastoma cell line (NB1691luc) was kindly provided by Dr A. Davidoff and was used *in vitro* and in a quantitative *in vivo* mouse model [26,27]. Irradiated K562 and K562 with expression of cell membrane-bound IL-15 and 4-1BBL (K562-mb15-41BBL, kindly provided by Dr. D. Campana, National University of Singapore) were used as feeder cells for NK cell activation and expansion [28].

*Phenotypic analysis*

The surface phenotype of overnight MGN-3/Biobran (100 µg/mL)-stimulated NK cells, overnight IL-15 (10 ng/mL)-stimulated NK cells, unstimulated NK cells and expanded NK cells from 3 healthy adult volunteers was determined using 6-color immunofluorescent staining. We stained $5 \times 10^5$ fresh NK cells from various conditions with appropriate mouse anti-human monoclonal antibodies for 30 min in the dark at 4°C. The cells were washed twice with cold PBS, resuspended in 0.5 mL of PBS and analyzed using a FACSCanto II flow cytometer (Becton Dickinson). The percentage of positive cells and mean fluorescence intensity (MFI) ratios were determined for each cell surface antigen. Controls were applied using appropriate isotype control antibodies.

*Cytotoxicity assays and NK cell stimulation*

The natural cytotoxicity of NK cells was monitored in a conventional 2-hour europium-2,2′:6′,2″-terpyridine-6,6″-dicarboxylic acid release assay

(Perkin-Elmer Wallac) as described previously [29]. K562, Jurkat, A673, NB1691, A-204, RD and RH-30 cell lines were used as the target cells. In brief, target cells were labeled with a fluorescence-enhancing ligand (bis(acetoxymethyl) 2,2′:6′,2″-terpyridine-6,6″-dicarboxylate). This hydrophobic ligand quickly penetrates the cell membrane. Within the cell, the hydrolysis of ester bonds results in the ligand becoming hydrophilic and therefore unable to pass through the cell membrane. Cytolysis, however, results in the release of the ligand and ultimately a reaction of the ligand with the europium to form a stable, fluorescing chelate, which is evaluated fluorometrically (Infinite F200 reader TECAN Group Ltd). The following formulas were used to calculate spontaneous and specific cytotoxicity:

$$\% \, Specific \, release$$
$$= (Experimental \, release - spontaneous \, release)/$$
$$(Maximum \, release - spontaneous \, release) \times 100$$

$$\% \, Spontaneous \, release$$
$$= (Spontaneous \, release - background)/$$
$$(Maximum \, release - background) \times 100$$

NK cells from healthy volunteers were stimulated overnight with 100 µg/mL MGN-3/BioBran, 10 ng/mL IL-15, 40 IU/mL or 1000 IU/mL IL-2 or with a combination of MGN-3/Biobran and 40 IU/ml IL-2. Cultures were performed in complete culture medium (RPMI 1640 supplemented with 10% of heat-inactivated fetal bovine serum, 100 IU/mL penicillin, 100 ng/mL streptomycin, and 2 mmol/L glutamine) in a humidified atmosphere of 5% $CO_2$ and 95% air. Cytotoxic activity was assessed as described earlier.

*Murine model*

NB-1691luc $2 \times 10^5$ neuroblastoma cells were injected intravenously into 12-week-old NOD-scid IL-2Rgnull mice. For the isolation of NK cells, we used PBMCs from healthy volunteers. NK cells were then enriched by magnetic bead selection (NK cell isolation KIT, Miltenyi Biotech). NK cells obtained were >90% CD3-CD56+. Fresh NK cells or NK cells activated with 100 µg/mL MGN-3/BioBran overnight were used. Intravenous NK cellular therapy began 7 days after the injection of tumor cells and was performed twice a week for 4 weeks. In 2 independent experiments (4 mice per group), we compared an untreated cohort (control group) with a cohort receiving $1 \times 10^6$ unstimulated NK cells (NK group) and a cohort receiving $1 \times 10^6$ NK cells

stimulated overnight with 100 μg/mL MGN-3/Biobran (NK-Biobran group). Bioluminescence imaging was performed after the initiation of NK cell therapy on days 7, 14, 28 and 42 after intraperitoneal injection of 100 μL of luciferin dissolved in PBS at a concentration of 15 mg/mL.

Five minutes after the administration of substrate, the animals were anesthetized using isofluorane (induction of anaesthesia at 3% and then maintained at 1.5%) and transferred to the Xenogen IVIS Lumina II (Quantitative Fluorescent and Bioluminescent Imaging, Xenogen Corporation). Images were captured at varied exposures and the analysis was performed using Xenogen Living Image Software (version 3.2). For bioluminescence imaging plots, a rectangular region of interest encompassing the entire thorax and abdomen was applied for each mouse and total flux (photons/s) calculated in ventral and prone positions at 180 s exposure. This value was scaled to a comparable background value (from a nontumor bearing, luciferin-injected control mouse). All experiments were conducted following the guidelines of the Institutional Animal Care and Use Committees according to criteria outlined in the National Institutes of Health Guide for Care and Use of Laboratory Animals.

### NK cell activation and expansion

Expansion was achieved by 14 days of culture with or without 100 μg/mL MGN-3/Biobran and cytokines (100 IU/mL IL-2 or 100 IU/mL IL-2 plus 10 ng/ml IL-15) or additional coculture with irradiated feeder cells consisting of K562 cells or K562-mb15-41BBL [28]. In brief, PBMCs were obtained from 5 healthy adult volunteers by density gradient centrifugation (Ficoll). PBMCs were incubated in a 6-well flat-bottom plate with or without MGN-3/Biobran and human cytokines (IL-2, IL-2 + IL-15) or cocultured at 1:1.5 ratio with sublethal irradiated K562 or K562-mb15-41BBL feeder cells. The culture medium was RPMI 1640 supplemented with 10% AB fresh frozen human plasma, L-glutamine and penicillin-streptomycin (Biochrom). Fresh medium was added every 2 days. After 14 days, cells were collected and analyzed for phenotype and *in vitro* NK cell cytotoxicity.

### Cytometric bead array and flow cytometer analysis to determine TLR agonist contamination in MGN-3/Biobran

The release of tumor necrosis factor (TNF)-α, IL-6 and IL-8 in human macrophages after exposure to LPS (10 ng/mL) or MGN-3/Biobran (at 10, 100, 1000 and 10,000 μg/mL) was detected by cytometric bead array technique Flex Set (BD Biosciences) following the manufacturer's protocol and then analyzed by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences). MGN-3/BioBran concentration (100 μg/mL) was determined to be the highest concentration that did not induce inflammation (elevation of TNF-α, IL-6 and IL-8, Figure 1). MGN-3/Biobran was screened for its potential agonistic effect on TLR-2, -3, -4, -5, -7, -8 and 9 by InvivoGen. Because traces of LPS in MGN-3/Biobran could increase NK cell cytotoxicity by TLR-4 signaling, the determination of contaminating lipopolysaccharide/endotoxin, the TLR-4 ligand, in MGN-3/Biobran (100 μg/mL) was carried out by BioChem GmbH. In addition, we quantified LPS/endotoxin by chromogenic assay (ToxinSensor Chromogenic LAL Endotoxin Assay Kit, GenScript). Functional *in vitro* cytotoxicity assays were performed against K562 and NB1691 cell lines as targets using LPS (10 ng/mL) as an NK cell stimulus and polymyxin B (100 μg/mL) as an inhibitor of LPS-induced activation of TLR-4. Finally, we performed cytotoxic assays against the NB1691 cell line using MGN-3/Biobran-stimulated NK cells with polymyxin B inhibition.

### Statistical analysis

Results are shown as means ± SD. Non-parametric Wilcoxon tests were used to compare MGN-3/Biobran effect on NK cell phenotype, cytotoxicity and expansion rate. In the mouse model, survival was estimated by the univariate Kaplan-Meier method and compared using the log-rank test. Statistical significance was defined as $P < 0.05$.

## Results

### NK phenotyping

The addition of MGN-3/Biobran-stimulated NK cells resulted in an increase in CD69 and CD25 expression from a median of 9%−88% and 6%−90%, respectively (MFI ratio increased 3.1-fold and 3.2-fold, respectively). The percentages and MFI of the other receptors studied was unchanged. IL-15-stimulated NK cells, used as a positive control, increased the median expression of CD25 significantly (6%−92%, MFI ratio increased 3.9-fold), CD69 (9%−98%, MFI ratio increased 35.5-fold), NKG2D (92%−97%, MFI ratio increased 2.1 fold), DNAM (81% to 96%, MFI ratio increased 1.7 fold) and NKp30 (54 to 81, MFI increased 3.8 fold). Table I and Figure 2A and B show the response of activating receptors on NK cells to overnight MGN-3/Biobran and IL-15 stimulation.
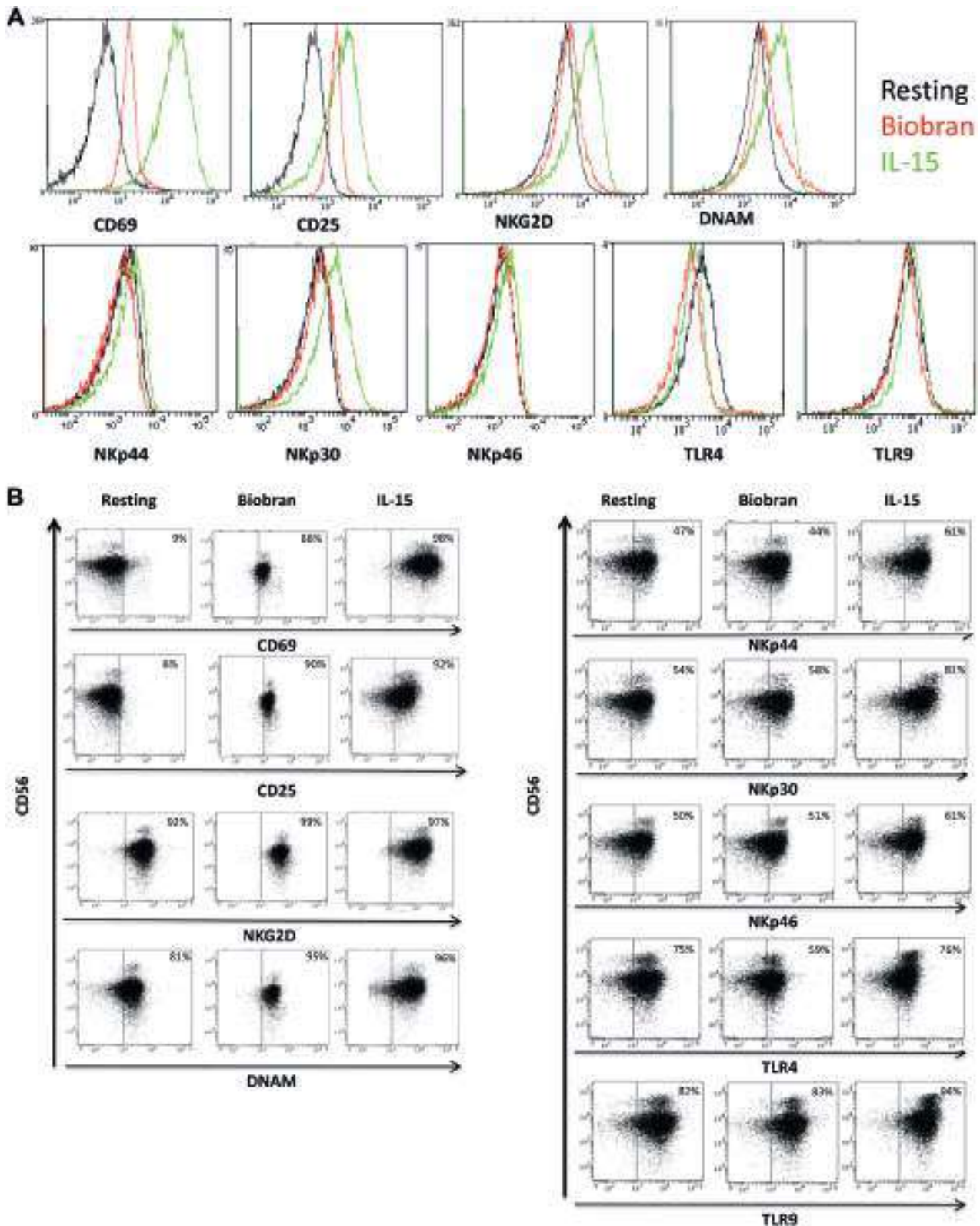
Figure 2. (A) Mean fluorescence intensity from activating NK cell receptors: at rest (black), MGN-3/Biobran (red) and IL-15 stimulated (green) in 3 healthy controls. (B) Percentages of activation markers expression on resting, MGN-3/Biobran- and IL-15-stimulated NK cells.

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*In vitro cytotoxicity assays*

Overnight stimulation with MGN-3/Biobran resulted in a significant increase in NK cell cytotoxicity against all tested cell lines at an E/T ratio of 8:1 (K562, NB1691, Jurkat, A673) or 10:1 (A-204, RD, RH-30) compared with resting NK cells (Figure 3A, K562 80% vs. 69%, $P = 0.03$, NB1691 41% vs. 23%, $P = 0.03$, Jurkat 40% vs. 19%, $P = 0.03$, A673 34% vs. 13%, $P = 0.02$, A204 34% vs. 18%, $P = 0.03$, RD 45% vs. 22%, $P = 0.002$, RH-30 34% vs. 18%, $P = 0.02$). Stimulation with IL-15 led to even higher percentages of lysis of the K562 (100%), NB1691 (61%), Jurkat (60%) and A673 (58%) cell lines (Figure 3B). To test the synergistic effect of IL-2 and MGN-3/Biobran, we compared stimulation with high-dose IL-2 (1000 IU/mL) with low dose IL-2 (40 IU/mL) and low dose IL-2 + MGN-3/Biobran. Adding MGN-3/Biobran to low dose IL-2 further enhanced the stimulatory effect of 40 IU/mL IL-2 and resulted in comparable cytotoxicity to that obtained with 1000 IU/mL IL-2 (Figure 3C). To test the safety profile of MGN-3/Biobran-stimulated NK cells, we performed cytotoxicity assays on negative controls (autologous CD56 negative cells), which revealed an absence of cytotoxicity (supplementary Figure 2 and supplementary Table I for reviewers).

*In vivo model*

To examine whether the stimulated effect of MGN-3/Biobran on NK cells *in vitro* has clinical significance, we then extended our investigation to an *in vivo* xenograft model of luciferase-transfected neuroblastoma. Figure 4A shows ventral and dorsal bio-images of 3 representative mice receiving PBS (control), $1 \times 10^6$ unstimulated NK cells and $1 \times 10^6$ MGN-3/Biobran-stimulated NK cells. There was a dramatic progression of the NB1691 tumors in the control group and unstimulated NK cell group, whereas significant neuroblastoma growth inhibition was observed in the cohort that received $1 \times 10^6$ MGN-3/Biobran-stimulated NK cells (Figure 4B and supplementary Table II for reviewers). We also observed that MGN-3/Biobran-stimulated NK cells significantly increased survival in the NOD/scid/IL-2Rγnull-hu model ($P < 0.05$; Figure 4C).

*Role of MGN-3/Biobran in expansion of NK cells*

After 2 weeks of culture, NK cells expanded more strongly when MGN-3/Biobran was added to the culture medium (supplementary Table III). In contrast, T-cell expansion was decreased when MGN-3/Biobran was added to the culture medium (Figure 5A). MGN-3/Biobran addition to IL-2
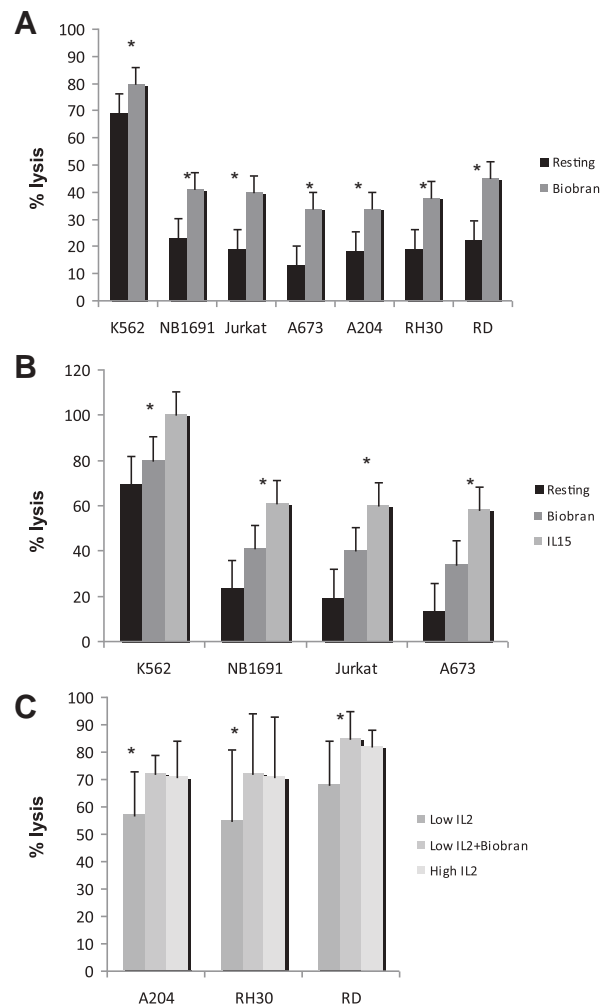


Figure 3.  (A) MGN-3-stimulated NK cell cytotoxic activity against K562, NB1691, Jurkat and A673 cell lines (effector/target ratio 8:1), A-204, RD and RH-30 (effector/target 10:1). (B) IL-15 and MGN-3-stimulated NK cell cytotoxic activity against K562, NB1691, Jurkat and A673 cell lines (effector/target ratio 8:1). (C) IL-2- and MGN-3-stimulated NK cell cytotoxic activity against A-204, RD and RH-30 (effector/target 10:1). Data include results from 3 healthy volunteers in 3 independent experiments. *Statistically significant.

and IL-2 + IL-15 cultures did not produce a statistically significant difference in NKT cells and B cells. The cytotoxic activity of expanded NK cells did not significantly change when MGN-3/Biobran was added to the culture medium (Figure 5B). In contrast, the addition of IL-15 enhanced cytotoxicity compared with IL-2 alone, even when using transfected K562 cell line.

*Mechanisms of MGN-3/Biobran stimulation on NK cells*

Because human NK cells can be stimulated by TLRs, we tested TLR triggering by MGN-3/Biobran using human macrophages as biosensors to identify the optimal dose of MGN-3/Biobran to stimulate NK cells without stimulate macrophages. Only high
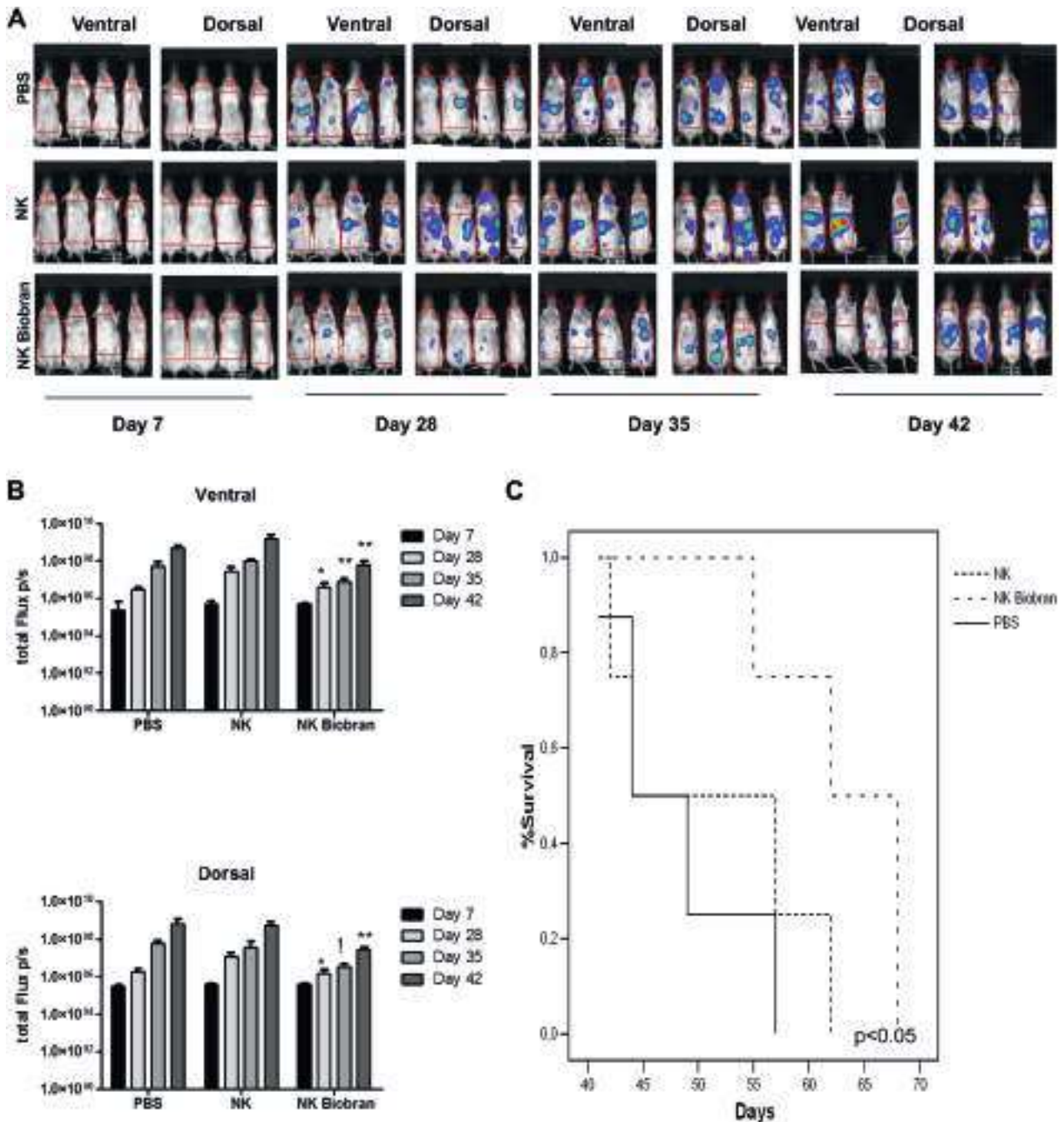
Figure 4. (A) Imaging of NB1691 tumors expressing luciferase illustrates the neuroblastoma burden in mice receiving PBS (control), $1 \times 10^6$ freshly isolated unstimulated NK cells and $1 \times 10^6$ MGN-3-stimulated NK cells (given by 8 intravenous injections, twice a week for 4 consecutive weeks). Three representative mice, ventral and dorsal, from each group are shown. ROI, region of interest. (B) The fold tumor volume relative to baseline values was significantly lower in the cohort of mice that received MGN-3-stimulated NK cells than in either the control group or the group that received resting NK cells. (C) Kaplan-Meier curves indicate the survival of each group of mice. [1]Statistically significant compared with control group. [*]Statistically significant compared with unstimulated NK cells group. [**]Statistically significant compared with both groups.

levels of MGN-3/Biobran (10 mg/mL) resulted in the release of IL-8, IL-6 and TNF-α (4776, 164 and 132 and pg/ml, respectively), see Figure 1. These measurements were significantly lower than those observed with LPS (10 ng/mL) stimulation (7487, 362 and 208 pg/mL, respectively).

We observed traces (Eu/mL = 1.68) of LPS contamination in MGN-3 Biobran in a limulus amebocyte lysate (LAL) assay. To investigate the role of LPS contamination of MGN-3/Biobran as a mechanism of stimulation, we determined *in vitro* cytotoxicity assays against NB1691. These assays

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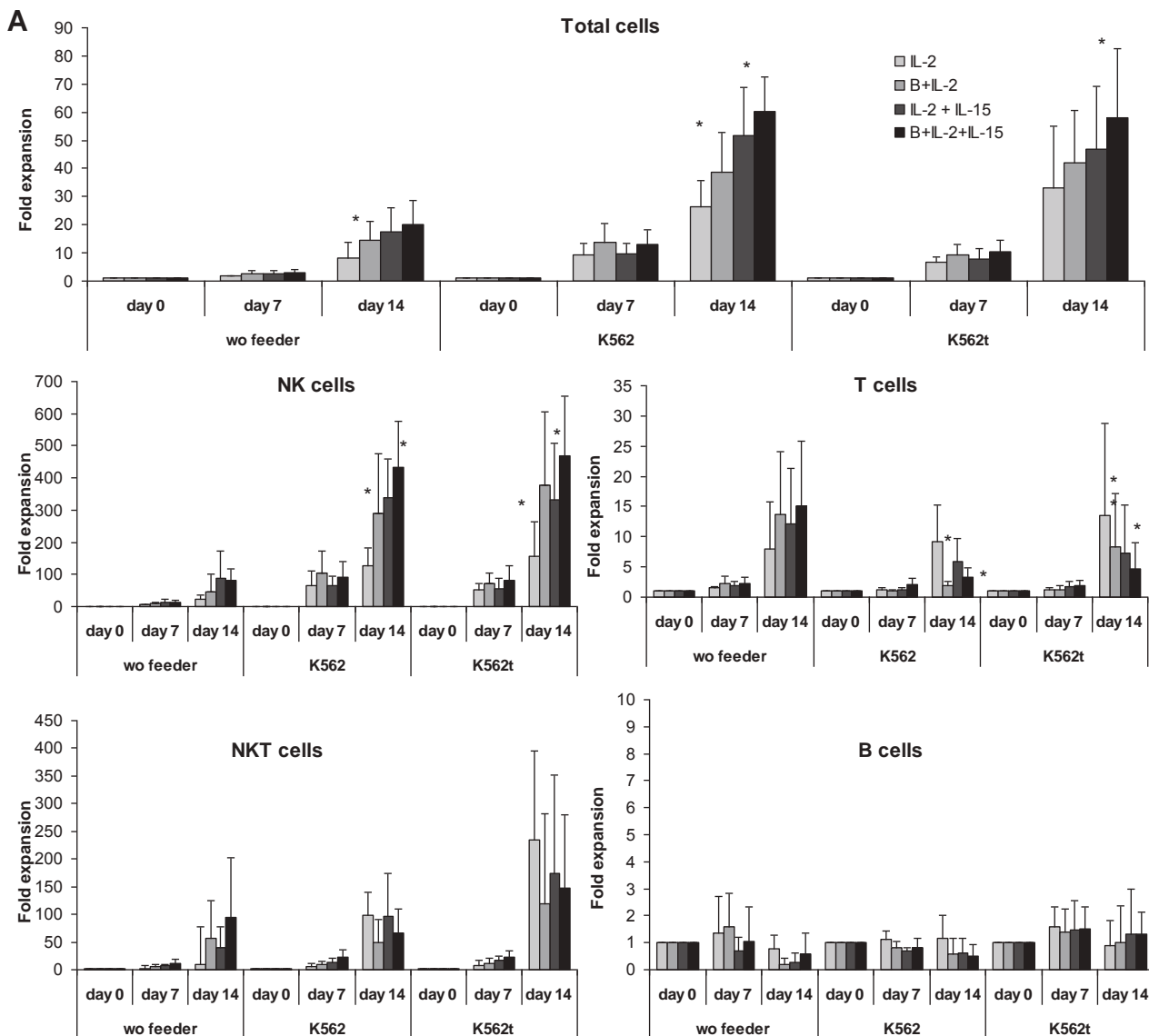


Figure 5. (A) Kinetics of total cells, NK cells, T cells, NKT cells and B cells after 14 days of culture expansion from 5 healthy donors with and without (wo) MGN-3/Biobran and cytokines (IL-2 or IL-2 + IL-15) or co-culture with irradiated feeder cells consisting of K562 cells or K562-mb15-41BBL. *Statistically significant. (B) Cytotoxic activity of expanded NK cells adding MGN-3/Biobran to the culture medium.

showed increased cytotoxic activity of LPS-stimulated NK cells compared with resting NK cells, whereas polymyxin B abrogated the effect of LPS stimulation (Figure 6A). In contrast, the stimulating effect of MGN-3/Biobran on NK activity against NB1691 could not be antagonized with polymyxin B (Figure 6B). The mechanism of NK stimulation is not mediated by LPS contamination in MGN-3/Biobran.

**Discussion**

Published findings have shown that the use of MGN-3/Biobran in cancer therapy can improve outcomes in some adult cancer patients [30,31]. A clinical trial of adult patients with hepatocellular carcinoma

showed that the addition of MGN-3/Biobran to interventional therapies including transarterial chemoembolization, percutaneous ethanol injection, radiofrequency ablation and cryoablation improved overall survival [21]. It has also been reported that the addition of MGN-3 stimulated innate immunity in multiple myeloma patients by increasing NK cell cytotoxic activity, levels of myeloid DCs and concentrations of T helper cell type 1−related cytokines [32]. There is no reported data regarding the use of MGN-3/Biobran with pediatric tumors.

Our study shows that MGN-3/Biobran stimulation of NK cells improved both *in vitro* and *in vivo* cytotoxic activity against various pediatric tumor cell lines. We demonstrated increased NK cell mediated killing

**B**
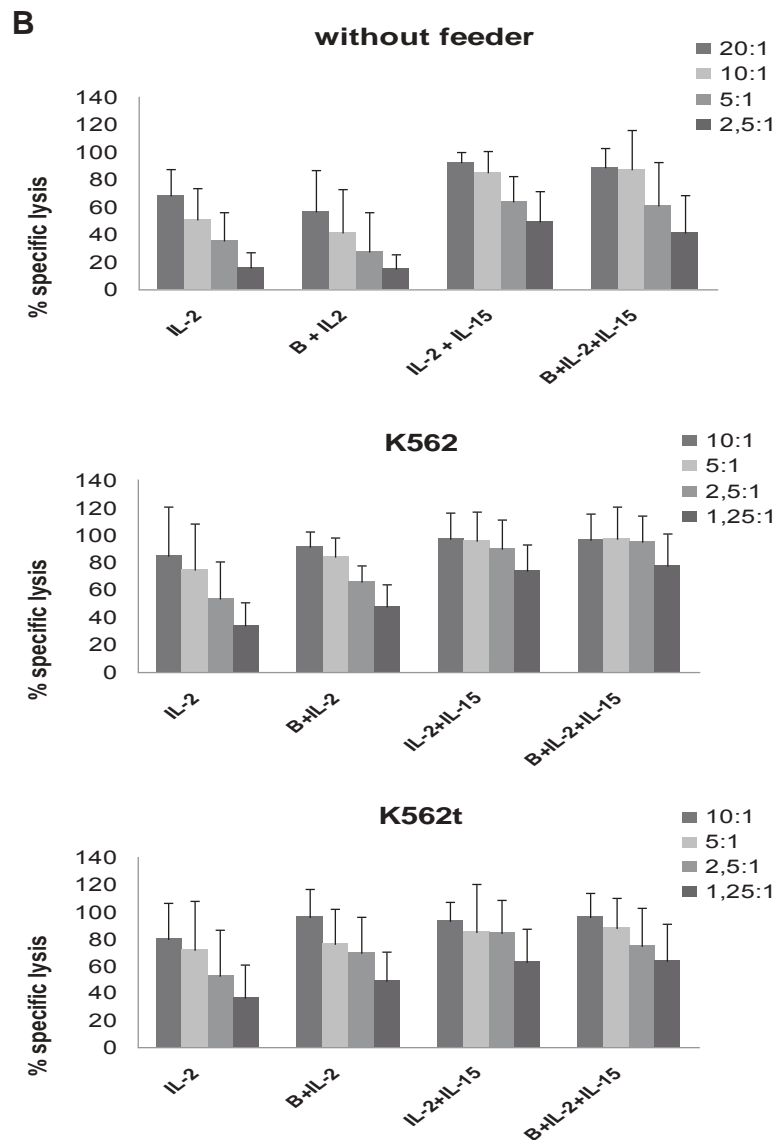


Figure 5. (*continued*).

of acute leukaemia, neuroblastoma, Ewing sarcoma, embryonic rhabdomyosarcoma and alveolar rhabdomyosarcoma cell lines *in vitro* after stimulation with MGN-3/Biobran. We also observed a significant inhibition of neuroblastoma growth and a significant improvement in survival in a NOD/scid/IL-2Rγnull neuroblastoma model when using MGN-3/Biobran-stimulated NK cells. These data are in agreement with previous data published on adult malignancies [20−25].

The mechanism and dose by which MGN-3/Biobran increases NK cell activity remains unknown. We suggest that a variety of immune mechanisms may be involved in the beneficial effect observed with MGN-3/Biobran treatment of NK cells. Because high doses of MGN-3/Biobran resulted in modifying macrophages from M0 to M1

releasing IL-6, IL-8 and TNF-α, we considered a low dose of MGN-3/Biobran to remove the NK cell activation caused by an inflammatory background. Because TLR agonists can stimulate human NK cells, we hypothesized that LPS contamination in MGN-3/Biobran could increase NK cell cytotoxicity by TLR-4 signaling. In our study, a small amount of LPS contamination was observed. However, neutralizing LPS with polymyxin B did not abrogate the stimulating effect of MGN-3/Biobran on NK activity, suggesting that LPS contamination is not the mechanism through which MGN-3/Biobran stimulates NK cells. According to our data, MGN-3/Biobran appears to activate resting NK cells, but it is unable to further activate cells undergoing expansion with IL-15, despite augmenting expansion

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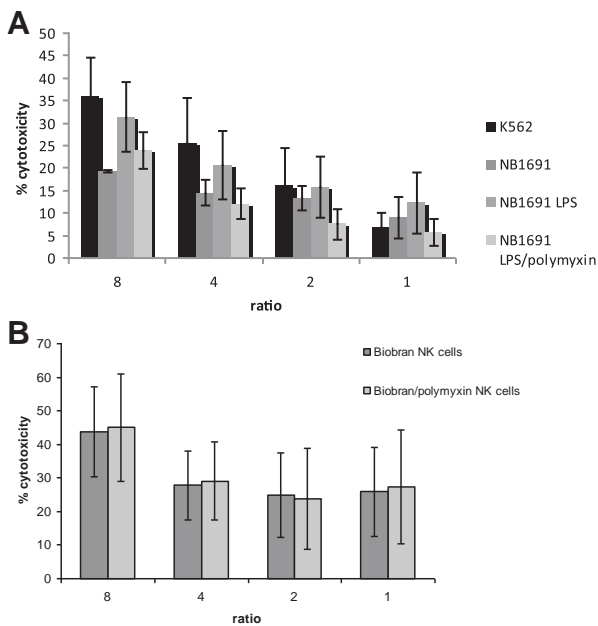


Figure 6. (A) LPS-stimulated NK cells increase resting NK cell cytotoxicity against NB1691 and polymyxin B abrogates LPS stimulation to a resting state. (B) MGN-3/Biobran stimulates NK cells against NB1691 that is not antagonized by polymyxin B.

during this period. This outcome suggests a mechanism that partially overlaps with IL-15. Another theory is an apoptotic effect mediated by activation of NK cells releasing TNF-α and IFN-γ [30,33]. This theory is supported by recently reported data that the addition of MGN-3/Biobran to chemotherapy had a synergistic effect, as evidenced by enhanced apoptosis and cell proliferation inhibition in breast cancer cells [34]. Another possible mechanism could be the augmentation of activating receptors on NK cells stimulated with MGN-3/Biobran. We observed an increase of the activation-associated receptors CD69 and CD25 on MGN-3/Biobran-stimulated NK cells of healthy donors. CD69 elevation on NK cells correlates with an increase in NK cell cytotoxicity [35−37]. In addition, proliferative potential is indicated by CD25 expression elevation on NK cells [38]. Lastly, MGN-3/Biobran interaction with other immune cells has been also reported [39,40].

The adoptive transfer of *in vitro*−activated NK cells is currently used for cancer therapy. Recent studies have demonstrated that NK cells can be expanded to large numbers *ex vivo* using various methods, including using K562-mb15-41BBL as feeder cells [28]. These expanded NK cells exerted antitumor activity *in vitro* on a variety of cell lines and malignancies including adult and pediatric cancers [41−43]. When we added MGN-3/Biobran in various expansion protocols, we observed an improvement in the expansion of NK cells, a retention of cytotoxic activity and a reduction in T-cell proliferation. These data could be important for

large-scale expansion of highly cytotoxic clinical-grade NK cells, especially in an allogeneic setting where T cells should be removed to avoid graft versus host disease. Additionally, using MGN-3/Biobran in combination with low-dose IL-2 increased NK cell cytotoxic activity to the same level as high dose IL-2. These data are in accordance with earlier studies [44]. Therefore, MGN-3/Biobran and low dose IL-2 act synergistically and this approach can avoid toxicities related to high dose IL-2 treatment *in vivo*.

Data from adult patient studies have suggested that the use of MGN-3/Biobran as an alternative or adjuvant treatment to various immunotherapeutic approaches may be beneficial in the treatment of malignancy [20,22,24]. Our results extend to the pediatric patient population, as demonstrated by an increase in NK cell cytotoxic activity with the addition of MGN-3/Biobran against a variety of pediatric tumors *in vitro* and neuroblastoma *in vivo*. We also observed that the addition of MGN-3/Biobran increased NK cell expansion/activation and, in combination with a low-dose of IL-2, has a beneficial effect on activating NK cells for the purpose of immunotherapy against neuroblastoma. Further studies are warranted in the pediatric clinical setting to elucidate the role of MGN-3/Biobran in combination with chemo-immune protocols.

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## Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcyt.2014.11.001.

# Modulation of the anticancer immunity by natural agents: inhibition of T regulatory lymphocyte generation by arabinoxylan in patients with locally limited or metastatic solid tumors

Research Article

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## Summary

In the last years, several immunomodulating antitumor agents have demonstrated in the nature, particularly from Aloe plant and rice bran. However, the major problem concerning the natural antitumor agents is to define their immune mechanisms of action in relation to the more recent advances in tumor immunobiology. At present, the main cause responsible for the lack of an effective antitumor response in advanced cancer patients is belived to be represented by the generation of a subtype of T helper lymphocytes (CD4$^+$) with suppressive activity on anticancer immunity, the so-called T regulatory lymphocytes (T reg), which may be clinically identified as CD4$^+$CD25$^+$ cells. On this basis, a study was planned to evaluate the effect of rice bran extract arabinoxylan on T reg cell count and percentage in solid tumor patients in relation to the various lymphocyte subpopulations. The study included 22 evaluable cancer patients, 16 of whom had an untreatable metastatic solid tumor. Arabinoxylan was given orally at a dose of 2000 mg/day for the first month, followed by a dose of 1000 mg/day for the next month. In each patient we evaluated by monoclonal antibodies the absolute number of lymphocytes, T lymphocytes (CD3$^+$), T helper (TH) lymphocytes (CD4$^+$), T cytotoxic lymphocytes (CD8$^+$), NK cells (CD16$^+$CD56$^+$), T reg lymphocytes (CD4$^+$CD25$^+$) and TH/T reg ratio before and after 2 months of therapy. No substantial change occurred on therapy in the mean number of lymphocytes, CD3$^+$, CD8$^+$ and NK cells. On the other hand, the mean number of TH cells increased, whereas that of T reg cell decreased on treatment, even though none of these differences was statistically significant. On the contrary, TH/T reg mean ratio significantly enhanced after arabinoxylan therapy. In addition to its previously demonstrated stimulatory action on NK function, this study shows that arabinoxylan may inhibit the production of T reg cells, which are responsible for cancer-related immunosuppression, with a following improvement in the anticancer immunity. If further studies will confirm these results, arabinoxylan could be successfully associated with chemotherapy to induce not only a cytotoxic destruction of cancer cells, but also an improvement in the immune status.

# I. Introduction

The recent advances in the definition of the mechanisms responsible for tumor progression have suggested the possibility to control cancer growth not only trough chemotherapy-induced cancer cell destruction, but also by stimulating the anticancer immunity. In addiction to the exisence of endogenous antitumor molecules, several agents capable of stimulating the anticancer immunity have alsso isolated from plants. However, the immunomodulatory effects of most natural immunomodulating agents need to be better investigated in an attempt to establish their mechanisms of action in relation to the most recent discoveries concerning the physiopathology of the anticancer immunity. At present, Aloe extracts (Lissoni et al, 1998) and arabinoxylan extract from rice bran (Ghoneum and Jewett, 2000) would represent some of the potential natural agents which could be utilized in the complementary therapy of human neoplasms. Today, it is known that the antitumor immune response is the end-result of several interactions involving cytokines and immune cells, provided by stimulatory or suppressive effects on the anticancer immunity (Atzpodien and Kirchner, 1990; Rosenberg, 1992). Therefore, the lack of an effective anticancer immune response in most cancer patients with advanced disease would simply depend on the prevalence of immunosuppressive mechansisms with respect to the immunostimulatory ones (Atzpodien and Kirchner, 1990). The anticancer immunity is mainly activated by T helper-type 1 lymphocytes by releasing IL-2 (Whittington and Faulds, 1993), and by dentritic cells, which act as antigen-presenting cells producing IL-12 (Banks et al, 1995), T cytotoxic lymphocytes and NK-LAK system, which are involved in the induction of the antigen-dependent and antigen-independent cytotoxicity, respectively (Atzpodien and Kirchner, 1990). Therefore, IL-2 and IL-12 would represent the main anticancer cytokines in humans. On the contrary, the suppression of the anticancer immune response is mediated by several cytokines, namely IL-10 (Moore et al, 1993), IL-6 (Matsuda and Hirano, 1990) and TGF-β (Shevach, 2002). Recently, however, it has been demonstrated that the various endogenous suppressive factors would exert their inhibitory immune effect through a common end-mechanism, consisting of the generation of a subtype of T helper lymphocytes (CD4$^+$cells), provided by a fundamental suppressive activity on the anticancer immunity, the so-called T regulatory lymphocyte (T reg) (Dieckmann et al, 2001), which at present seems to constitute the main mechanism responsible for cancer-related immunosuppressive status. T reg cells may be identified by the simultaneous expression of the alpha-chain of IL-2 receptor (CD25) and CD4 antigen (Dieckmann et al, 2001). Then, T reg cells may be clinically recognized as CD4$^+$CD25$^+$ lymphocytes. Therefore, each eventual natural immunomodulating agent would have to be investigated in relation to its possible effect on T reg generation since, at least from a theoretical point of view, each natural agent capable of counteracting T reg activity could positively influence the prognosis of the neoplastic disease by improving the efficacy of the anticancer immune response. Moreover, our previous preliminary studies have suggested that the percentage of T reg cells with respect to the total number of T helper cells, as expressed as CD4/CD4CD25 ratio, may represent an optimal synthetic immune index to investigate the functional status of the anticancer immunity in the single cancer patient, by representing the synthesis of the actions of the great number of immunostimulating and immunosuppressive factors involved in the modulation of the anticancer immunity (Dieckmann et al, 2001). Within the great number of natural agents derived from plants and potentially usefull to be employed in the complementary therapy of cancer, arabinoxylan would seem to represent one of the potential natural agent, because of its efficacy in improving the clinical status of cancer patients (Ghoneum and Jewett, 2000; Ghoneum and Gollapudi, 2005; Markus et al, 2006; Ghoneum et al, 2007). The immunomodulating properties of this nautral substances extracted from plants have been confirmed by experimental studies, but unfortunately most experiments have been limited to the investigations of they effects on non-specific immune parameters for the anticancer immunity, such as NK cell cytotoxicity. In contrast, since reg cells play a fundamental role in suppressing the generation of the anticancer immunty, each potential antitumor immunomodulatory natural substances, would have to be investigated also in relation to their eventual influence on T reg cell system. On the basis of the recent discoveries in tumor immunobiology (Dieckmann et al, 2001; Shevach, 2002), a study was planned to investigate the possible influence of arabinoxylan on both absolute number of T reg cells and their ratio with respect to the total CD4$^+$ T cells in a group of solid tumor patients, affected by locally limited or metastatic disease.

## II. Materials and methods

The study included 24 consecutive patients, 18 of whom had a metastatic solid tumor, which did not respond to the conventional anticancer chemotherapies and for whom no other effective standard treatment was available, while the remaining 6 patients had been surgically treated for a locally limited neoplasm. Patients were followed at Biological Medical Institute of Milan and the protocol was approved by the Director of the Institute. Eligibility criteria were, as follows:histologically proven locally limited or metastatic solid tumor, no double tumor, no chronic therapy with corticosteroids because of their immunosuppressive effects and no concomitant treatment with other immunomodulating agents,such as interferons,interleukins and monoclonal antibodies. At the time of the start of arabinoxylan therapy, patients with untreatable metastatic cancer were under treatment with the only supportive care, consisting of anti-inflammatory agents for pain, anti-dopaminergic drugs for nausea and vomiting and with the pineal hormone melatonin for the therapy of the neoplastic cachexia (Banks et al, 1995). Patients were considered as fully evaluable when they had received arabinoxylan therapy for at least 2 consecutive months. Arabinoxylan was given orally at a dose of 1000 mg twice/day for the first month, followed by a dose of 1000 mg/day for the next month. Arabinoxylan was supplied by DAIWA Pharmaceutical (Tokyo, Japan). It was derived from rice bran treated enzymatically with an extract of the shiitake mushrooms. It is a polysaccharide containing β-1,4-xylopironase hemicellulose, commercially available and known as Biobran. For the immune investigation, venous blood samples were collected in the morning after an overnight fast before the onset

of arabinoxylan therapy and after 2 consecutive months of treatment. In each blood sample, we evaluated the absolute number of total lymphocytes, T lymphocytes (CD3^{+}), T helper (TH) lymphocytes (CD4^{+}), T cytotoxic lymphocytes (CD8^{+}), NK cells (CD16^{+} CD56^{+} and T regulatory (T reg) lymphocytes (CD4^{+} CD25^{+}). The different lymphocyte subsets were measured with a flow cytometric assay by using specific monoclonal antibodies supplied by Becton-Dickinson (Milan, Italy). Moreover, because of the importance not only of their absolute number, but also of their percentage with respect to the other lymphocyte subsets, namely to that of CD4^{+} cells, CD4/CD4CD25 ratio, corresponding to TH/T reg ratio, was also determined before and after therapy. Normal values (95% confidence limits) of T reg number and TH/T reg ratio observed in our laboratory were below 240/mm^{3} and above 4.0, respectively. Data were reported as mean +/- SE and statistically analyzed by the Student's t test, the analysis of variance and the chi-square test, as appropriate.

## III. Results

Evaluable patients were 22/24, while the remaining 2 patients, both affected by untreatable disseminated liver metastases due to colorectal cancer, rapidly died for disease progression before concluding the two planned months of arabinoxylan therapy. The clinical characteristics of the evaluable patients are reported in **Table 1**. **Figure 1** illustrates changes in the mean number of total lymphocytes, T lymphocytes, T cytotoxic lymphocytes and NK cells occurring after 2 months of arabinoxylan therapy. No substantial variation was found in the mean number of lymphocytes, T lymphocytes, T cytotoxic lymphocytes and NK cells under arabinoxylan treatment. In contrast, as illustrated in **Figure 2,** TH and T reg mean numbers increased and decreased, respectively, after arabinoxylan therapy, without, however statistically significant differences with respect to the values seen prior to therapy. On the contrary, a statistically significant increase in TH/T reg mean ratio was achieved after

arabinoxylan therapy ($p<0.025$). The increase in TH/T reg ratio under arabinoxylan therapy was more pronounced in patients with an abnormally low ratio prior to therapy with respect to that occurring in those with normal pre-treatment ratio, however without statistically significant differences ( 2.3 +/- 0.4 *vs* 1.7 +/- 0.5). In more detail,

**Table 1.** Clinical characteristics of 22 evaluable cancer patients treated by arabinoxylan.

| Characteristics | n |
|---|---|
| M/F | 14/8 |
| Median Age (years) | 62 (24-82 ) |
| Median performance status (Karnofsky's score) | 90 (70-100) |
| Tumor histotypes: | |
| colorectal cancer | 6 |
| lung cancer | 4 |
| prostate cancer | 4 |
| breast cancer | 3 |
| renal cell cancer | 2 |
| pancreatic cancer | 2 |
| soft tissue sarcoma | 1 |
| Disease extension: | |
| Locally limited disease | 6 |
| Metastatic disease | 16 |
| bone | 2 |
| lung | 5 |
| liver | 3 |
| lung + liver | 3 |
| brain | 2 |
| peritoneum | 1 |
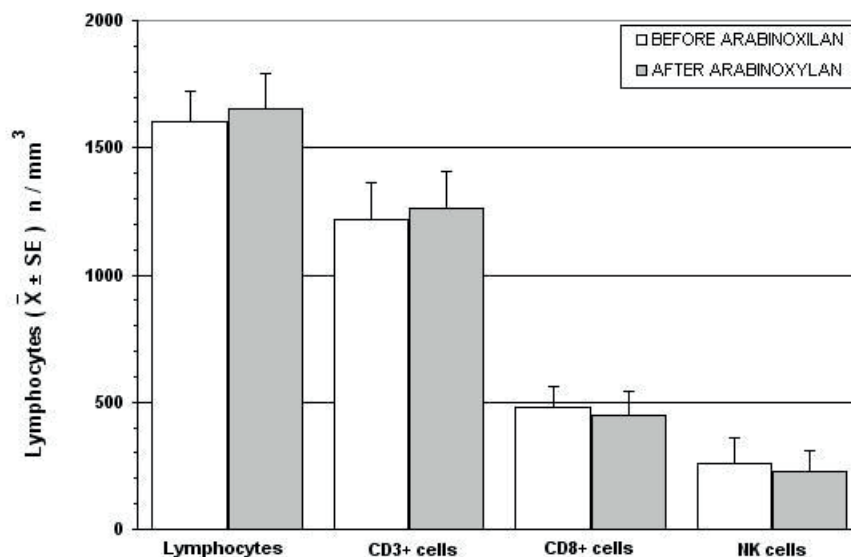


**Figure 1.** Changes in the number of lymphocytes, Tlymphocytes (CD3), T cytotoxic lymphocytes (CD8) and NK cells (CD16 CD56) after 2 months of arabinoxylan therapy.
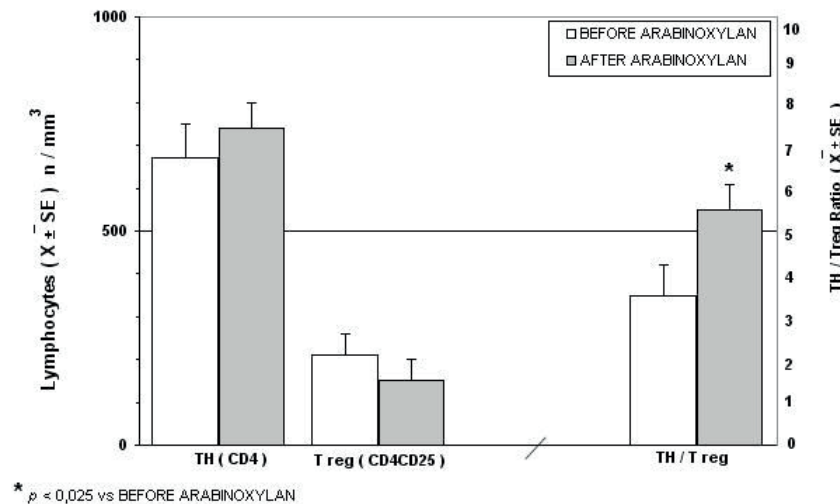
**Figure 2.** Changes in the mean number of T helper (TH) lymphocytes (CD4) and T regulatory lymphocytes (cd4 cd24) and in TH/T reg mean ratio.

before arabinoxylan therapy,an abnormally low TH/T reg ratio was present in 12/22 (55%) evaluable patients. Arabinoxylan treatment induced a normalization of TH/T reg ratio in 5/12 (42%) patients with an abnormally low ratio prior to therapy. The percentage of arabinoxylan-induced TH/T reg normalization obtained in lymphocytopenic patients was not significantly different from that achieved in patients with normal pre-treatment lymphocyte count ( 3/7(43%) vs 2/5(40%) ). No toxicity was observed under arabinoxylan treatment, which was well tolerated in all patients. Asthenia was present in 8/22 (36%) evaluable patients. An evident relief of asthenia, as assessed by a specific patient report, was obtained under arabinoxylan therapy in 5/8 (63%) patients.

## IV. Discussion

Previous experimental studies had already demonstrated some immunomodulating properties of arabinoxylan, in particular consisting of stimulation of NK cytotoxic function (Ghoneum, 1998), whereas NK cell number did not seem to be influenced by arabinoxylan administration. However, it has to be remarked that NK cells were belived to be fundamental in the antitumor immunity until some years ago, before the discovery of the essential role played by the antitumor cytokines, such as IL-2 and IL-12 (Whittington and Faulds, 1993) and dendritic cells, because of their function as antigen-presenting cells (Banks et al, 1995). In fact, it has to be considered that the cytotoxic activity of NK cells is effective only against artificial laboratory cancer cell lines, whose biological malignant properties are different from those presented by fresh human tumor cells (Whittington and Faulds, 1993). In addition, NK cells have been proven to be also able to destroy fresh human cancer cells only after the activation of their cytotoxic function by IL-2 (Atzpodien and Kirchner, 1990). From this point of view, arabinoxylan had been already proven to amplify the stimulatory effect of IL-2 on NK-mediated antitumor cytotoxicity (Ghoneum and Jewett, 2000). In contrast, no study has been performed up to now

to evaluate the possible influence of arabinoxylan not only on the mechanisms responsible for the generation of an effective anticancer immune response, but also on those involved in the suppression of anticancer immunity. The results of this preliminary study, carried out to evaluate the influence of arabinoxylan on T reg cells, which represent the most important cells involved in the suppression of the antitumor cytotoxic immune response, demonstrates that arabinoxylan may counteract T reg cell generation by reducing their number and percentage with respect to the total amounts of CD4+ cells and circulating lymphocytes. Since NK cell function is inhibited by T reg activation (Shevach, 2002), the previously demonstrated arabinoxylan-induced stimulation of NK cell cytotoxic function might depend at least in part on its capacity of counteracting T reg generation (Dieckmann et al, 2001). Moreover, this study would suggest that the inhibitory action of arabinoxylan on T reg generation is more pronounced in patients with an abnormally high percentage of T reg cells prior to therapy, with a following pre-treatment abnormally low TH/T reg ratio before therapy, whereas its effect was less evident in patients with a pre-treatment value of TH/T reg ratio within the normal range. Therefore, the influence of arabinoxylan on T reg generation would consist of a modulatory action rather than an inhibitory activity. This finding could explain a potential favourable immunomodulatory effect of arabinoxylan also in patients with autoimmune diseases (Ghoneum, 1998), who in contrast to cancer patients would tend to present abnormally low amounts of T reg cells. In any case, the importance of the inhibition of T reg generation in the induction of an effective anticancer immune response has been recently confirmed by the evidence that the block of T reg activity by specific monoclonal antibodies may induce objective tumor regressions in humans (Yang et al, 2007). Obviously, the major problem is the exact identification of he T reg cell population. Even though T reg cells may express other immune markers, namely FOX-p2 cytoplasmatic antigen, most clinicians are in agreement to identify the CD4+CD25+ cells as T reg

lymphocytes (12). In any case, further studies, by evaluating other immune markers, will be required to better identify T reg cells population, namely FOX-p3, even though recently some Authors have shown that FOX-p3 expression by T reg cells is associated with a lower suppressive activity (Dieckmann et al, 2001; Shevach, 2002). Moreover, it has to be remarked that several patients included in the present study were concomitantly under palliative therapy with the anti-cachectic pineal hormone melatonin (Brzezinski, 1997), which may also play immunomodulating effects (Maestroni, 1993). Therefore, further randomized studies with arabinoxylan alone versus arabinoxylan plus melatonin will be required to better define the immunomodulating action of arabinoxylan. If further clinical and experimental studies will confirm the inhibitory action of arabinoxylan on T reg cell system, it could be included in cytokine-based immunotherapies to enhance their efficacy by counteracting T reg cell generation.

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# Arabinoxylan Rice Bran (MGN-3) Enhances the Effects of Interventional Therapies for the Treatment of Hepatocellular Carcinoma: A Three-year Randomized Clinical Trial

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**Abstract.** *Background and Aims: This study examined the efficacy of arabinoxylan rice bran (MGN-3) in conjunction with an interventional therapy (IT) for the treatment of hepatocellular carcinoma patients. Patients and Methods: A total of sixty-eight patients with hepatocellular carcinoma (stages I and II) participated in the study. Patients were randomized to receive IT (30 patients, control group) or IT+MGN-3 (38 patients), and randomly divided into two groups using a computer-generated randomization list. Patients and investigators were blinded. IT included transarterial oily chemoembolization (TOCE) or a combination of TOCE and percutaneous ethanol injection treatment (PEIT). Results: Patients in the IT+MGN-3 group showed: (i) lower recurrence of the disease, 31.6% (12/38), as compared to 46.7% (14/30) for the control; (ii) higher survival after the second year, 35%, as compared to 6.7% for the control; (iii) significantly lower alpha-fetoprotein level, a 38% decrease (p=0.0001), as compared to baseline value, while the control showed no significant change; and (iv) a significant decrease in tumor volume, in contrast to the control, which showed no significant change. When the results were analyzed according to each IT modality, MGN-3+IT sub-groups displayed a greater response to treatment, in every aspect examined, than the IT sub-groups alone. However, the patients in the MGN-3+TOCE+PEIT sub-group demonstrated greater reduction in AFP levels and longer survival time than the MGN-3+TOCE sub-group. Conclusion: MGN-3 in conjunction with IT may be useful for the treatment of hepatocellular carcinoma and warrants further investigation in multiple clinical trials.*

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide with an estimated 626,000 new cases per year (1, 2). The prognosis for this cancer is poor, with a median survival time of less than six months. The etiology of HCC may be related to three major epidemiological factors, namely hepatitis B and C infection, aflatoxin exposure and cirrhosis (1, 3). Interventional therapies (ITs) include transarterial oily chemoembolization (TOCE), percutaneous ethanol injection therapy (PEIT), radiofrequency ablation (RFA) and cryoablation (4). However, the survival of patients who undergo these therapies is still limited to only two to three years (5). Although it has been suggested that TOCE is an effective treatment for inoperable HCC, an enhanced survival has not yet been validated in randomized trials. Nonetheless, it remains a widely used palliative treatment for HCC cases that are not amenable to resection or ablative therapies. Similarly, several non-randomized studies have demonstrated a beneficial effect of transarterial chemoembolization (TACE), though this was also not confirmed in randomized trials (6). In addition, the side-effects and complications of TACE treatment are severe (7). PEIT has gained wide acceptance as a treatment for HCCs, but is applicable only to a minority of cases.

The poor prognosis for HCC patients coupled with the low efficacy of the available treatments highlights the necessity to find new treatments, or to modify current modes of IT. The present study examined whether combining IT, namely TOCE, PEIT and RFA, with MGN-3, an arabinoxylan rice

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bran, may increase the survival rate of HCC patients. MGN-3 has been demonstrated to be a potent biological response modifier (BRM) (8-11) and a chemo-sensitizer that sensitizes cancer cells to both death receptor (CD95)-induced apoptosis (12) and to daunorubicin treatment (13). The results demonstrated that HCC patients given IT+MGN-3 showed a higher percent survival than the patients given IT alone.

## Patients and Methods

*MGN-3*. MGN-3 is a denatured hemicellulose that is obtained by reacting rice bran hemicellulose with multiple carbohydrate hydrolyzing enzymes from Shiitake mushrooms. It is an arabinoxylan with a xylose in its main chain and an arabinose polymer in its side chain (8). Patients were treated with MGN-3 at a dose of 1g per day aliquoted in packets that were taken orally with meals for 12 months simultaneously with IT (see below). MGN-3 was provided by Daiwa Pharmaceuticals Co. Ltd., Tokyo, Japan and is commonly known as Biobran or Lentin Plus 1000.

*Patients*. Sixty-eight patients (54 males, 14 females) with HCC, aged 30-68 years, participated in the study. The patients were admitted to the 108 Military Central Hospital in Hanoi, Vietnam and were randomly divided into two groups using a computer-generated randomization list: the IT group and the IT+MGN-3 group. Both patients and investigators were blinded. The IT group (30 patients, age 51±17 years: 24 males, 6 females) was treated with IT alone, while the IT+MGN-3 group (38 patients, age 49±19 years: 30 males, 8 females) was treated with IT+MGN-3 for three years (age values represent mean±standard deviation).

Informed consent was obtained from all participants. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in the a priori approval by the 108 Military Central Hospital General Secretary of VASLD Hanoi, Vietnam and by the Institutional Review Board at Charles Drew University of Medicine and Science, Los Angeles, CA, USA.

Prior to treatment, clinical characteristics, tumor characteristics and IT modalities were determined for the patients in each group. The clinical characteristics of the HCC patients prior to treatment were investigated for hepatitis infection and alpha-fetoprotein (AFP) levels. The number of patients with hepatitis B and/or C infection for the IT group was 26 and for the IT+MGN-3 group was 35. The number of patients with AFP levels in three categories (<20, 20-500, and >500 ng/ml) were 11, 7, and 12, respectively, for the IT group and 12, 10 and 16, respectively, for the IT+MGN-3 group. Further clinical findings for HCC patients prior to treatment were examined. The numbers of patients for each symptom in the two treatment groups (IT and IT+MGN-3, respectively) were as follows: fatigue (19 *vs.* 23), right upper-quadrant pain (22 *vs.* 27), fever (5 *vs.* 5), digestive disorder (3 *vs.* 5), weight loss (8 *vs.* 9), jaundice (2 *vs.* 3), hepatomegaly (11 *vs.* 15) and no symptoms (4 *vs.* 4). The tumor characteristics of the HCC patients were documented prior to treatment with respect to the number of tumors, tumor site, size and differentiated levels. Table I summarizes the number of patients in the IT group and the IT+MGN-3 group for each of the aforementioned characteristics. Notably, the majority of patients in each group had only one tumor, which was located in the right lobe with a tumor diameter greater than 3 cm. The differentiation levels of the tumors were

characterized as well, moderate and poor in each of the groups. The number of patients in each category was not significantly different between the two groups (*p*>0.05).

*Interventional Therapies (IT)*. IT modalities for the HCC patients included PEIT, TOCE, and RFA. The number of patients in the two groups (IT and IT+MGN-3, respectively) for each modality was as follows: TOCE (11 *vs.* 13), TOCE+PEIT (14 *vs.* 20), PEIT (3 *vs.* 3), TOCE+RFA (2 *vs.* 2). The number of patients in each modality was not significantly different between the two groups (*p*>0.05). The treatment protocols were dependent on tumor size, the outcome after first treatment and the time to recurrence. For TOCE, adriamycin (20-60 mg/treatment; mean=40 mg/treatment) was mixed with lipiodol (5-20 ml/treatment; mean=12 ml/treatment) and patients were treated every 1-2 months (2-5 treatments/patient; mean=3 treatments/patient). PEIT treatment consisted of ethanol injections (99.5%; 3-15 ml/treatment; mean=8 ml/treatment) given twice per week (mean=6 treatments/patient). For tumors with radius r under 2.5 cm, the total ethanol volume (V) was calculated according to the following formula: $V=4/3\pi(r+0.5)^3$ (14). For RFA treatment, the radiofrequency energy was emitted at a power setting of 60 W for five minutes, 2-4 cycles per treatment, 1-2 times/week. For tumors smaller than 2 cm in length, the patients were treated once; for tumors with length 2-4 cm, the patients were treated 2-3 times, while for larger tumors, the patients were treated 3-5 times. RF data were not included due to the small sample of patients.

*Outcome assessment*. Patients response to treatment was determined by assessing overall response to treatment, AFP levels, tumor volume, recurrence, and survival.

*Overall response to treatment*. Patients were assessed for overall response to treatment. A positive response was defined when the patient did not feel fatigued, had a good appetite, had no pain in the liver area, had no fever and a weight gain of 3-5 kg. In addition, for positive response, physical examination would reveal a decrease in liver size and no signs of jaundice or ascites. Patients who did not experience any significant changes in energy levels, pain, fever, weight, jaundice or ascites were placed in the no-response group. An adverse response was defined as a response to treatment opposite of the aforementioned positive response. In addition, most patients in the adverse response group experienced an increase in tumor size or recurrence, as observed *via* imaging, and developed metastasis.

*AFP levels*. Patient AFP levels were analyzed at six-month intervals for 36 months using an AFP Elisa Kit.

*Tumor volume*. Tumor volumes were determined at six-month intervals for 36 months using computerized tomography (CT) scanning, and the final tumor measurements are displayed in Table IV.

*Recurrence*. The secondary outcome measurement was recurrence. Patients were examined for recurrence at six-month intervals for 36 months. If patients had previously experienced disease remission, the presence of tumors was considered recurrent disease. Data were presented as the percentage of patients with recurrent disease.

*Survival*. The primary outcome measurement was survival and was examined in 2 to 3-month intervals for 36 months.

Table I. *Tumor characteristics of patients before treatment.*

| Characteristics | IT | | IT+MGN-3 | |
|---|---|---|---|---|
| | n=30 | % | n=38 | % |
| Number of tumors | | | | |
| Number of patients with 1 tumor | 23 | 76.7 | 31 | 81.6 |
| Number of patients with 2 tumors | 7 | 23.3 | 7 | 18.4 |
| Total number of tumors | 37 | 100 | 45 | 100 |
| Site of tumor | | | | |
| Right lobe | 28 | 75.7 | 32 | 71.1 |
| Left lobe | 5 | 13.5 | 7 | 15.6 |
| Both | 4 | 10.8 | 6 | 13.3 |
| Size (diameter) | | | | |
| <3 cm | 4 | 10.8 | 5 | 11.1 |
| 3-6 cm | 20 | 54.1 | 23 | 51.1 |
| > 6 cm | 13 | 35.1 | 17 | 37.8 |
| Differentiaton | | | | |
| Well | 9 | 24.3 | 11 | 24.5 |
| Moderate | 22 | 59.5 | 24 | 53.3 |
| Poor | 6 | 16.2 | 10 | 22.2 |

Table II. *Effect of treatment on AFP levels (whole groups). Data represent the mean AFP level. % Change=100 × [(value after treatment) – (value before treatment)] / (value before treatment).*

| Treatment | Tumor volume (cm$^3$) | Patients | | AFP level (ng/ml) | | % Change |
|---|---|---|---|---|---|---|
| | | Number | % | Before treatment | After treatment | |
| IT | ≤200 | 17 | 57 | 292.8 | 316.5 | +8% |
| | >200 | 13 | 43 | 431.7 | 456.1 | +6% |
| | 10-1320† | 30 | 100 | 353.0 | 376.97 | +7% |
| IT+MGN-3 | ≤200 | 22 | 58 | 509.0 | 356.8 | −30% |
| | >200 | 16 | 42 | 511.4 | 260.4 | −49% |
| | 12-1200† | 38 | 100 | 510.0 | 316.2 | −38% |

†Range of tumor volumes in group.

*Statistical analysis.* Descriptive statistics were used to characterize the subjects (mean, median and standard deviation for continuous variables, and percentages for categorical variables). The chi-square test for categorical variables was used to test the statistical difference between the groups. Significant differences between the groups for the continuous variables were determined using the non parametric median test, Mann Whitney *U*-test, and Wilcoxon test for the non normally distributed data and by two-sided *t*-tests, and paired *t*-tests for normally distributed data when appropriate. The Kaplan-Meier procedure was used to estimate time to death. The Kaplan-Meier method estimated the conditional probabilities at each time point when death occurred and, by taking the product limit of those probabilities, it estimated the survival rate at each point in time. *P*-values <0.05 were considered significant. Data were analyzed using SPSS version 15 (SPSS, Inc., Chicago, IL, USA).

Table III. *Effect of treatment on AFP levels (sub-groups). Data represent the mean AFP level. % change=100 × [(value after treatment) - (value before treatment)] / (value before treatment).*

| Treatment | Number of patients | AFP level (ng/ml) | | % Change |
|---|---|---|---|---|
| | | Before treatment | After treatment | |
| TOCE | 11 | 433.5 | 360.9 | −16% |
| TOCE+MGN-3 | 13 | 726.1 | 506.9 | −30% |
| TOCE+PEIT | 14 | 242.2 | 382.6 | +58% |
| TOCE+PEIT+MGN-3 | 20 | 450.0 | 218.9 | −51% |

Table IV. *Effect of treatment on tumor volume (whole groups). Data represent mean±standard deviation. % Change=100 × [(value after treatment) – (value before treatment)] / (value before treatment).*

| Treatment | Tumor volume (cm$^3$) | Patients | | Treatment | | % Change |
|---|---|---|---|---|---|---|
| | | Number | % | Before | After | |
| IT | ≤200 | 17 | 57 | 92.7±12.3 | 97.6±35 | +5% |
| | >200 | 13 | 43 | 460.4±76.1 | 454.9±106.7 | −1% |
| | 10-1320† | 30 | 100 | 252.0±258.7 | 252.5±324.3 | +0.2% |
| IT+MGN-3 | ≤200 | 22 | 58 | 125.9±13.6 | 94.1±22.3 | -25% |
| | >200 | 16 | 42 | 481.7±56.6 | 288.3±47.9 | −40% |
| | 12-1200† | 38 | 100 | 275.7±234.1 | 175.8±174.9 | −36% |

†Range of tumor volumes in group.

## Results

*Response to IT treatment.* Figure 1 shows that 89% of patients in the IT+MGN-3 group and 80% of patients in the IT group demonstrated a positive response to treatment. In contrast, 5% of patients in the IT+MGN-3 group experienced adverse side-effects compared to 13% for the IT group (*p*<0.05).

*AFP levels.* AFP levels in HCC patients were examined before and after treatment according to the total tumor volume, and the patients were clustered into sub-groups with tumor volume ≤200 cm$^3$ or >200 cm$^3$ in the IT group and the IT+MGN-3 group, and the final AFP level measurements are displayed in Table II. Using the non-parametric Mann Whitney *U*-test, the IT group as a whole showed an increase in APF levels of 7% (*p*=0.2), relative to the before treatment value of APF. Specifically, an increase in APF levels ranging from 6 to 8% was noted in patients with a total tumor volume ≤200 cm$^3$ or >200 cm$^3$. In contrast, the IT+MGN-3 group showed a significant decrease of APF levels with a

Figure 1. Overall response to treatment. The patients in IT and IT+MGN-3 groups were examined for an overall positive response, no response or adverse response to treatment. Parameters measured included patient fatigue, appetite, liver pain, liver size, fever, weight gain or loss, jaundice and ascites. Response classification is described under Patients and Methods. *p<0.05 and **p<0.01, as compared to the IT group.



Figure 3. Spiral multi-slice CT scan and angiograms of a IT+MGN-3 patient. A: A multi-slice CT scan before treatment showed that the patient had a large tumor in right liver measuring 14×13 cm² in plane. B: After six months of treatment the CT scan showed a decrease in tumor size to 5×6 cm² in plane. C: Angiography showed liver artery and portal angiography of the same patient before treatment. D: The angiography taken just after treatment with TOCE showed that the right liver artery had complete embolization.



Figure 2. Multi-slice CT scans of an IT+MGN-3 patient. A: Before treatment, the patient had a large tumor in right liver measuring 12×10 cm² in plane. B: At three months after treatment, the tumor size decreased to 7×8 cm² in plane and had a very high lipiodol marking (arrows). C: At six months after treatment, the tumor size had decreased further to 6×7 cm² in plane with lower lipiodol marking, showing liver regeneration.

Table V. Effect of IT modality on tumor volume (sub-groups). % Change=100 × [(value after treatment) – (value before treatment)] / (value before treatment).

| Treatment | Number of patients | Treatment Before | After | % Change |
|---|---|---|---|---|
| TOCE | 11 | 272.8 | 262.5 | −4% |
| TOCE+MGN-3 | 13 | 301.5 | 175.5 | −42% |
| TOCE+PEIT | 14 | 303.4 | 313.4 | +3% |
| TOCE+PEIT+MGN-3 | 20 | 349.5 | 222.4 | −36% |

change of 38% relative to values before treatment (p<0.001). In particular, a significant decrease of AFP levels ranging from 30 to 49% (p<0.05) was observed in patients with tumor volumes ≤200 cm³ and >200 cm³ (Table II).

The patients were also sub-grouped according to the IT modality and the changes in AFP levels post-treatment are illustrated in Table III. Patients in the TOCE sub-group demonstrated a 16% decrease in AFP, while patients in the TOCE+MGN-3 sub-group experienced a 30% decrease in AFP. Furthermore, patients in the TOCE+PEIT group had a 58% increase in AFP, while those in the TOCE+PEIT+MGN-3 sub-group demonstrated a 51% decrease in AFP (p<0.01).

Tumor volume. Tumor volumes were examined in HCC patients post-treatment and the results were categorized into two sub-groups according to the whole tumor volume, ≤200 cm³ or >200 cm³. Surprisingly, the IT group had almost no change in tumor volume (0.2%). In contrast, the IT+ MGN-3 group had an overall reduction of tumor volume by 36% (Table IV). Specifically, patients in the IT+MGN-3 group with tumor volumes ≤200 cm³ and >200 cm³ experienced a significant decrease in average tumor volume by 25% (p<0.05) and 40% (p<0.05), respectively. However, the patients in the corresponding IT groups showed no significant change in tumor

Figure 4. *Recurrence of tumors in HCC patients post-treatment for the two groups compared as a whole (IT and IT+MGN-3).*
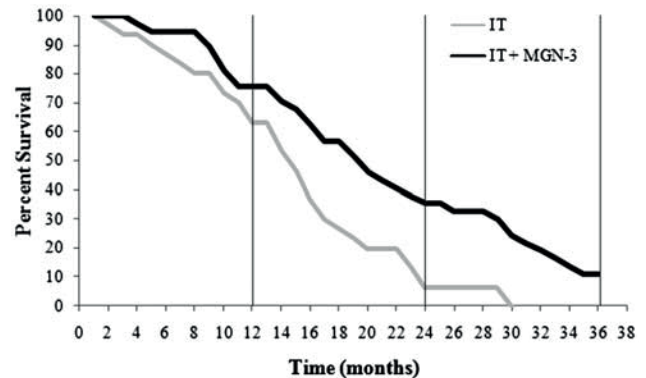


Figure 5. *Percent survival in HCC patients post-treatment for the two groups compared as a whole (IT and IT+MGN-3).*

volume. Using the Mann Whitney *U*-test, patients in the IT+MGN-3 group also showed a significant change in tumor volume compared with the control group ($z=2.5$ and $p=0.01$).

The patients were also sub-grouped according to the IT modality and the changes in tumor volume post-treatment are illustrated in Table V. The patients in the TOCE sub-group demonstrated a 4% decrease in tumor volume, while those in the TOCE+MGN-3 sub-group experienced a 42% decrease in tumor volume ($p=0.01$). In addition, patients treated with TOCE+PEIT showed a 3% increase in tumor volume, while those in the TOCE+PEIT+MGN-3 sub-group demonstrated a 36% decrease in the tumor volume ($p<0.01$).

*Imaging studies.* HCC tumors were analyzed using multi-slice CT and angiography. Imaging results from representative patients of the IT+MGN-3 group are shown in Figures 2 and 3. The multi-slice CT scan in Figure 2 clearly demonstrates that the tumor mass steadily decreased from a size of $12\times10$ cm$^2$ to $6\times7$ cm$^2$ within a period of six months. The angiogram in Figure 3 shows a decrease in tumor size, from $14\times13$ cm$^2$ to $4\times5$ cm$^2$ in a period of six months.

*Recurrence.* Tumor recurrence in the IT and IT+MGN-3 groups was examined every six months for three years. As shown in Figure 4, the recurrence in the IT+MGN-3 group was decreased and maximized at 30 months post-treatment (32%) compared to the IT group (47%) at the same time point. When the patients were sub-grouped according to the IT modality, the statistical analysis demonstrated an insignificant difference in tumor recurrence between the TOCE and TOCE+MGN-3 sub-groups. However, 43% of patients in the TOCE+PEIT sub-group experienced recurrence, compared to 20% in the TOCE+PEIT+MGN-3 sub-group.

*Survival.* Percentage survival was assessed every 2-3 months in the HCC patients. As shown in Figure 5, patients in the IT group demonstrated a sharp decline in survival. While 63% had survived at the end of the first year, this number dropped significantly to 6.7% by the end of the second year and at 30 months no patients remained alive. In contrast, the IT+MGN-3 group maintained significantly higher survival rates, being 76% at the end of the first year, 35% at the end of the second year, and 11% at the end of the third year. Half of the IT+MGN-3 group survived at 20 months compared with 15 months for the IT group. When the patients were sub-grouped according to the IT modality, the patients in the TOCE sub-group survived for an average of 14.8 months as compared to 16.5 months for the TOCE+MGN-3 sub-group. Notably, patients treated with TOCE+PEIT survived an average of 14.0 months as compared to 23.8 months for the TOCE+PEIT+MGN-3, showing, therefore, an increase in survival time by 10 months.

## Discussion

The inability of IT to cause significant necrosis of cancer cells and the low survival rates among HCC patients (3-5%) reflects the inadequacy of ITs in treating this disease (15-17). Therefore, many attempts have been made to improve the efficacy of IT. For example, the use of interferon-alpha (IFN-α) in combination with IT was a focus of many studies, however the results were contradicting. While some studies reported that treatment with 5-FU+IFN-α enhances the survival of patients with advanced HCC (18-20), other studies reported that such treatment is not beneficial for advanced HCC (21, 22). Additionally, other BRMs such as PSK, lentinan, and OK-432, have been shown to have no effect when combined with 5-FU for the treatment of HCC (23).

Realizing the need for further studying on the potential of combination BRM treatments for HCC, the present study

investigated IT in combination with MGN-3. MGN-3 has been previously demonstrated to be a potent BRM (8-11), and may inhibit the production of T regulatory (T reg) cells responsible for cancer-related immunosuppression (24). Furthermore, MGN-3 has demonstrated anticancer activity (11, 25) and improved the survival in cancer patients treated simultaneously with MGN-3 and anticancer drugs (26).

In the current study, the overall results showed that the addition of MGN-3 to IT enhances its anti-neoplastic effect to cause necrosis, and causes a beneficial effect in terms of tumor response rates, recurrence and survival. When the results were analyzed for each IT modality separately, the IT+MGN-3 sub-groups displayed a greater response to treatment, in all aspects compared to the respective IT sub-groups. Notably, the patients in the MGN-3+TOCE+PEIT sub-group demonstrated a greater reduction in AFP levels and longer survival times than those in the MGN-3+TOCE sub-group. However, recurrence, survival, and tumor volume did not significantly differ between the TOCE and TOCE+PEIT sub-groups, suggesting that the combination of the two IT modalities did not increase anticancer efficacy. The exact mechanism underlying the potent anticancer effect of the IT+MGN-3 combinations are not yet fully understood.

Possible mechanisms of MGN-3 action on HCC may be attributed to its immune-modulatory effects. There is much evidence suggesting a major role of the natural killer (NK) cells and dendritic cells in the immune surveillance of neoplastic disease including HCC (27-29). The role of MGN-3 in the potent activation of NK cell activity in cancer patients (30), healthy humans (9, 31) and mice (10) is well documented and was associated with growth suppression of different murine and human malignancies, including HCC (11, 25, 26). MGN-3-induced NK cell activation was mediated through an increase in the NK cell granular content (10) and the expression of key NK cell surface receptors, including CD69, CD25 and ICAM-1 (CD54) (31). Recent studies also showed that MGN-3 is a potent enhancer of human dendritic cell maturation *in vitro* (32) and can suppress T reg lymphocytes in patients with locally limited and metastatic solid tumors including those in the liver (24).

The earlier studies carried out by Suto *et al.* (23) showed no change in survival time or tumor diameter when using the various BRMs with 5-FU. Our contrasting results show changes in survival and tumor growth when treated with MGN-3 +IT. This suggests that the immune modulatory effect by MGN-3 is more potent than previously studied BRMs. In addition, MGN-3 may exert its effect on HCC *via* sensitizing the cancer cells to TOCE and PEIT. Earlier studies showed that MGN-3 has the ability to sensitize human leukemia cells to both death receptor (CD95)-induced apoptosis (12) and human breast cancer cells to the chemotherapeutic agent daunorubicin (13).

Treatment with MGN-3 may reduce the side-effects of TOCE. Earlier studies showed a potential for MGN-3 in reducing chemotoxic effects. These include the protection of animals against gross pathological changes and weight loss produced by chemotherapy (33, 34). Additionally, studies in humans with progressive cancer showed that MGN-3 prolongs life span and improves the quality of life (26). MGN-3 has been shown to be a nontoxic agent and is safe for human consumption (9, 35).

In conclusion, HCC therapy that includes MGN-3 represents a new approach with great clinical potential that could be used in conjunction with other IT modalities to enhance treatment efficacy. The present study provided a rationale for further future clinical studies of this combination of therapies.

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ORIGINAL ARTICLE

# MGN-3 arabinoxylan rice bran modulates innate immunity in multiple myeloma patients

**Dana Cholujova · Jana Jakubikova · Branislav Czako · Michaela Martisova · Luba Hunakova · Jozef Duraj · Martin Mistrik · Jan Sedlak**

**Abstract** Dendritic cells (DCs) and natural killer (NK) cells are central components of innate immunity for controlling tumor growth. The therapeutic effects of certain anti-myeloma drugs are partially mediated by targeting the innate immune response. In addition, novel types of natural compounds have been developed that efficiently modulate the activity of both the cellular and humoral compartments of immunity. MGN-3 is known as an activator of natural killer cells, inducer of apoptosis and cytokine production, and modulator of dendritic cell maturation and differentiation in vitro. We have performed a randomized, placebo-controlled study to examine the effects of MGN-3 on innate immune system parameters in 48 multiple myeloma patients. We performed immunophenotypic analysis of peripheral blood samples, determined NK cell activity, and assessed the cytokine profiles of plasma before and during 3 months of treatment. The results demonstrate a clear increase in NK activity in MGN-3-treated patients compared to the placebo group, an increased level of myeloid DCs in peripheral blood, and augmented concentrations of T helper cell type 1-related cytokines. The present study suggests that MGN-3 may represent an immunologically relevant product for activating innate immunity in multiple myeloma patients and warrants further testing to demonstrate clinical efficacy.

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## Introduction

Different cell types within the bone marrow (BM), including cells of the immune system, mesenchymal stem cells, and BM stromal cells, can contribute to the development of the disease multiple myeloma (MM). Patients with MM suffer from a generally diminished immune capacity that is likely due to the generation of a suppressive environment. Dendritic cells are central to innate and adaptive immunity because they interact with cells from both systems. DCs are pivotal for T cell priming, but tumor burden can impair DC differentiation, causing them to become dysfunctional [1]. A lack of stimulation within the tumor environment as well as defects in DC differentiation may be responsible for the low activity of the immune system [2]. In MM patients at diagnosis, the levels of myeloid DCs (mDC) and plasmacytoid DCs (pDC) are reduced compared to control donors, but normal mDC levels tend to be restored upon remission [3]. The interaction between DCs and NK cells can trigger NK cell activation. Importantly, DCs also require external stimuli to trigger NK cells [4] because immature DCs are poor inducers of interferon (IFN)-$\gamma$ secretion by NK cells. Once activated, NK cells can either kill or promote DC maturation, depending on the DC/NK cell ratio [5].

Myeloma cells themselves can affect host immunity. These cells prime DCs toward a maturation state that favors the generation of T cells with regulatory rather than

effector phenotypes [6]. In contrast to normal plasma cells, primary MM cells express CD1d antigen and can activate invariant natural killer T (iNKT) cells [7]. In MM patients, a marked polarization toward T helper cell type 2 (Th2) cytokines exists, while T helper cell type 1 (Th1) cytokines remain suppressed [8]. Both interleukin (IL)-6 and IL-10 enhance the proliferation of MM cells [9]. The MM prognostic marker $\beta$2-microglobulin is a negative regulator of the immune system, and high $\beta$2-microglobulin concentrations inhibit the generation of functional DCs in vitro [10].

A significant proportion of common chemotherapeutic agents used at sub-cytotoxic concentrations augment the stimulatory capacity of DCs [11]. A preclinical study has demonstrated that the novel pan-histone deacetylase inhibitor LBH589 decreased the production of cytokines during Toll-like receptor-induced DC activation and significantly impaired the phenotype and function of DCs [12]. Drugs can influence other cell types present in the tumor microenvironment in addition to DCs. Gemcitabine specifically reduces the number of myeloid suppressor cells found in the spleens of animals bearing large tumors [13]. The new anti-MM drug lenalidomide augments the anti-tumor effect of iNKT cells in part by increasing Th1 and reducing Th2 cytokine production [7]. Lenalidomide and thalidomide abrogate the stimulatory effect of stromal cells and significantly decrease the percentage of stem-like clonogenic MM cells [14]. The small-molecule pharmacologic agent bortezomib is a proteasome inhibitor that has received FDA approval for the treatment of MM, which has subsequently been extended to other hematologic malignancies. Proteasome inhibitors decrease the presentation of antigenic peptides and reduce tumor cell recognition by cytotoxic T cells but unexpectedly increase tumor cell recognition by NK cells [15]. The interaction between the activating NK cell receptors and their ligands represents a crucial part of the innate immune response against several malignancies, including MM. Although drug-induced potentiation of NK cell-mediated lysis is accompanied by an enhancement of ligand expression [16–18], the potentially suppressive effect of ligand upregulation on cytotoxic activity in MM should be considered [19]. The curcumin derivative FLLL32 selectively inhibits STAT3 phosphorylation and STAT3 DNA binding, reduces cell viability, and induces apoptosis in multiple myeloma and other carcinoma cancer cells with constitutively activated STAT3 signaling [20]. Recently, a synergistic apoptosis-inducing potential of rice bran arabinoxylan and curcumin was observed in the human MM cell line U266 [21].

Previous MGN-3 research has suggested that this compound may have immunomodulatory properties, mainly by acting on NK cells and enhancing their activity [22, 23]. We have shown that MGN-3 induces the maturation of human monocyte-derived DCs in vitro [24]. In this randomized placebo-controlled study, we evaluated the potential modulatory effects of MGN-3 on innate immune system parameters in patients with MM.

## Materials and methods

### Patients

For MM diagnoses, BM aspirates were assessed to determine the percentage of BM plasma cells by morphology and electrophoresis of serum for the presence of monoclonal immunoglobulin (Ig). The pretreatment evaluation included complete blood counts, biochemical tests for renal and liver function, and an analysis of $\beta$2-microglobulin and C-reactive protein. A radiologic skeletal survey was performed to assess the presence of bone disease. The study was approved by the University Hospital ethics committee, consistent with the Helsinki Declaration on the use of human subjects for research. The patients were diagnosed and treated at the Hospital of St. Cyril and Method (Bratislava, Slovakia). All patients gave written informed consent. A total of 48 patients with a diagnosis of MM (of which 27, i.e., 56 %, presented the IgG subtype) were evaluated before and after MGN-3 treatment in this randomized, double-blind, placebo-controlled study (one-third of recruited patients received placebo), and their baseline characteristics are summarized in Table 1. The treated patients were given alternating courses of chemotherapy based on a combination of alkylating agents (melphalan, cyclophosphamide), anthracyclines (doxorubicin, idarubicin), and glucocorticoids (dexamethasone). Patients that were positive for monoclonal Ig, exhibited less than 20 % myeloma plasma cells in the BM aspirate, and were negative for CRAB criteria did not receive chemotherapy treatment and were under observation. The patients received 2 g per day of MGN-3 granule powder or an equivalent amount of placebo dissolved in water. The contents of the placebo and MGN-3 sachets were indistinguishable in taste and appearance. The patients were monitored for 1 week before treatment to obtain the baseline values of all of the analyzed parameters, followed by 3 months of treatment. Peripheral blood (15 ml) was collected for analysis in heparin-containing tubes every 4 weeks.

### MGN-3

MGN-3 is a nutritional supplement derived from rice bran hemicellulose that has been enzymatically treated with multiple hydrolyzing enzymes from Lentinus edodes mycelia (Shiitake mushrooms). The active component is an

**Table 1** Baseline characteristics of patients in placebo and MGN-3 group

| | MGN-3 group (N = 32) | Placebo group (N = 16) |
|---|---|---|
| Age, years median (range) | 65 (36–82) | 63 (50–80) |
| Sex ratio, male/female | 10/22 | 7/9 |
| *Myeloma subtype* | | |
| IgG | 17 | 10 |
| IgA | 7 | 2 |
| IgM | 1 | |
| Light chains | 2 | 2 |
| Non-secretory | 3 | 1 |
| NA | 2 | 1 |
| *Stage* | | |
| Durie–Salmon disease stage I/II/III | 3/8/17 | 2/5/9 |
| Solitary plasmacytoma | 2 | |
| NA | 2 | |
| *Treatment* | | |
| Without CHT, patients under observation | 11 | 6 |
| CHT only before MGN-3 | 7 | 3 |
| CHT during MGN-3 | 14 | 7 |

*NA* not analyzed, *CHT* chemotherapy

arabinoxylan that contains a xylose in its main chain and an arabinose polymer in its side chain. MGN-3 was provided by Daiwa Pharmaceuticals Co. Ltd, Tokyo, Japan.

Flow cytometry-based cytotoxicity assay

Effector peripheral blood mononuclear cells (PBMC) were isolated from the samples collected in heparin-treated tubes by Pancol (1.077 g/ml, PAN-Biotech, Aidenbach, Germany) density gradient centrifugation. The mononuclear cells from the interface were collected, washed twice with PBS, and resuspended in 10 ml of complete culture medium (CM; RPMI 1640 medium, 10 % heat-inactivated FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) before use in the cytotoxicity assay. Target K-562 cells, an erythroleukemia cell line, were maintained in CM and split every 3–4 days. For the assays, the cells were washed once with PBS and loaded with 0.1 μM calcein probe (CAM; Molecular Probes, Eugene, OR, USA) in FCS-free RPMI medium for 15 min at 37 °C in the dark.

To determine the absolute number of viable and dead cells, equal volumes (100 μl) of Flow-Count Fluorospheres (Beckman Coulter, Brea, CA, USA) and cells were mixed and analyzed with a Coulter Epics Altra flow cytometer.

The target and effector cell viability was determined by propidium iodide (PI) negativity (4 μl PI per sample, 1 mg/ml stock). Only populations with a viability of >95 % were further analyzed. The cell concentrations were calculated according to the following formula: cells/μl = (counts$_{viable cells}$ × concentration$_{beads}$)/(counts$_{beads}$).

The NK cytotoxic activity in PBMCs from MM patients was determined against target K-562 cells using the CAM assay as previously described [25]. Briefly, CAM-labeled target K-562 cells were mixed with effector PBMCs to obtain six twofold serial dilutions of the *E/T* ratios beginning at 50:1. Triplicates of the samples and controls were seeded in 96-well V-bottomed microplates, centrifuged at $200 \times g$ for 3 min, and incubated in CM for 3 h at 5 % $CO_2$, 37 °C. Next, the samples were transferred into cytometric tubes, and PI (4 μl of a 1 mg/ml stock per sample) was added to identify dead cells. The samples were analyzed with a Coulter Epics Altra four-color flow cytometer equipped with an argon laser operating at 488 nm. Gating was performed using side scatter (SSC; ordinate) versus the log-scale green fluorescence of the CAM probe (abscissa) to separate target cells from effector cells. To measure target cell death, green fluorescence-positive events were gated, and the PI positivity was analyzed. An average of 3,000 target cells were collected per sample. The data were analyzed with FCS Express software (De Novo Software, Los Angeles, CA, USA).

The percentage of specific lysis (PSL) was calculated at each *E/T* ratio as follows: % specific lysis = (CT–TE/CT) × 100 (where CT is the percentage of viable fluorescent target cells in the control tubes and TE is the percentage of viable fluorescent target cells in the experimental (target + effector) tubes). A lytic unit (LU) is defined as the number of effector cells required to lyse 20 % of a predetermined standard number (TSTD = $2 \times 10^4$) of target cells. The LU calculation was performed by fitting the curve on a semi-log$_2$ plot of the logarithmically transformed *E/T* values versus the specific lysis, according to Bryant et al. [26] and Pross et al. [27]. The results are reported as the number of LUs in $10^7$ effector cells.

Immunophenotypic analysis of the cells

The following mouse antihuman monoclonal antibodies (mAbs) were used to analyze mDCs, pDCs, and NK cell subsets in the peripheral blood of MM patients: CD11c-FITC, CD16-FITC, CD123-PE, CD56-PE, HLA-DR-ECD, CD45-ECD, CD3-PC5, CD14-PC5, CD16-PC5, and CD19-PC5 (Immunotech, Marseille, France). The circulating mDC and pDC subsets were defined by the concomitant lack of lineage markers (CD3$^-$, CD14$^-$, CD16$^-$, and CD19$^-$), HLA-DR expression, and mutually exclusive

membrane expression of CD11c or CD123, respectively. The results are expressed as the percentage of mDC or pDC among the HLA-DR$^+$Lin$^-$ cells. The subpopulation of NK cells was evaluated as the percentage of CD56$^{dim}$CD16$^+$ and CD56$^{bright}$CD16$^-$ cells from the CD3$^-$CD45$^+$ peripheral lymphocytes.

For immunophenotyping, whole-blood staining and a lyse/no wash method were utilized. Briefly, whole-blood aliquots (50 µl/well) were stained with 2 µl of the relevant fluorochrome-conjugated mAbs in 96-well V-bottomed microplates at room temperature for 30 min in the dark. OptiLyse B lysis (50 µl) solution was added to each well and incubated for 10 min. The samples were then transferred to cytometric tubes and further incubated with deionized H$_2$O (500 µl) for 10 min. The samples were measured by flow cytometry (1 × 10$^5$ events counted), and the data were analyzed with FCS Express software (De Novo Software, Los Angeles, CA, USA).

### Multiplex microbead-based cytokine immunoassay

Plasma samples were prepared from heparinized peripheral blood (1 ml) by centrifugation at 5,000 rpm for 10 min. The supernatants were collected and filtered through sterile 0.22-µm-pore-size filters, and aliquots were stored at −80 °C until analysis. The plasma levels of cytokines, including IL-1$\beta$, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, IFN-$\gamma$, and tumor necrosis factor (TNF)-$\alpha$, were analyzed using the Bio-Plex Suspension Array System (Bio-Rad Laboratories Inc., Hercules, CA, USA). The multiplex microbead-based cytokine immunoassay was performed in 96-well filter microplates according to the manufacturer's instructions. The cytokine standards and samples (50 µl) were diluted in plasma dilution buffer and incubated with the fluorescently labeled microspheres coupled to specific monoclonal antibodies (50 µl) for 30 min on a plate shaker (300 rpm) in the dark. After one wash step, the beads were incubated with the biotinylated detection antibody cocktail (25 µl/well) for 30 min followed by streptavidin-PE (50 µl/well) for 10 min. Finally, 125 µl of assay buffer was added to each well before reading the plate on a Bio-Plex system. The cytokine concentrations were calculated with Bio-Plex Manager Software.

### Statistical analysis

Statistical significance was calculated using the SigmaPlot ver. 11 software package (Systat Software Inc., Erkrath, Germany). The paired $T$ test, Wilcoxon signed rank test, or Mann–Whitney rank sum test was used for data evaluation.

## Results

### MGN-3 augments NK cytolytic activity in MM patients

The baseline percentage of cytolytic CD56$^{dim}$CD16$^+$ NK cells and cytokine-producing CD56$^{bright}$ CD16$^-$ NK cells among the CD45$^+$CD3$^-$ peripheral lymphocytes did not differ between MGN-3- and placebo-treated patients (24.5 ± 2.8 % vs. 17.5 ± 3.5 %, $p = 0.128$; and 1.4 ± 0.2 % vs. 2.2 ± 1.0 %, $p = 0.683$, respectively). No statistically significant changes were observed in the percentages of CD56$^{dim}$ and CD56$^{bright}$ subpopulations of NK cells during the treatment (data not shown).

The NK cell cytolytic activity against susceptible K-562 targets was analyzed in PBMCs from MM patients receiving MGN-3 ($N = 32$) or placebo ($N = 16$) with a flow cytometry-based CAM assay. Blood samples were collected a week before treatment (baseline) and after 1, 2, and 3 months of treatment. The NK cytolytic activity was evaluated as PSL at $E/T$ ratios of 50:1, 25:1, and 12.5:1 (Fig. 1a) and by calculating the number of LUs yielding 20 % cytotoxicity per 10$^7$ effector cells (Fig. 1b).

No statistically significant difference was found between the MGN-3 and placebo groups with regard to the baseline levels of NK activity when PSL was compared at $E/T$ ratios of 50:1, 25:1, and 12.5:1 ($p = 0.297$, $p = 0.257$, and $p = 0.307$, respectively) or the LUs ($p = 0.814$). The NK cytolytic activity significantly increased above baseline levels (27 ± 4.1 % at 50:1; 17.1 ± 3.1 % at 25:1; 9.7 ± 1.9 % at 12.5:1) in the MGN-3 group at all three $E/T$ ratios after 1 month (39.8 ± 5.8 %, $p \leq 0.001$ at 50:1; 25 ± 3.6 % at 25:1, $p = 0.003$; 13.9 ± 2.2 % at 12.5:1, $p = 0.008$) and 2 months of treatment (39.3 ± 5.7 %, $p = 0.038$ at 50:1; 25.3 ± 4.1 % at 25:1, $p = 0.021$; 14.4 ± 2.6 % at 12.5:1, $p = 0.044$; Fig. 1a). This increase in NK cell activity was also confirmed by comparing the lytic units; a significant increase over baseline levels (30.8 ± 7.4 LU) was observed after 1 month (47.0 ± 8.5 LU, $p = 0.045$) and 2 months (56.6 ± 12.2 LU, $p = 0.029$; Fig. 1b) of MGN-3 treatment. No significant changes in NK activity were observed in the placebo group during the treatment (Fig. 1a, b).

### MGN-3 treatment increases levels of circulating myeloid DCs

The frequencies of CD11c$^+$CD123$^-$ myeloid DCs and CD11c$^-$CD123$^+$ plasmacytoid DCs as well as the mDC/pDC ratio were examined in the MGN-3-treated patients ($N = 20$) and placebo group ($N = 15$) before treatment (baseline) and after 1, 2, and 3 months of treatment (Fig. 2). There was no statistically significant difference in the percentage of mDC in the placebo and MGN-3 groups

**Fig. 1** Effect of MGN-3 treatment on NK cytolytic activity in MM patients. NK cell lytic activity against target K-562 cells in PBMC from MM patients receiving MGN-3 ($N = 32$) and those receiving placebo ($N = 16$) was assessed using 3-h FC-based CAM cytotoxicity assay before treatment (*baseline*), and after 1, 2, or 3 months of treatment. Statistical significance: *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$ versus baseline. **a** The mean percentage of specific lysis ± SEM is shown at *E/T* ratio 50:1, 25:1, and 12,5:1. **b** Lytic units (LU, mean ± SEM) per $10^7$ of effector cells
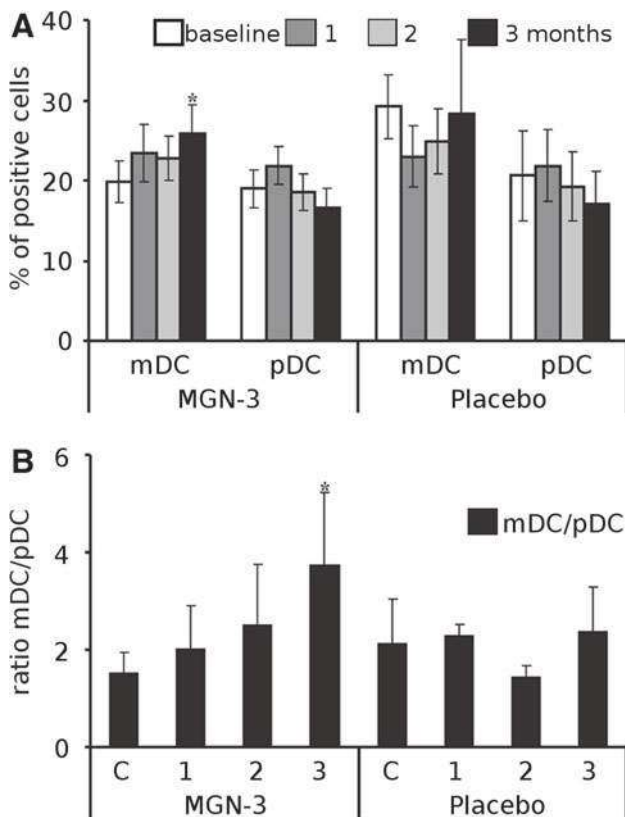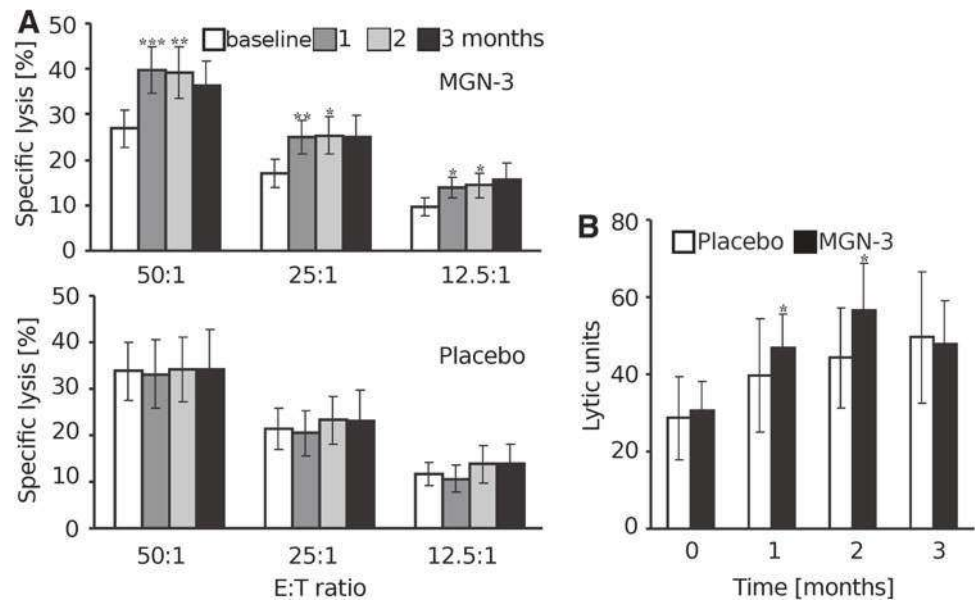


**Fig. 2** Changes in circulating DC subsets after MGN-3 treatment. The levels of CD11c$^+$CD123$^-$ mDC and CD11c$^-$CD123$^+$ pDC in peripheral blood of MM patients receiving MGN-3 ($N = 20$) and placebo ($N = 15$) were analyzed by flow cytometry before treatment (*baseline*) and after 1, 2, and 3 month of treatment. *$p < 0.05$, using Wilcoxon signed rank test. **a** The percentage of mDC and pDC from HLA-DR$^+$Lin$^-$ cells (mean ± SEM) is shown. **b** mDC/pDC ratio values (mean ± SEM) in MGN-3 and placebo group

at baseline ($p = 0.103$). The percentage of circulating mDCs among the HLA-DR$^+$Lin$^-$ peripheral leukocytes significantly increased after 3 months of MGN-3 treatment when compared to the baseline levels ($25.8 ± 3.6$ % vs. $17.6 ± 2.6$ %, $p = 0.036$), while there was no significant change in the placebo group over time. The baseline values of the circulating pDCs were similar between the MGN-3 ($16.6 ± 2.4$ %) and placebo groups ($24.9 ± 5.6$ %), with no significant changes during treatment (Fig. 2a). The mDC/pDC ratio (Fig. 2b) significantly increased over time after 3 months of MGN-3 treatment ($p = 0.030$). In the placebo group, no significant changes in the mDC/pDC ratio were observed over time.

Characterization of the Th1/Th2 profile in MM patients

Patients with advanced cancer often have impaired cell-mediated immunity associated with a switch from a Th1 to Th2 cytokine pattern in the local tumor environment and peripheral blood. Among the Th1 cytokines analyzed, only IFN-γ was significantly increased ($p = 0.034$) in MM patients ($N = 45$) compared to healthy controls ($N = 30$; Table 2). By contrast, we observed significant differences in the levels of six of the seven Th2 cytokines analyzed. The plasma levels of IL-4, IL-5, IL-6, and IL-13 were significantly higher in MM patients compared to healthy controls (the medians, interquartile range (IQR), and *p* values are presented in Table 2). However, IL-9 and IL-10 were present at significantly lower concentrations in MM patients than in healthy controls (the medians, IQR, and *p* values are presented in Table 2).

**Table 2** Comparison of Th1 and Th2 cytokine levels between MM patients and healthy donors

| | Cytokine | Healthy donors (N = 30) | | MM patients (N = 45) | | Difference p value |
|---|---|---|---|---|---|---|
| | | Median (pg/ml) | IQR (pg/ml) | Median (pg/ml) | IQR (pg/ml) | |
| Th1 | IFN-γ | 44.8 | 16.9–74.6 | 82.4 | 24.1–137.9 | 0.034* |
| | IL-1β | 1.4 | 0.7–2.0 | 1.8 | 0.2–2.8 | 0.231 |
| | IL-2 | 2.4 | 0.0–7.6 | 4.2 | 0.0–9.3 | 0.175 |
| | IL-12 | 3.6 | 2.4–9.2 | 3.1 | 0.8–6.3 | 0.284 |
| | IL-15 | 1.5 | 0.0–5.2 | 0.7 | 0.0–3.1 | 0.550 |
| | IL-17 | 0.0 | 0.0–3.9 | 1.6 | 0.0–4.4 | 0.189 |
| | TNF-α | 0.0 | 0.0–0.0 | 0.0 | 0.0–0.0 | 0.571 |
| Th2 | IL-1ra | 71.6 | 35.6–114.3 | 76.4 | 13.9–220.1 | 0.837 |
| | IL-4 | 0.6 | 0.0–0.9 | 0.9 | 0.0–2.0 | 0.040* |
| | IL-5 | 0.3 | 0.0–0.5 | 1.4 | 0.0–2.9 | 0.002** |
| | IL-6 | 1.9 | 0.0–7.3 | 6.4 | 0.0–13.0 | 0.027* |
| | IL-9 | 38.9 | 21.5–93.5 | 4.5 | 0.0–23.7 | <0.001*** |
| | IL-10 | 3.3 | 1.7–7.7 | 1.8 | 0.9–4.4 | 0.008** |
| | IL-13 | 1.2 | 0.0–2.7 | 4.5 | 0.0–23.7 | 0.012* |

Plasmatic concentration (pg/ml) of Th1 and Th2 cytokines in MM patients (N = 45) and healthy donors (N = 30) was assessed by multiplex microbead-based immunoassay

Results are reported as medians with interquartile range (IQR; 25–75th percentile). p values (* p < 0.05, ** p < 0.01, *** p < 0.001) were calculated using Mann–Whitney rank sum test



**Fig. 3** Th1/Th2 cytokine ratios in healthy donors (HD) and MM patients. The values of 5 Th1 cytokines (rows: IFN-γ, IL-1β, IL-2, IL-12, and IL-15) and 6 Th2 cytokines (columns: IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13) ratios (totally 30 Th1/Th2 ratios) are shown. The ratio values were calculated using the following equation: $value_{row, column} = sum ((c_{row}/c_{column})_{pat1}, (c_{row}/c_{column})_{pat2}, \dots (c_{row}/c_{column})_{patN})/N$. The values greater than 1.0 are depicted in white, the ratios less than one in *gray rectangles*

We also analyzed the Th1/Th2 ratios of 5 Th1 cytokines (IFN-γ, IL-1β, IL-2, IL-12, and IL-15) and 6 Th2 cytokines (IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13) in MM patients and healthy donors (Fig. 3). In healthy individuals, 20 of the 30 analyzed Th1/Th2 ratios were greater than 1.0, and only 10 were less than 1.0 (Th1/Th2 score 20:10). In MM patients, 14 Th1/Th2 ratios were greater than 1.0, and 16

were less than 1.0 (Th1/Th2 score 14:16), which suggests that Th2 immunity may be predominantly active in patients with MM. Specifically, a shift toward the Th2 pattern was observed for the IL-12/IL-6, IL-12/IL-13, IL-2/IL-6, IL-2/IL-13, IL-15/IL-4, IL-15/IL-5, and IL-15/IL-13 ratios of MM patients when compared to healthy donors.

### Effect of MGN-3 treatment on Th1 and Th2 cytokine levels

We also assessed the plasma concentrations of Th1 and Th2 cytokines in patients treated with MGN-3 (N = 30) or placebo (N = 15; Fig. 4). No statistically significant differences were observed between the groups when the baseline values of Th1 and Th2 cytokines were compared. After 1 month of MGN-3 treatment, we observed significantly elevated levels of IL-1β (p = 0.047), IL-12 (p = 0.011), IL-17 (p = 0.036), and TNF-α (p = 0.01) compared to the baseline levels before treatment (Fig. 4a). After 3 months, we observed increased levels of IL-12 (p ≤ 0.001), IL-17 (p = 0.032), IFN-γ (p = 0.018), and TNF-α (p = 0.007) in the MGN-3 group (Fig. 4a).

Among the Th2 cytokines, the levels of IL-5 and IL-9 were significantly increased in the MGN-3 group (p = 0.014 and p = 0.031, respectively). However, IL-10 levels were significantly reduced in the placebo group after 1 month of treatment (p = 0.037), and IL-4 levels were elevated in both the MGN-3 and placebo groups compared

**Fig. 4** Changes in Th1 and Th2 cytokine profile after MGN-3 treatment. Plasmatic concentration (pg/ml) of a Th1 cytokines and IL-17 and b Th2 cytokines was measured using multiplex microbead-based immunoassay in patients treated with MGN-3 ($N = 30$) or placebo ($N = 15$). Statistical significance: $*p < 0.05$; $**p < 0.01$, $***p < 0.001$
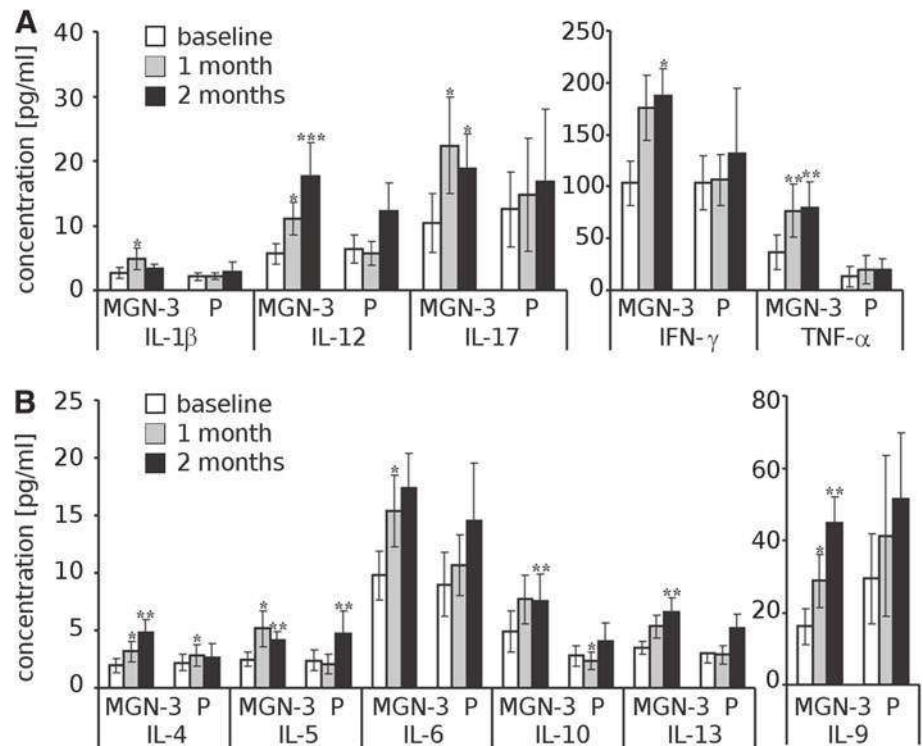
to the baseline levels ($p = 0.034$ and $p = 0.024$, respectively). After 3 months of MGN-3 treatment, IL-4 ($p = 0.003$), IL-6 ($p = 0.031$), IL-9 ($p = 0.006$), IL-10 ($p = 0.010$), and IL-13 ($p = 0.002$) levels were significantly increased compared to the placebo, whereas IL-5 levels increased in both the MGN-3 and placebo groups ($p = 0.006$ and $p = 0.004$, respectively; Fig. 4b).

## Discussion

Recent evidence suggests that NK cells play an important role in MM immunosurveillance by exerting anti-MM cytotoxicity. The differential expression of NK cell surface receptors and their ligands on myeloma cells during disease progression reflects immune editing and the selection of more aggressive myeloma clones that are resistant to NK-mediated lysis [19, 28, 29]. The effect of anti-MM cytotoxicity can be enhanced by novel therapies, such as the adoptive transfer of in vitro-activated NK cells with increased anti-myeloma activity [30] or comprehensive treatment with proteasome inhibitors [31] and immunomodulatory drugs [32, 33], which have been demonstrated to increase NK cell numbers and function.

We evaluated NK cell activity in the peripheral blood of MM patients during treatment with MGN-3. Consistent with the previously published data [22], we observed a statistically significant increase in NK-mediated cytotoxicity

during the first 2 months of MGN-3 treatment, while no statistically significant differences in NK cytotoxicity were detected in the placebo-controlled group. However, the phenotypic analysis performed in MM patients did not reveal changes in the percentage of NK cell subsets. This effect is similar to the results observed in humans during treatment with other types of natural products [34–36].

In our previous in vitro study, we demonstrated that MGN-3 augmented the maturation of monocyte-derived DCs and induced a pDC-like phenotype switch [24]. Dendritic cells are a key component of the immune system and play a critical role in priming naïve T cells and inducing tumor-specific protective immune responses [37]. Patients with MM suffer from general impaired immunity involving deficiencies in DC frequencies and functions. Both the mDC and pDC subsets of DCs were significantly reduced in the peripheral blood of MM patients compared to healthy age-matched controls [3, 38, 39]. In addition, DCs in MM patients exhibited phenotypic abnormalities as well as an altered pattern of inflammatory cytokine secretion [39–41]. Our phenotypic analysis of the peripheral blood from MM patients revealed a significant increase in the relative concentration of mDCs. Similarly, an increase in the mDC/pDC ratio was observed after MGN-3 treatment but not in the placebo group, while there were no significant changes in circulating pDC levels in either group. Because our study was limited to peripheral blood analysis, further studies are needed to determine whether

the phenotypic pDC switch observed in vivo and the increase in mDCs in peripheral blood is due to the preference of pDCs for bone marrow [42].

Immune DC dysfunction has been linked to high levels of soluble factors, including multiple cytokines, such as VEGF, IL-6, and IL-10, which interfere with DC differentiation and maturation [43, 44]. As mediators of the immune response, cytokines are often classified as Th1-type cytokines, which mainly induce cell-mediated immunity, and Th2-type cytokines, which predominantly induce humoral immunity [45].

We analyzed the concentrations of Th1 and Th2-related cytokines in the plasma of MM patients compared to healthy donors. The plasma cytokine levels and Th1/Th2 profiles observed in healthy controls were consistent with the published data [46, 47]. In MM patients, we observed a statistically significant increase in Th1-related IFN-$\gamma$ along with increases in the Th2 cytokines IL-4, IL-5, IL-6, and IL-13, while levels of IL-9 and IL-10 were significantly lower in MM patients compared to healthy controls. These results indicate deregulated immune homeostasis and a shift toward systemic Th2 cytokine dominance, as previously described in patients with cancers including MM [8].

Along with Th1 cytokines, an increased concentration of IL-1($\alpha$,$\beta$) and IL-6 is consistently associated with successful anti-myeloma immunosurveillance [48] and possible cancer eradication. In our study, we observed increased levels of several important Th1 cytokines, in particular IL-1$\beta$, IL-12, IFN-$\gamma$, and TNF-$\alpha$, in MM patients receiving MGN-3, in contrast to the placebo group. In addition, the concentration of IL-17 was significantly increased upon MGN-3 treatment. Very recently, a similar pattern of in vitro production of proinflammatory and immuno-regulatory cytokines in DCs stimulated by MGN-3 was observed to induce CD4 + cell proliferation, and the production of IFN$\gamma$, IL-10, and IL-17 was observed [49].

The study results demonstrate a clear increase in NK activity, an increased level of mDCs in the peripheral blood, and augmented levels of Th1-related cytokines in the plasma of MM patients treated with MGN-3 compared to the placebo group. To elucidate the underlying mechanisms, the immunomodulatory effects of MGN-3 merit further study involving focused attention on the bone marrow environment.

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# A Randomized Study of Chemotherapy *Versus* Biochemotherapy with Chemotherapy plus *Aloe arborescens* in Patients with Metastatic Cancer

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**Abstract.** *Background: The recent advances in the analysis of tumor immunobiology suggest the possibility of biologically manipulating the efficacy and toxicity of cancer chemotherapy by endogenous or exogenous immunomodulating substances. Aloe is one of the of the most important plants exhibiting anticancer activity and its antineoplastic property is due to at least three different mechanisms, based on antiproliferative, immunostimulatory and antioxidant effects. The anti-proliferative action is determined by anthracenic and antraquinonic molecules, while the immunostimulating activity is mainly due to acemannan. Patients and Methods: A study was planned to include 240 patients with metastatic solid tumor who were randomized to receive chemotherapy with or without Aloe. According to tumor histotype and clinical status, lung cancer patients were treated with cisplatin and etoposide or weekly vinorelbine, colorectal cancer patients received oxaliplatin plus 5-fluorouracil (5-FU), gastric cancer patients were treated with weekly 5-FU and pancreatic cancer patients received weekly gemcitabine. Aloe was given orally at 10 ml thrice/daily. Results: The percentage of both objective tumor regressions and disease control was significantly higher in patients concomitantly treated with Aloe than with chemotherapy alone, as well as the percent of 3-year survival patients. Conclusion: This study seems to suggest that Aloe may be successfully associated with chemotherapy to increase its efficacy in terms of both tumor regression rate and survival time.*

The recent formulation of chemo-biochemotherapeutic regimens could represent a very simple but promising strategy in the treatment of human neoplasms (1-3). The chemo-biochemotherapeutic combinations have been developed to associate the cytotoxic action of cancer chemotherapy with molecules capable of modulating the antitumor biological response and to counteract the suppressive effect of cancer chemotherapy on host immunobiological responses, which plays a fundamental role in the control of tumor progression and dissemination (4-7). Hence, the rationale of the association between cancer chemotherapy and biological response modifier agents consists of the prevention of chemotherapy-induced damage of host anticancer immunobiological reaction. A great variety of natural molecules with immunostimulatory activity have been isolated from plants commonly used in traditional medicine in an empirical manner, in particular from Aloe, *Cannabis indica* and myrrh (8-10). The immunobiological information available up to now may justify the clinical use of these three plants in the palliative therapy of human neoplasms, at least to improve the efficacy and tolerability of the common standard anticancer therapies, including chemotherapy and radiotherapy. Despite differences in the chemical structure of their molecules, the anticancer activity of aloe, cannabis and myrrh is based on very similar mechanisms, consisting of antiproliferative, immunostimulatory, anti-inflammatory and antioxidant effects (8-10). In cannabis and myrrh, both the antiproliferative and immunoinflammatory-modulating effects are attributed to the same molecules, tethrahydrocannabinol and cannabinol for cannabis (11) and the sesquiterpene T-cadinol for myrrh (12). On the contrary, the antiproliferative and the immunomodulating effects of aloe are mediated by separate molecules. More specifically, the antitumor and antiproliferative effects of aloe are mainly exerted by aloenin-like substances, namely aloe-emodine, whose oncostatic action has been shown to be particularly evident against neuroendocrine cancer cell lines (13). On the other hand, the immunostimulatory properties of aloe are mainly dependent on acemannan and glycomannan (14), whose stimulatory action on anticancer immunity is mediated, at least in part, by the inhibition of interleukin (IL)-l0 secretion, with a resulting increase in the production of IL-2, which plays a fundamental role in the generation of the anticancer immunity

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(15). The anticancer properties of aloe have been confirmed by several experimental *in vitro* and *in vivo* studies (16, 17), revealing that the anticancer activity of aloe does not depend only on its immunomodulatory effect, as believed until recently, but also on a direct inhibition of cancer cell proliferation through aloenin-like molecules.

This finding is not surprising, since aloenin and other similar molecules may be classified within the group of anthracenic and anthraquinonic substances, whose antiproliferative cytotoxic effects are well known. A considerable number of clinical investigations with aloe extracts have been performed, however, these have yelded controversial results. Aloe therapy has been particularly investigated in the treatment of psoriasis, hyperlipidemia and diabetes mellitus (18-21) and it may exert anticholesterolemic and antidiabetic effects (18). Moreover, it stimulates wound repairing, however, no efficacy has been observed in the treatment of radiotherapy-induced skin damage (21).

Finally, aloe has been used for the tratment of human neoplasm (22), although only preliminary data are available. Despite all of this work, most studies are very limited from a methodological point of view, due to the low number of patients and lack of randomization. Therefore, the present study was planned in an attempt to investigate the influence of a concomitant aloe administration on the efficacy and tolerability of chemotherapy in patients with advanced cancer and poor clinical status.

## Patients and Methods

*Patients.* The study included 240 consecutive patients with metastatic solid tumor, who were treated with chemotherapy with or without aloe treatment. The study was performed by using the variety *Aloe arborescens*. Eligibility criteria were as follows: histologically proven metastatic solid tumor; histological diagnosis of lung cancer or gastrointestinal tract tumor; measurable lesions, no previous chemotherapy for the metastatic disease; no possibility to tolerate the most aggressive polychemotherapies because of low performance status (PS), age and/or concomitant important medical illnesses other than cancer; no brain metastasis and no double tumor. The metastatic disease was established by CT scan and/or NMR or PET. Moreover the diagnosis of poor clinical status was established on the basis of low PS and/or concomitant relevant medical diseases other than cancer. The experimental protocol was explained to each patient, and written consent was obtained.

*Treatments.* According to tumor histotype, sites of metastases and type of chemotherapy, patients were randomized to receive chemotherapy alone or chemotherapy plus aloe. Chemotherapy consisted of cisplatin (CDDP) plus etoposide (VP16) or weekly vinorelbine (VNR) for non-small cell lung cancer (NSCLC) in patients with good or poor clinical status respectively; CDDP plus VP-16 for small cell lung cancer (SCLC); low-dose oxaliplatin (OXA) plus 5-fluorouracil (5-FU) for colorectal cancer; weekly 5-FU for gastric cancer, and weekly gemcitabine (GEM) for pancreatic adenocarcinoma.

Table I. *Clinical characteristics of 240 evaluable patients withs metastatic solid tumors treated with chemotherapy (CT) alone or CT plus aloe.*

| Characteristics | CT | CT + Aloe |
|---|---|---|
| No. | 121 | 119 |
| Male/female | 67/54 | 65/54 |
| Median age (years) | 64 (61-77) | 65 (58-79) |
| Median performance status (Karnofsky's score) | 80 (60-100) | 80 (60-100) |
| Dominant metastasis sites: | | |
| Soft tissues | 7 | 6 |
| Bone | 20 | 16 |
| Lung | 26 | 25 |
| Liver | 37 | 35 |
| Liver + lung | 24 | 25 |
| Liver + peritoneum | 7 | 12 |

CDDP and VP-16 were given *i.v.* at 20 mg/m$^2$ and at 100 mg/m$^2$ for 3 consecutive days every 21 days, corresponding to one complete chemotherapeutic cycle. OXA was given *i.v.* at 85 mg/m$^2$ on days 1 and 8, in association with 5-FU and folates (FA) at a dose of 500 mg/m$^2$ and 10 mg/m$^2$ respectively, at days 18 and l5, by repeating the cycle every 28 days. VNR was given weekly at 25 mg/m$^2$. Weekly 5-FU consisted of 375 mg/m$^2$, in association with FA at a dose of 10 mg/m$^2$.

Finally, GEM was given weekly at 1,000 mg/m$^2$. *Aloe arborescens* was given orally at a dose of 10 ml thrice daily of a mixture consisting of 300 g of Aloe fresh leaves in 500 g of honey plus 40 ml of 40% alcohol, every day without interruption, either during or after chemotherapy, until the progression of disease, starting 6 days prior to the onset of chemotherapy. Aloe mixture was supplied by Deca (Isernia, Italy). The clinical response and toxicity were assessed according to WHO criteria. PS was evaluated by Karnowsky's score. The clinical responses were radiologically evaluated after at least three cycles of chemotherapy by repeating the same radiological investigation used prior to the onset of chemotherapy, including CT scan, NMR and PET. Patients were monitored weekly by routine laboratory tests. Lymphocyte counts were determined by hemochromocytometric analysis. The evaluation of subjective symptoms, such as fatigue and asthenia, was assessed by an individual report.

Statistical analysis. The results were statistically analyzed by the chi-square test, Student's *t*-test and analysis of variance, as appropriate.

The survival curves were plotted by the Kaplan-Meier method and statistically evaluated by the log-rank test. The differences were considered to be statistically significant when *p*-values were <0.05.

## Results

The clinical characteristics of patients are reported in Table I. As shown, the two groups of patients treated with chemotherapy alone, or chemotherapy plus aloe were well comparable for the main prognostic variables, including

Table II. *Clinical response (WHO criteria) in 240 metastatic solid tumor patients treated with chemotherapy (CT) or CT plus Aloe.*

| Histotypes | CT | | | | | | | CT + ALOE | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | CR | PR | CR+PR | SD | DC | PD | n | CR | PR | CR+PR | SD | DC | PD |
| Small cell lung cancer<br>CDDP/VP16 | 22 | 2 | 6 | 8 (36% ) | 7 | 15 (68% ) | 7 | 23 | 6 | 8 | 14 (61% )* | 4 | 18 (78% )** | 5 |
| Non-small cell lung cancer | 36 | 1 | 6 | 7 (19% ) | 11 | 18 (50% ) | 18 | 38 | 4 | 8 | 12 (32% ) | 14 | 26 (68% ) | 12 |
| Weekly VNR | 17 | 0 | 3 | 3 (18% ) | 4 | 7 (41% ) | 10 | 17 | 2 | 3 | 5 (29% ) | 6 | 11 (65% ) | 6 |
| CDDP/VP | 19 | 1 | 3 | 4 (21% ) | 7 | 11 (58% ) | 8 | 21 | 2 | 5 | 7 (33% ) | 8 | 15 (71% ) | 6 |
| Colorectal cancer<br>OXA/5-FU/FA | 21 | 1 | 5 | 6 (29% ) | 8 | 14 (67% ) | 7 | 21 | 2 | 6 | 8 (38% ) | 7 | 15 (71% ) | 6 |
| Gastric cancer<br>Weekly 5-FU/FA | 22 | 0 | 0 | 0 | 9 | 5 (28% ) | 13 | 19 | 0 | 3 | 3 (16% ) | 7 | 10 (59% ) | 9 |
| Pancreatic adenocarcinoma<br>Weekly GEM | 20 | 0 | 2 | 2 (7% ) | 10 | 8 (50% ) | 8 | 18 | 0 | 3 | 3 (17% ) | 8 | 11 (73% ) | 7 |
| Overall | 121 | 4 | 19 | 23 (19% ) | 37 | 60 (50% ) | 61 | 119 | 12* | 28 | 40 (34% )** | 40 | 80 (67% )** | 39 |

CDDP: Cisplatin; VP16: etoposide; VNR: vinorelbine; OXA: oxaliplatin; 5-FU: 5-fluorouracil; FA: folinic acid; GEM: gemcitabine; CR: complete response; PR: partial response; SD: stable disease; DC: disease control (CR+PR+SD); PD: progressive disease. *$p<$(0.025 *vs.* CT; **$p<0.01$ *vs.* CT

histotype, sites of metastasis, PS and age. The observed clinical response in the two groups of patients are reported in Table II.

By considering the overall tumor histotypes, the percentages of complete responses (CR) and partial responses (PR) achieved in patients concomitantly treated with aloe were significantly higher than in those who received chemotherapy alone (40/119 (34% ) *vs.* 23/121 (19% ), $p<0.01$). A CR occurred in 12/119 (10% ) patients concomitantly treated with aloe and in only 4/121 (3% ) patients treated with chemotherapy alone. This difference was statistically significant ($p<0.01$). Stable disease (SD) was achieved in 37/121 (31% ) patients treated with chemotherapy alone and in 40/119 (34% ) patients who received a concomitant aloe administration. The disease control (DC=CR+PR+SD) obtained in patients concomitantly treated with aloe showed a significantly higher percentage than that found in patients who received chemotherapy alone (80/119 (67% ) *vs.* 60/121 (50% ), $p<0.01$).

As far as the clinical response in relation to tumor histotype is concerned, the objective tumor response rate (CR+PR) achieved in the group of SCLC patients concomitantly treated with aloe was significantly higher than that found in the group of chemotherapy alone (14/23 (61% ) *vs.* 8/22 (36% ), $p<0.05$). Moreover, the percentage of CR was also significantly higher in SCLC patients concomitantly treated with aloe (6/23 (26% ) *vs.* 2/22 (9% ), $p<0.05$). Similarly, the objective tumor response (CR+PR) observed in the remaining tumor histotypes, including colorectal

cancer, gastric cancer and pancreatic adenocarcinoma, was consistently higher in patients concomitantly treated with aloe, without statistically significant differences.

Figure 1 illustrates the 3-year survival curves achieved in patients treated with chemotherapy alone or chemotherapy plus aloe. As shown, the percentage of 3-year survival obtained in patients concomitantly treated with aloe was significantly higher than that found in the group of chemotherapy alone ($p<0.01$). As far as the survival in relation to tumor histotype are concerned, the percentage of 3-year survival achieved in both SCLC and NSCLC patients concomitantly treated with aloe was significantly higher than that obtained in those treated with chemotherapy alone ($p<0.05$). The survival was also longer in all other tumor histotypes treated with chemotherapy plus aloe, without statistically significant differences. Aloe was well tolerated in all patients and no metabolic undesirable effects were observed. In addition, no aloe-related toxicity occurred, including vomiting and diarrhoea.

From an immunobiological point of view, mean numbers of lymphocytes decreased and increased after chemotherapy in patients treated with chemotherapy alone or chemotherapy plus aloe, respectively, even though none of these differences were statistically significant. However, as illustrated in Figure 2, the mean lymphocyte mean number observed after therapy in patients concomitantly treated with aloe was significantly higher than that observed in the group treated with chemotherapy alone ($p<0.05$), while no difference was seen before the onset of treatment.
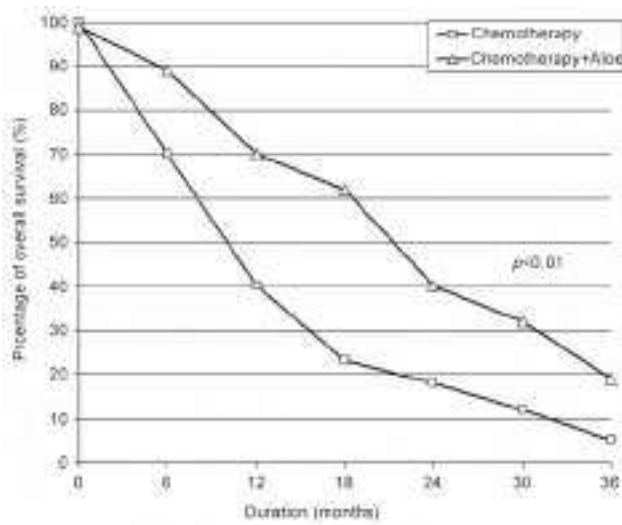
Figure 1. *3-Year survival curves observed in 240 patients with metastatic solid tumor treated with chemotherapy alone or chemotherapy plus aloe.*



Figure 2. *Mean number of lymphocytes observed before and after the chemotherapeutic treatment in 240 patients with metastatic solid tumor treated with chemotherapy alone or chemotherapy plus aloe. *p<0.05 vs. Chemotherapy.*

Finally, chemotherapy was substantially better tolerated in patients concomitantly treated with aloe. In particular, the occurrence of asthenia and/or fatigue was significantly less frequent in patients concomitantly treated with aloe than in those who received chemotherapy alone (31/119 (26%) *vs.* 56/121 (46%), *p<0.01*). Similarly, VNR-induced constipation was significantly less frequent in the aloe group than in patients treated with VNR alone (3/17 (18%) *vs.* 12/17 (71%), *p<0.0l*). In addition, OXA-induced neurotoxicity, with paresthesic disturbances, was also less frequent in patients who received aloe with respect to those treated with chemotherapy alone (6/21 (29%) *vs.* 9/21(43%)), without statistically significant differences. No other important difference in the occurrence of side-effects was found.

**Discussion**

The results of this study confirm previous preliminary clinical investigations which had already shown the efficacy of aloe extracts in the palliative therapy of patients with untreatable metastatic cancer, either to improve their quality of life, or to prolong the survival time (22). In addition to these previous results, this study demonstrates the efficacy of aloe in association with cancer chemotherapy, at least in patients with poor clinical status because of low PS or important medical diseases, in whom the therapeutic activity of chemotherapy alone is generally low.

Thus, aloe extracts may exert not only a direct oncostatic effect, but also enhance the efficacy of chemotherapy in terms of both tumor regression rate and survival time as well as reducing some toxicities. Moreover, aloe-induced prolonged survival time was constantly associated with a better quality of life, at least in terms of relief of asthenia and fatigue. Aloe-induced increase in chemotherapy cytotoxic efficacy appear to be particularly evident in SCLC, because of its neuroendocrine nature. This evidence is not surprising, since experimental studies had already shown that the oncostatic properties of aloe substances are more pronounced against neuroendocrine cancer cell lines (13). In any case, aloe-induced increase in chemotherapy anticancer efficacy would depend not only on molecules provided by antiproliferative action, but also on the activity of immunomodulating substances, such as acemannan (8, 14). A particularly interesting combination could be represented by the association between VNR and aloe in the treatment of NSCLC, since aloe seemed either to increase VNR cytotoxic potency, or to correct the most frequent side-effect of VNR, that of severe constipation. The biochemotherapeutic combination of VNR plus aloe could thus constitute a very well tolerated and active therapy for NSCLC patients, including those with poor clinical status. Obviously, the low number of patients for the single tumor histotype does not allow definitive conclusions to be drawn in the treatment of the various solid tumor histotypes by aloe and chemotherapy combination therapy. The relatively low percentage of responses shown by this study for a single histotype with respect to that reported in the literature could depend on the poor clinical status of patients. In any case, further studies will be required to better investigate the real impact of a concomitant aloe therapy on the life of chemotherapy-treated patients with advanced cancer by using more appropriate scales for the quality of life. Moreover, since the study was not blinded, multiple bias may occur. Hence, double-blind randomized studies will be necessary to

confirm these promising results. Finally, further studies should be performed to establish whether aloe extracts may also enhance the efficacy of chemotherapy in patients with good clinical status. Future clinical studies with single aloe molecules, such as aloe-emodine and acemannan for their immuno-modulating and antiproliferative properties, respectively, could allow further benefits in the treatment of human neoplasms. Several recent studies (23-27) have contributed to better define the mechanism of the anticancer activity of aloe. However, the exact mechanism of its immunomodulatory antitumor effect has still to be established in detail. Hence, successive studies, by evaluating the most important immune biomarkers, namely IL-2, IL-12, IL-6, IL-10, TGF-β and T regulator lymphocytes, will be essential to establish the influence of aloe on the anticancer cytokine network.

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# A phase II study of anastrozole plus the pineal anticancer hormone melatonin in the metastatic breast cancer women with poor clinical status

Research Article

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**Key words**: Anastrozole, breast cancer, melatonin, pineal gland
**Abbreviations:** melatonin, MLT; estrogen receptor, ER;

## Summary

The recent advances in the psychoneuroendocrinology have suggested the possibility to modulate tumor hormone dependency through a neuroendocrine approach. In particular, it has been proven that the pineal neurohormone melatonin (MLT) may stimulate estrogen receptor (ER) expression in breast cancer cells and inhibit the aromatase activity. On this basis, a study was planned to evacuate the efficacy of a concomitant treatment with the aromatase inhibitor anastrozole plus MLT in metastatic breast cancer. The study included 14 metastatic breast cancer women of poor clinical conditions with ER positive or unknown. Both anastrozole and MLT were given orally at a dose of 1 mg at noon and of 20 mg in the evening, respectively. The clinical response consisted of complete response in 2 and partial response in 6 patients. Then, an objective tumor regression was achieved in 8/14 (57%) patients, with a median duration of 26 months. No neoplastic cachexia occurred on treatment. This preliminary study shows that a neuroendocrine strategy with anastrozole plus the pineal hormone MLT may represent a new effective and well tolerated regimen in the treatment of metastatic breast cancer women, including those with poor clinical status, with therapeutic results apparently superior to those reported in the literature with the only aromatase inhibitor. Then, these results would justify further randomized studies of aromatase inhibitors with or without a concomitant administration of MLT, in an attempt to establish whether the pineal hormone may enhance the efficacy of the aromatase inibibitors in the treatment of human advanced breast cancer.

## I. Introduction

Recent experimental studies have demonstrated that the hormone dependency is at least in part under a psychoneuroendocrine regulation (Cos et al, 2008;Grant et al, 2009). In particular, it has been shown that the pineal hormone melatonin (MLT), whose anticancer properties have been well demonstrated (Bartsch et al, 1981; Maestroni, 1993; Reiter et al, 2002), may in vitro stimulate estrogen receptor (ER) expression on breast cancer cell lines (Molis et al,1995). Therefore, the

hormone dependency of breast cancer cells would not depend only on intrinsic characteristics of cancer cells themselves, but also on host neuroendocrine regulation of tumor cell proliferation and differentiation (Bartsch et al, 2000). Moreover, cancer progression has been proven to be associated with pineal alterations, consisting of a progressive decline in MLT nocturnal production. (Maestroni, 1993). Therefore the advanced cancer would require a substitutive endocrine therapy with MLT (Bartsch et al, 1981; Maestroni, 1993). Previous preliminary clinical studies had already suggested that the

concomitant administration of the pineal hormone MLT may apparently increase the efficacy of tamoxifen therapy in the treatment of metastatic breast cancer (Lissoni et al, 1995). Moreover, experimental studies have shown that the activity of aromatase enzyme, which is responsible for the peripheral production of estrogens from testosterone (Bagatell et al, 1994), is under a light/dark circadian rhythm (Bhatnagar et al, 1992). Because of the fundamental role of the pineal hormone MLT in the regulation of the daily photoperiod (Bartsch et al, 1981), it is possible to hypothesize that MLT may be involved in the control of the aromatase activity. In fact, recent studies have demonstrated an inhibitory action of MLT on the aromatase activity (Cos et al, 2005). This finding could reserve a prosiming application in the treatment of both early and advanced breast cancer. This statement is justified by the fact that the aromatase inhibitors represent a new class of agents in the endocrine treatment of breast cancer Plourde et al, 1994), with a potential efficacy superior to that achieved by the previous hormonal therapies with anti-estrogens, such as tamoxifene, even though tumor response rate obtained by the aromatase inhibitors are generally not greater than 40%. On this basis, a phase II study was planned in an attempt to evaluate the efficacy of a neuroendocrinotherapeutic regimen consisting of a concomitant administration of the aromatase inhibitor anastrozole and the pineal hormone MLT in metastatic breast cancer women with poor clinical conditions.

## II. Materials and methods

The study included 14 consecutive metastatic breast cancer women (median age: 72 years, range 51-82), who were followed at Biological Medicine Institute in Milan, or at Health Local Unit 2 of Avellino, from Feb. 2002 to Sept. 2003. Eligibility criteria were, as follows: histologically proven metastatic breast cancer, measurable lesions, ER positive or unknown, no ability to tolerate chemotherapy because of age, low performance status (PS), important clinical illnesses other than cancer and/or heavy chemotherapeutic pre-treatments, no previous endocrine therapies for the metastatic disease, no double tumor and life expectancy less than 1 year. Previous heavy chemotherapeutic treatment consisting of at least 3 chemotherapeutic lines was made in 11/14 (79 %) patients. Dominant metastasis sites were, as follows: soft tissues:1; bone:1; lung:7 (neoplastic lymphangitis:2); liver:1; lung + liver:1; bone marrow:3. Time-span since first diagnosis of the primary tumor was 44 months (31-66 months). All patients had an acceptable social conditions. The minimum and median follow-up periods were 60 months and 72 months respectively. In all patients, in the case of disease progression, at least to other endocrine therapeutic lines with other aromatase-inhibitors were planned. The experimental protocol, wich was approved by the Health Direction of Biological Medicine Institute of Milan, was explained to each patient and informed consent was obtained. The treatment consisted of anastrozole at a dose of 1 mg/day orally at noon, plus MLT at 20 mg/day orally in the evening, generally half-hour before sleeping, to correct cancer progression-related decline in MLT night secretion(10). Patients were considered to be evaluable when they were treated for at least 3 consecutive months. The clinical response was evaluated according to WHO criteria. Complete response (CR) was the complete disappearance of all neoplastic lesions for at least 1 month. Partial response (PR) was a reduction greater than 50 % of the sum of all neoplastic lesions, for at least 1 month. Stable disesase (SD) was no increase or decrease greater tha 25 % of tumor volume. Progressive Disease (PD) was an increase in tumor volume greater than 25 % or the appearance of new neoplastic lesions. PS was assessed according to Karnofsky's score, consisting of the evaluation of the quality of life in relation to patient activity and bed-rest period. ER was positive in 10 and unknown in the remaining 4 patients. The median PS was 80% (range 70-100). Data were statistically evaluated by the chi-square test and the Student's t test, as appropriate.

| Cases | Age | PS | ER | Metastasis sites | Response | Clinical Duration (months) |
|---|---|---|---|---|---|---|
| 1 | 81 | 80 | ? | Lung lymphangitis | PR | 23 |
| 2 | 76 | 90 | + | Lung | PR | 42 |
| 3 | 72 | 70 | + | Lung lymphangitis | CR | 38 |
| 4 | 66 | 100 | + | Bone marrow | SD | 23 |
| 5 | 82 | 90 | ? | Bome marrow | PR | 16 |
| 6 | 64 | 80 | + | Lung, bone | PD | - |
| 7 | 51 | 100 | ? | Bone marrow | SD | 10 |
| 8 | 77 | 90 | + | Liver | SD | 27 |
| 9 | 59 | 80 | + | Lung, bone | SD | 13 |
| 10 | 81 | 90 | ? | Bone | SD | 9 |
| 11 | 62 | 80 | + | Lung | RP | 39 |
| 12 | 65 | 80 | + | Lung,bone | PD | - |
| 13 | 72 | 90 | + | Liver,lung | PR | 27 |
| 14 | 75 | 90 | + | Soft tissues | CR | 42+ |

**Table 1**: Clinical characteristics of metastatic breast cancer women and their clinical response (WHO criteria) to a neuroendocrine regimen consisting of anastrozole plus the pineal hormone melatonin.

## III. Results

All patients were fully evaluable for the clinical response. The clinical characteristics of patients and their individual clinical response to the treatment are reported in **Table 1.** As reported, a complete response (CR) was achieved in 2/14 (14%) (soft tissues:1; lung lymphangitis:1). A partial response (PR) was obtained in other 6/14 (43%) (bone:1; lung:3; liver:1; bone marrow:1). Then, an objective tumor response (CR + PR) was reached in 8/14 (57%) patients. The median duration of response was 26 months(range 9-42 months). A stable disease (SD) was seen in other 4/14 (29%), with a median duration of 25 months (range 10-27). Therefore, a disease-control (DC:CR + PR + SD) was achieved in 12/14 (86%) patients, whereas the remaining 2/14 (14%) patients had a progressive disease (PD). No significant difference in tumor response rate was observed between patients with positive or unknown ER ( 6/10(60%) vs 2/4(50%) ). An overall survival at 1 year and at 3 year was achieved in 11/14 (79 %) and in 5/14 (36 %) patients, respectively. Moreover, 3/14 (21%) patients were still alive at 5 years. The treatment was well tolerated in all patients. Moreover, most patients experienced a relief of asthenia under the treatment and in no patient the neoplastic cachexia occurred. Finally, an evident increase in PS mean values was achieved under treatment, even though it did not reach the statistical significance (86 ±5 vs 93 ± 4, mean ± SE).

## IV. Discussion

The results of this preliminary phase II study, by showing a percentage of 1-year survival greater than 70% in patients with live expectancy less than 1 year, would suggest that a neuroendocrine regimen consisting of the aromatase inhibitor anastrozole plus the pineal neurohormone MLT may represent a new effective therapeutic strategy in the treatment of metastatic breast cancer women, also in patients with poor clinical conditions, who would not be able to tolerate the most aggressive therapies. The concomitant administration of the pineal hormone would seem to enhance the efficacy of the aromatase inhibitor in terms of objective tumor regressions with respect to the results commonly reported in the literature with the only aromatase inhibitor (Plourde et al, 1994), which are generally lower than 40%. The time to progression would seem to be apparently increased by the concomitant treatment with MLT.This finding is not surprising, since MLT could enhance the therapeutic anticancer acitivity of the aromatase inhibitors by either exerting direct antiproliferative antitumor effects (Bartsch et al, 1981; Maestroni, 1993; Reiter et al, 2002), or further inhibiting the aromatase activity by acting on gene and oncogene expression (Molis et al,1995; Cos et al, 2005). In addiction, MLT appeared to stimulate ER expression of breast cancer lines, by transforming ER negative into ER positive breast cancer, as observed in experimental conditions (Danforth et al, 1983). Since the prognosis of ER positive breast cancers is clearly better than that of ER negative ones, MLT could per se improve the clinical couse of mammary tumors. Finally, because of its interesting therapeutic efficacy as a supportive care (Reiter et al, 2002), MLT would be responsible for the evident improvement in the relief of asthenia and in preventing the occurrence of the neoplastic cachexia. On the other hand, because of the inhibitory effect of MLT (Grant et al, 2009; Reiter et al, 2002) on cancer cell proliferation, the anticancer activity of this polyendocrine regimen would be due not only to an indirect effect, depending on a diminished estrogen production following aromatase enzyme inhibition, but also on a direct inhibition of cancer cell growth, due to MLT itself. Therefore, the results of this preliminary study may justify further clinical randomized investigations with the only aromatase inhibitor versus the concomitant treatment with MLT, in an attempt to confirm the ability of the pineal hormone to enhance the antitumor properties of the aromatase inhibitors in the treatment of metastatic breast cancer women with poor clinical conditions.

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# Biotherapy with the pineal hormone melatonin plus aloe and myrrh tincture in untreatable metastatic cancer patients as an essence therapy of cancer

Research Article

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## Summary

**Background: The recent advances in understanding the immunobiological interactions responsible for cancer progression have allowed us to define the mechanisms of action of some plants, whose antitumor properties were already known by the popular Medicine, in particular Aloe and Myrrha, whose mixture was already therapeutically utilized more than 2000 years ago by the Essence medicine. Moreover, some endogenous natural substances, namely the main hormone produced by the pineal gland melatonin (MLT) may also play anticancer activity. On this basis, a study was performed with a biological regimen consisting of MLT, Aloe and Myrrha in untreatable metastatic cancer patients with life expectancy lower than 1 year. Methods: The study included 35 patients. MLT was given orally at 20 mg/day in the evening and a mixed Aloe and Myrrha tincture was administered at a dose of 5 ml/thrice daily. Results: The clinical response consisted of complete response (CR) in 1, partial response (PR) in 2, stable disease (SD) in 19 patients, whereas the remaining 13 patients had a progressive disease (PD). Thus, a disease control (CR + PR + SD) was achieved in 22/35 (63%)patients. Moreover, a survival longer than 1 year was achieved in 17/35 (49%) patients. Finally, DC was associated with an evident improvement in the immune status, namely consisting of a decrease in the number of T regulatory lymphocytes, which are the main cells responsible for the suppression of the anticancer immunity. Conclusion: This preliminary study shows that a biological anticancer regimen consisting of the pineal hormone MLT in association with Aloe and Myrrha mixture, already known at the times of the Essence medical tradition, may induce a control of the neoplastic disease by stimulating the anticancer immunity, in a relevant percentage metastatic cancer patients, who did not respond to the conventional anticancer treatments and for whom no other standard therapy was available.**

## I. Introduction

The recent better definition of the biochemical mechanisms responsible for cancer cell proliferation and for immune system-mediated tumor cell destruction has allowed the possibility to establish the biochemical actions of several plants already known by the popular Medicine to be provided by empiristic potential anticancer properties, namely Aloe, Myrrha, Cannabis Indica, Turmeric and Hyssopus (Davis et al, 1991; Capasso et al,

1998; Vogler et al, 1999; Claeson et al, 1991; Qureshi et al, 1993; Blazquez et al, 2003; Grotenhermen et al, 2004; Aggarwall et al, 2003; Lodha et al, 2000). In more detail, the anticancer activity of Aloe is due to several therapeutically active molecules capable of inhibiting cancer cell proliferation, such as aloenine, aloesine and aloe-hemodin, or stimulating the anticancer immunity, such as acemannane and glycomannane (Davis et al, 1991; Capasso et al, 1998; Vogler et al, 1999). On the same way, the antitumor therapeutic properties of Myrrha extracts have been proven to exert both anticancer antiproliferative and immunostimulating effects, which are mediated by T-cadinol and muzumboic acid, respectively (Claeson et al, 1991; Qureshi et al, 1993). The therapeutic biological properties of a mixture of Aloe and Myrrha were well known by the Essence medical tradition at Qumran, near to the Death Sea, as reported by John's Gospel (John's Gospel), referring that men connected to the Essence community, such as Nycodemus and Joseph of Arimathea, prepared a mixture of Aloe and Myrrha for the burial of Christ. Together with the Ellenic medical sciences, the Essence medicine represented the most advanced medical tradition in the ancient world. With respect to the Ellenic medicine, which was founded by Hippocrates, the Essence medical science was more symbolic and spiritual, by considering the treatment of the human diseases as a simultaneous chemical, psychic and spiritual regeneration of man, mediated by humans, but originating from God. The Essence philosophy interpreted the Universe, the human History and the individual life of men and women as the expression of a war between two opposite principles, the Light and Dark, and the single human disease was considered to be the consequence of the prevalence of the principle of Darkness, as the unconscious aspect of the human life, on the principle of Light, which in contrast is the expression of the spiritual consciousness. The philosophic and spiritual characteristics of the Essence medical tradition were further amplified by the Islamic Medicine, by affirming the existence in the Nature of a therapeutic remedy for the overall human illnesses, as the manifestation of Love and harmonies of God. The fundamental importance of the light/dark circadian rhythm in regulating the living organisms, including humans, has been recently confirmed by the investigation on the physiology of the pineal gland, which has appeared to regulate the most important biological functions and systems, such as cell proliferation, DNA expression and immune reactions in relation to the light/dark rhythm through the circadian secretion of its most known hormone melatonin (MLT), with high production during the darkness and low secretion during the light period of the day (Iguchi et al, 1982; Attanasio et al, 1985; Jankovic et al, 1997; Brzezinski et al, 1997). As well as Aloe and Myrrha, MLT also has been proven to play an anticancer action and the antitumor properties of MLT have been confirmed by several experimental and clinical studies (Bartsch et al, 1981; Regelson et al, 1987; Lissoni et al, 2002; Sze et al, 1993). The anticancer action of MLT is due to both direct antiproliferative effects and stimulation of IL-2- dependent anticancer immunity (Maestroni 1993; Lissoni et al, 2008).

Because of its dependency on the Light/Dark universal rhythm, whose importance was already known by the Essence tradition, the knowledgements of the functions of the pineal gland, including its anticancer fundamental role, may be considered as the last contribution of the Essence science to the treatment of the human diseases, namely cancer, since the Essence medicine was the first to discover the therapeutic properties of the mixture of Aloe and Myrrha. Moreover, preliminary data would suggest the possibility to amplify the anticancer action of MLT by Aloe extracts (Lissoni 2002). On these bases and in agreement with the well experimentally documented anticancer activity of its overall compounds (Davis et al, 1991; Claeson et al, 1991; Bartsch et al, 1981), in this preliminary study we have evaluated the clinical efficacy of a biological regimen, consisting of Aloe, Myrrha and the pineal hormone MLT, which could be symbolically defined as an Essence therapy, in the treatment of metastatic cancer patients, who failed to respond to the conventional antitumor therapies, including chemotherapy, endocrine therapy and anti-angiogenic treatment, or who were unable to tolerate the conventional therapies and for whom no other standard treatment was available. The objective of the study was to establish whether the association of other natural anticancer agents such as Aloe and Myrrh might further enhance the antitumor efficacy of MLT in the treatment of human neoplasm, with respect to the historical ones achieved with MLT alone.

## II. Materials and methods

The study included 35 consecutive metastatic cancer patients, who were followed at the Institute of Biological Medicine of Milan. The therapeutic protocol was explained to each patient and informed consent was obtained. Eligibility criteria were, as follows: histologically proven metastatic solid tumor, measurable lesions, no double tumor, lack of response top the conventional anticancer therapies or poor clinical conditions unable to subtain a chemotherapeutic approach, a life expectancy less than one year, no chronic concomitant therapy with corticosteroids because of their immunosuppressive effects and a minimum follow-up of 12 months. The clinical characteristics of patients are reported in **Table 1**. The treatment consisted of MLT at 20 mg/day orally during the dark period of the day according to its light/dark circadian rhythm (Iguchi et al, 1982; Attanasio et al, 1985; Jankovic et al, 1997; Brzezinski et al, 1997), plus a mixture of Aloe Vera and Myrrha tincture, containing 60% of Aloe and 40% of Myrrha, which was administered orally at a dose of 5 ml thrice/day at 8- hour intervals. The treatment was continued until the progression of disease. Both MLT (Melaton-Med) and mixed Aloe and Myrrha tincture (Mirral) were supplied by Natur-Spiritual (Milan, Italy). The clinical response was evaluated according to WHO criteria. The treatment was also evaluated in relation to its possible immunomodulating effects on the anticancer immunity, by measuring the absolute number of the most important anticancer lymphocyte subset and that of the main immunosuppressive lymphocyte subpopulation, consisting of T helper lymphocyte (TH) and T regulatory lymphocyte (T reg), respectively (Shevach et al, 2002). Lymphocyte subsets were measured by a flow cytometric assay and monoclonal antibodies supplied by Becton-Dickinson (Milan, Italy). TH and T reg lymphocytes were identified as $CD4^+$ cells and $CD4^+$ $CD25^+$ cells, respectively. CD4/CD4CD25 cell ratio was also established. Normal values of CD4/CD4CD25 ratio observed in our laboratory (95% confidence limits) was

greater than 4.0. The immune analysis was made before the onset of treatment and after three months of therapy. Finally, patients were also clinically evaluated from a psychological point of view by the Rorschach test (Rorschach et al, 1921) and spiritually investigated by a specific patient spiritual questionnaire, previously reported in literature (Lissoni et al, 2008). Moreover, patients, who asked a psychospiritual therapeutic approach, were followed through a specific psychospiritual herapeutic method, consisting of an educational program carried out to stimulate the concomitant rediscovery of the perception of pleasure and the

spiritual sensitivity. In more detail, according to previous studies (Lissoni et al, 2008), patients were stimulate to become conscious that both pleasure repression and self-punishment may suppress the anticancer immunity and promote cancer cell dissemination. Data were reported as mean ± SE and statistically analyzed by the chi-square test, the Student's t test and the analysis of variance, as appropriate. Moreover, the 1-year survival curves were plotted according to Kaplan-Meier method and statistically analyzed by the log-rank test.

**Table 1:** Clinical characteristics of 35 untreatable metastatic cancer patients.

| Characteristics | N |
|---|---|
| Male / Female | 19/16 |
| Median age (year s) | 63 (52-81) |
| Median Performance status (Karnofsky's score) | 90 (70-100) |
| Tumor histotypes: | |
| Lung cancer | 10 |
| Nonsmall cell lung cancer | 7 |
| Small cell lung cancer | 3 |
| Colorectal cancer | 5 |
| Pancreatic cancer | 4 |
| Ovarian cancer | 4 |
| Prostate cancer | 4 |
| Gastric cancer | 3 |
| Biliary tract cancer | 3 |
| Malignant melanoma cancer | 2 |
| Dominant metastasis sites: | |
| Soft tissues | 2 |
| Bone | 3 |
| Lung | 10 |
| Liver | 7 |
| Lung + liver | 5 |
| Peritoneum | 6 |
| Brain | 2 |
| Previous Chemotherapies | 31 / 35 |

**Table 2:** Clinical results in response to Melatonin plus Aloe and Myrrh in relation to tumor hitotypes.

| Tumor Histotype | N | CR | PR | CR+PR | SD | DC (CR+PR+SD) | PD |
|---|---|---|---|---|---|---|---|
| Overall patients | 35 | 1 | 2 | 3 (9%) | 19 (54%) | 22 (63%) | 13 (37%) |
| Nonsmall cell lung cancer | 7 | 0 | 0 | 0 | 5 | 5 | 2 |
| Small cell lung cancer | 3 | 0 | 0 | 0 | 2 | 2 | 1 |
| Colorectal cancer | 5 | 0 | 0 | 0 | 4 | 4 | 1 |
| Pancreatic cancer | 4 | 0 | 1 | 1 | 1 | 2 | 2 |
| Ovarian cancer | 4 | 0 | 0 | 0 | 2 | 2 | 2 |
| Prostate cancer | 4 | 0 | 0 | 0 | 2 | 2 | 2 |
| Gastric cancer | 3 | 0 | 0 | 0 | 2 | 2 | 1 |
| Biliary tract cancer | 3 | 0 | 1 | 1 | 1 | 2 | 1 |
| Malignant melanoma cancer | 2 | 1 | 1 | 1 | 0 | 1 | 1 |

## III. Results

As shown in **Table 2**, an objective tumor regression was achieved in 3/35 (9%) patients, consisting of a complete response (CR) in one patient with node metastases due to malignant melanoma and 2 partial responses (PR), the former in a patient with liver metastases due to pancreatic adenocarcinoma and the latter in a patient with biliary tract cancer-induced liver involvement. The median duration of the response was 11 months (Aggarwall et al, 2003; Lodha et al, 2000; John's Gospel; Iguchi et al, 1982; Attanasio et al, 1985; Jankovic et al, 1997; Brzezinski et al, 1997; Bartsch et al, 1981). A stable disease (SD) was observed in 19/35 (54%) patients (non-small cell lung cancer: 5; small cell lung cancer: 2; colorectal cancer: 4; gastric cancer: 2; pancreatic cancer: 1; biliary tract cancer: 1; prostate cancer: 2; ovarian carcinoma: 2). Then, a disease control (DC), consisting of CR, PR and SD, was achieved in 22/35 (63%) patients. On the contrary, the remaining 13/35 (37%) patients had a progressive disease (PD). The median duration of DC was 8 months (Qureshi et al, 1993; Blazquez et al, 2003; Grotenhermen et al, 2004; Aggarwall et al, 2003; Lodha et al, 2000; John's Gospel; Iguchi et al, 1982; Attanasio et al, 1985; Jankovic et al, 1997; Brzezinski et al, 1997; Bartsch et al, 1981; Regelson et al, 1987). A survival longer than 1 year was achieved in 17/35 (49%) patients and the percentage of 1-year survival observed in patients with DC was significantly higher with respect to that found in those who had a PD (15/22(68%) vs 2/13(15%), P < 0.01). As far as the ratio was found in 21/35 (60%) patients. The mean numbers of TH and T-reg lymphocytes increased and decreased on therapy, respectively, without however statistically significant differences with respect to the pre-treatment values (TH: $592 \pm 46$ vs $544 \pm 38$/mm$^3$; T reg: $226 \pm 28$ vs $277 \pm 22$/mm$^3$). On the same way, CD4$^+$/CD4$^+$ CD25$^+$ mean ratio increased on therapy, without however significant differences ($3.1 \pm 0.4$ vs $2.8 \pm 0.3$). On the contrary, by evaluating the immune variations in relation to the clinical response, a significant decrease in T-reg mean number and a significant increase in CD4+/CD4$^+$ CD25$^+$ mean ratio were observed in patients with DC (T-reg: $189 \pm 14$ vs $268 \pm 217$/mm$^3$, p<0.05; CD4$^+$/CD4$^+$ CD25$^+$: $5.9 \pm 0.3$ vs $2.2 \pm 0.4$, p < 0.01), whereas T-reg mean count enhanced ($309 \pm 28$ vs $284 \pm 25$/mm$^3$) and CD4+/CD4$^+$ CD25$^+$ mean ratio diminished ($2.6 \pm 0.5$ vs $2.9 \pm 0.3$) in patients with PD, even though none of these differences was statistically significant. TH means number enhanced ($686 \pm 38$ vs $584 \pm 41$/mm$^3$) in patients with DC and decreased ($576 \pm 46$ vs $598 \pm 37$/mm$^3$) in patients with PD, without however significant differences. A lack of both spiritual sensitivity and pleasure feeling at the Rorschach test was observed in 21/35 (60%) patients. Moreover, the percentage of DC obtained in patients expressing pleasure and spiritual sensitivity at the Rorschach test was significantly greater with respect to that achieved in patients with suppression of both pleasure and spirituality (12 /14(86%) vs 10/21(48%), p<0.05). On the same way, the mean values of the spiritual score were significantly higher in patients who achieved a DC than in those who had a PD ($72 \pm 4$ vs $53 \pm 3$, p<0.025). The

treatment was well tolerated in all patients. A mild transient diarrhoea, due to the laxative action of aloine, occurred in only 4/35 (11%) patients. Moreover, a clear improvement in the well being was reported in 14/22 (64%) patients with DC and in only 3/13 (23%) patients with PD. This difference was statistically significant (P < 0.05). Finally, in none of the patient the neoplastic cachexia occurred.

## IV. Discussion

This preliminary biotherapeutic study shows that a biological strategy consisting of the pineal hormone MLT, Aloe and Myrrha, each of who has been proven to play antitumor activity (Davis et al, 1991; Claeson et al, 1991; Bartsch et al, 1981), may induce a control of the neoplastic growth in a relevant percentage of metastatic cancer patients, for whom no other standard antitumor therapy was available. Moreover, this study demonstrates that the control of the neoplastic disease achieved by this biological strategy may influence the clinical course of the neoplastic disease, a prolonged survival with respect to that observed in patients, who had no benefit from the treatment. In particular, by comparing these results with those historically obtained with MLT alone (Lissoni 2002; Maestroni 1993) it seems that Aloe and Myrrh association further amplify the anticancer action of MLT (Lissoni 2002; Maestroni 1993). Therefore these preliminary data would justify successive randomized trials with MLT alone vs. MLT plus Aloe and Myrrh to confirm the greater efficacy of a polytherapy with several biological natural agents, with respect to single agent. In addition, this study would suggest that the therapeutic efficacy of this natural biological regimen is mainly mediated by the immune system by piloting in an antitumor way the host immunobiological reaction and in particular it seems to be able to counteract advanced cancer-related abnormally enhanced function of T-reg cell system, which would represent the main cause responsible for the lack of an effective anticancer immune reaction in the disseminated neoplastic disease (Shevach 2002). Finally, this study would seem to suggest that the efficacy of an anticancer immunobiological regimen, consisting of MLT, Aloe and Myrrha, may be influenced by both psychological and spiritual status of patients and in particular the evidence of a suppression of both pleasure and spiritual feeling may predict a reduced efficacy of the treatment in terms of control of the neoplastic growth. Generally, the Oncologists subdivide the medical treatments of cancer into curative and palliative therapies, by commonly considering as antitumor curative drugs the only chemotherapeutic agents. From this point of view, a clinical approach with natural biological anticancer agents, which is generally considered as a complementary medicine, cannot be simply defined as palliative treatment, because of its capacity of counteracting cancer cell proliferation also in patients for whom there was no other standard anticancer therapy. Further promising results in terms of control of the neoplastic progression could be achieved by considering that MLT is not the only anticancer hormone produced by the pineal gland (Bartsch

et al, 1981; Regelson et al, 1987; Lissoni et al, 2002; Sze et al, 1993). In fact, at least another pineal hormone, the 5 -methoxytryptamine, may play an anticancer action, with in vitro antiproliferative effects superior to those of MLT itself (Sze et al, 1993). Retinoids play also anticancer effects through cytodifferentiating and anti-angiogenic activities. In addition, at least five other plants could be successfully employed in the treatment of human neoplasms (Blazquez et al, 2003; Grotenhermen et al, 2004; Aggarwall et al, 2003; Lodha et al, 2000), including Hyssopus, Cannabis Indica, Turmeric and Incense may play anticancer effects. Moreover, Hyssopus, whose potential anticancer activity would be due to diosmine, could be particularly useful in the treatment of lung cancer patients, because of its very potent expectorating activity (Lodha et al, 2000). Cannabis Indica contains several cannabinoid agents provided by direct anticancer antiproliferative and anti-angiogenic actions (Blazquez et al, 2003; Grotenhermen et al, 2004). Finally, according to preliminary studies (unpublished data), curcumin, the main active anticancer molecule produced by turmeric (Aggarwall et al, 2003), would be particularly useful in the treatment of cancer of pancreas. Therefore, further studies will be required to establish which may be the best biological natural anticancer combination, by considering the therapeutic and the supportive care effects, the toxicity and the social coast of the various potential both endogenous and exogenous natural antitumor substances.

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# A PsychoNeuroEndocrineImmune (PNEI) Approach to Enhance the Efficacy of Radiochemotherapy in Glioblastoma

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## Abstract

GBM would represent perhaps the only tumor, whose prognosis had achieved no evident benefits in terms of survival from the main oncological therapies, including chemotherapy, immunotherapy and anti-angiogenic treatments. According to the recent advances in the Psychoneuroendocrinology, an improvement in GBM therapy could arise from the knowledge of the psychoneuroendocrine mechanisms responsible for GBM cancer cell growth, and, at present, it has been proven that GBM cells may express opioid receptors, whose activation stimulate cancer proliferation, whereas melatonin (MLT) and other pineal indole hormones, namely the 5-methoxytryptamine (5-MTT), may suppress GBM growth. In addition, several plants, such as Aloe, Myrrh, Boswellia, Magnolia and Cannabis Indica, have appeared to exert an anticancer activity on several tumor histotypes, including GBM. On these bases, a study was planned by associating a neuroendocrine and phytotherapeutic combination to the standard therapy of GBM with radiotherapy (RT) plus chemiotherapy of temozolomide (TMZ). The study included 30 consecutive patients with histologically proven GBM after radical or palliative surgery. The neuroendocrine regimen consisted of an oral administration of MLT at 100 mg/day in the dark period plus 5-MTT at a dose of 5 mg/day in the light period of the day plus the opioid antagonist naltrexone (NTX) at escalating doses until a maximal dosage of 50 mg/day in the morning. The phytotherapeutic regimen included Aloe, Myrrh, Magnolia and Boswellia. Finally, patients were randomized to received also Cannabis infusion. A disease control, including partial response and stable disease, was achieved in 16/30 (53%) patients, and it was associated with a survival longer than 1 year in 17/30 (57%) patients. At the end, the 3-year survival achieved in patients concomitantly treated by Cannabis was significantly higher that that found in patients, who received no Cannabis therapy. This preliminary study would suggest that a neurorendocrine approach, carried out to biologically counteract GBM growth, in association with the standard therapy with RT plus TMZ may increase the overall survival of GBM patients.

## Introduction

Brain glioblastoma (GBM) still remains the most untreatable neoplastic disease. Several factors have been taken into consideration to identify possible subtypes of GBM with different prognostic behaviour, but, at present, the main prognostic factors would be represented by age, performance status (PS) and methyl-guanine DNA-methyltransferase (MGMT). The prognosis is worse in aged patients and in those with low PS. In patients 60 year older the overall survival time is generally less than 6-9 months [1,2]. In contrast, patients positive for MGMT expression would have a longer survival and a better response to chemotherapy [3]. Almost all clinical therapeutic studies, performed up to now, have been carried out with the only radiotherapy (RT) and chemotherapy (CT). Only the temozolomide (TMZ) has been substantially used as potentially active chemotherapeutic agent, without taking into consideration the possible existence of endogenous growth factor stimulating GBM cancer cell growth, as well as estrogens for breast cancer and androgens for prostate cancer, and, on the other hand, pos-

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sible endogenous inhibitory factors on GBM cancer cell proliferation. In fact, it is known since many years that GBM cells may express mu-opioid receptors and that mu-opioid agents may stimulate GBM cancer cell growth [4].

Therefore, the evidence of tumor mu-opioid receptors expression would be associated with a poor prognosis, because of the stimulatory action of mu-opioid agonists, such as beta-endorphin, on cancer growth. On the contrary, the pineal indole hormones [5] and the cannabinoid agonists from Cannabis Indica [6] have been proven to inhibit GBM cell proliferation. Melatonin (MLT) represents the most investigated pineal hormone provided by a well documented anticancer activity on several tumor histotypes, including GBM [5], but at least another pineal indole hormone, the 5-methoxytryptamine (5-MTT), has appeared to exert in vitro an anticancer action superior to that of MLT itself [7]. In addition, cancer progression has been proven to be associated with a progressive decline in MLT secretion, mainly during the night [8], and most in general with a diminished pineal endocrine function [9]. MLT exerts its effects by acting on specific MLT receptors MT 1 and MT 2 ([10], and it has been demonstrated that tumor expression of MLT receptors are associated with a better prognosis in cancer patients [11]. Pineal deficiency may be corrected by an exogenous administration of the main pineal indole hormones, whereas the stimulatory activity of brain opioid system may be counteracted by the administration of the long-acting opioid antagonist naltrexone (NTX) [12].

On the basis of these data and according to a neuroendocrine strategy, it seems to be justified the employment of pineal hormones and opioid antagonists in the treatment of GBM in association with the standard radiochemotherapeutic regimen or after progression on RT plus CT with TMZ. This preliminary phase 2 study was carried out to evaluate the impact of a neuroendocrine schedule with pineal hormones and opioid antagonists in association with the standard therapy by RT plus CT in the treatment of GBM.

## Materials and Methods

The study included 30 consecutive GBM patients, who underwent the standard treatment with RT plus CT with TMZ in association with a neuroendocrine regimen consisting of the oncostatic pineal hormones MLT and 5-MTTplus the mu-opioid antagonist NTX. Eligibility criteria were as follows: histologically proven GBM, measurable lesions, macroscopically radical or palliative surgery, and life expectancy less than 1 year. The experimental protocol was explained to patients, and their consent was obtained. The clinical characteristics of patients are reported in Table 1. The standard treatment consisted of RT 60 Gy in 2-Gy 30 fractions plus TMZ at 75 mg/m$^2$/day orally during RT, followed by 6 cycles of TMZ at 200 mg/m$^2$/day for 5 consecutive days every 28 days. The pineal endocrine therapy consisted of an oral administration of MLT at 100 mg/

day during the dark phase of the day plus 5-MTT at a dose of 10 mg/day during the light phase of the day, corresponding to the time of their circadian secretion. Moreover, in case of progression, because of the evidence of a dose-dependency in its antitumor activity [13], MLT dose was increased of 100 mg/day every time, until a maximal dosage of 500 mg/day. NTX was given orally at a daily dose of 50 mg, starting with a dose of 20 mg/day in the morning by slowly increasing the dose of 10 mg every month in an attempt to reduce liver toxicity of NTX [12]. The supportive care with natural agents consisted of the oral administration of antitumor plants, including a mixture of Aloe arborescens [14] plus Myrrh [15] ( 60/ 40% ratio) at a dose of 10 ml thrice/day, Magnolia cortex at 500 mg twice/day [16], and Boswellia [17], also provided by an anti-oedema activity, at 1000 mg twice/day. At the end, according to their free adhesion and compliance, patients were randomized to receive also Cannabis flos (19% tetra-hydro-cannabinol) as an infusion of Cannabis 0.5 mg/ liter of water, by drinking it at 100 ml three times/day. The clinical response was evaluated by WHO criteria. Data were statistically analyzed by the chi-square test, the Student's test and the log-rank test, as appropriate.

| M / F | 20 / 10 |
|---|---|
| Median age (years) | 65 (range 21-75) |
| Median PS (ECOG) | 1 (range 0-3) |
| Tumor sites | |
| - Frontal cortex | 13 |
| - Temporal cortex | 7 |
| - Temporo- parietal cortex | 5 |
| - Occipital cortex | 2 |
| - Corpus callosum | 3 |

**Table 1.** Clinical characteristics of 30 GBM patients.

## Results

The clinical response (WHO) is shown in Table 2. A macroscopically radical surgery was obtained in no patient. No complete response (CR) was achieved after RT plus CT. A partial response (PR) was obtained in 3/13 (23%) patients treated also by Cannabis and in none of the 17 patients, who received no Cannabis infusion. A stable disease (SD) occurred in 6/17 patients treated without Cannabis and in 7/13 patients under Cannabis treatment. Therefore, the percentage of disease control (DC) (PR + SD) obtained in patients concomitantly treated with Cannabis was significantly higher with respect to that found in patients, who did not receive Cannabis infusion (10/13 (77%) vs 6/17 (35%), P < 0.05). The percent of 3-year survival is illustrated in Figure 1. A survival longer than 1 year and than 3 years was achieved in 17/30 (57%) and in

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5/30 (17%) patients, respectively. Moreover, the percentage of 3-year survival achieved in patients concomitantly treated by Cannabis was significantly longer than that found in patients who did not receive Cannabis infusion (4/13 (31%) vs 1/17 (6%), P < 0.05).

| CLINICAL RESPONSE + | | | | | | |
|---|---|---|---|---|---|---|
| PATIENTS | n | CR | PR | SD | DC | PD |
| ALL PATIENTS | 30 | 0 | 3 | 13 | 16 | 14 |
| CANNABIS | 13 | 0 | 3 | 7 | 10 * | 3 |
| NO CANNABIS | 17 | 0 | 0 | 6 | 6 | 11 |

+ CR: complete response; PR: partial response; SD:stable disease; DC (CR +PR+SD): disease control; PD: progressive disease.

* P <0.05 vs no Cannabis

**Table 2.** Clinical response (WHO) to radio-chemotherapy plus neuroendocrin approach plus or without Cannabis infusion in GBM patients.
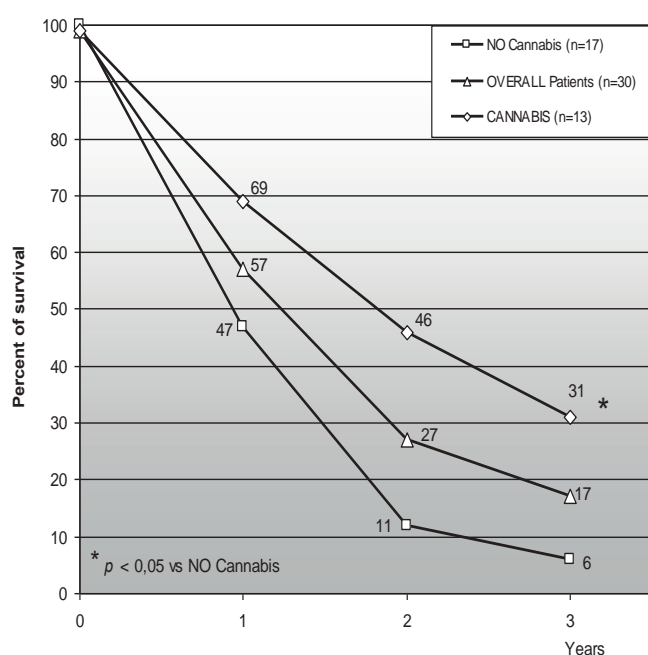


**Figure 1.** Survival curves in GBM patients on neuroendocrine therapy with or without Cannabis infusion.

The neuroendocrine treatment was well tolerated in all patients. No biological toxicity occurred, and the only side-effect was sleepiness or paradoxical excitation for few days in 5/30 (17%) under high-dose MLT administration. On the contrary, most patients referred an improvement in their mood and a mild relief of asthenia. Finally, no cancer progression-related cachexia occurred.

## Discussion

With respect to the expected survival time and by considering that most GBM patients included in the clinical investigation were 60-year older, therefore with an expected survival time generally less than 9 months [1, 2], the results of this preliminary study would show that the survival of GBM patients may be improved by associating to the standard radio-chemotherapy schedule the administration of an oncostatic neuroendocrine regimen, consisting of antitumor pineal hormones plus opioid antagonists in association with plants with well documented anticancer antiproliferative immunomodulating properties. Obviously, randomized clinical studies will be required to confirm the therapeutic efficacy of a concomitant neuroendocrine phytotherapeutic combination in association with the standard radio-chemotherapy in the treatment of GBM. However, the survival achieved by this combination has been clearly superior to that described by previous clinical studies of GBM patients treated with MLT alone after progression under RT [18]. Moreover, this study would suggest that the further association of cannabinoids may prolong the survival time with respect to GBM patients, who did not received Cannabis infusion. This evidence is not surprising, since cannabinoid have been proven to exert direct antiproliferative and anti-angiogenic effects on several tumor histotypes, including brain GBM [6]. The evaluation of mu-opioid [4] and MLT receptor expression [10, 11] on GBM cells could identify possible subgroups of tumors with different prognostic profiles, and in more detail cancer expression of MT receptors could predict a better prognosis [11], whereas that of mu-opioid receptors would be associated with a poor prognosis, because of the stimulatory role of opioids on GBM cancer cell proliferation [4]).Therefore, the identification of MLT and opioid receptor expression on GBM cells could allow to identify possible subgroups of patients, who could obtain more benefits from a neuroendocrine approach with pineal hormones and opioid antagonists.

In conclusion, this study would simply represent only the first suggestion to further explore the therapeutic efficacy of a neuroendocrine strategy in the treatment of GBM, consisting of the administration of the same endogenous hormones provided by an anticancer activity on GBM cell growth, namely the pineal hormones, in association with opioid antagonists to inhibit the brain opioid system, which would play a stimulatory role on GBM development [4].

Further therapeutic results could be achieved by associating the neuroendocrine-approach in GBM therapy to the more recent immunotherapeutic techniques with anti-immune check-point monoclonal antibodies, mainly those against CTLA-4 and PD-1 [19-21].

## Acknowledgements

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# The Psychoneuroimmune Pathogenesis of Cancer: Therapeutic Strategy to Normalize Cancer-Related Brain Unbalance Between Hyperfunction of Opioid System and Hypofunction of Cannabinoid-Pineal Axis by Antitumor Pineal Indoles, and the Mu-Opioid Antagonist Naltrexone in Untreatable Advanced Cancer Patients

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**Abstract**

Today it is known that *in vivo* the immune reactions cannot be separated from their neuroendocrine regulation, which is mainly mediated by the brain opioid system and by the functional unit constituted by brain cannabinoid system and pineal gland. The opioid system is active in stress and depression conditions, and it mediates the suppression of the anticancer immunity. On the contrary, the pineal-cannabinoid functional system, which is involved in the perception of pleasure and mind spiritual expansion, stimulates the anticancer immunity, by playing a fundamental role in the natural resistance against cancer. Then, cancer progression would be due to an unbalance between hypoactivity of the cannabinoid-pineal system and hyperactivity of the opioid system, which could be corrected by a substitute therapy of the main antitumor pineal hormones, including Melatonin (MLT) and 5-Methoxytryptamine (5-MTT) in association with cannabinoids to normalize the cannabinoid-pineal function, and by the administration of opioid antagonists, such as Naltrexone (NTX) to counteract the opioid hyeractivity. The present study was carried out to evaluate the influence of a concomitant NTX administration in advanced cancer patients, for whom no other conventional anticancer therapy was available, and who had progressed under a complementary therapy with the only pineal hormones. The study included 14 untreatable solid tumor cancer patients. All drugs were given orally every day without interruption according the following schedule: MLT at a dose of 100 mg/day in the dark period of the day, 5-MTT at 10 mg/day in the light period of the day, and NTX at 20 mg in the evening. A control of tumor growth was achieved in 8/14 (57%) patients, and it was associated with an improvement in Lymphocyte-To-Monocyte Ratio (LMR). These preliminary results would suggest that the concomitant block of the opioid system by NTX may allow a control of tumor growth superior to that, which may be obtained with the only pineal antitumor hormones, and this effect would be mediated at least in part by an improvement in the immune status, as suggested by the rise in LMR values. Further promising antitumor results could be achieved with the association of cannabinoid agonists, or with Fatty Acid Amide Hydrolase (FAAH) inhibitors to enhance brain cannabinoid content.

Keywords: Cancer; Cannabinoid system; Melatonin; Naltrexone; Opioid system; Pinealgland

## Introduction

The different hypotheses concerning the possible influence of the psychological and spiritual status on cancer onset and development[1-3], have finally found their confirmation on scientific bases after the discovery of the fundamental role of the immunity in tumorcell growth inhibition[4], and the existence of a psychoneuroendocrine regulation of the immune responses, including the anticancer immunity[5-8]. Then, the main responsible for the natural biological resistance against cancer is the immune system, whose function however, is under a neuroendocrine regulation. Despite the great complexity of the NeuroImmunoModulation (NIM), it is possible to identify two major brain interneuronal immune modulatory systems, consisting of the opioid system[5,6], and the endocannabinoid system[7,8] through its functional connections with the pineal gland[9,10]. The opiod system, namely through a mu-opioid receptor, may inhibit the antitumor immunity[5,6], whereas the pineal-cannabinoid system axis stimulates the antitumor immunity[7,8,11]. The opioid system is active in stress, depression, and anxiety conditions, whereas the pineal-cannabinoid axisis operating in the perception of pleasure and spiritual expansion of mind. This evidence may constitute the explanation of the protumoral influence of stress and depression, and on the other side the preventing antitumor effect of pleasure and spirituality on tumor growth[3]. In more detail, the mu-opioid agonists, such as beta-endorphin and morphine, have been proven to play a pro-tumoral action through several mechanisms, including a direct proliferative activity, an angiogenic action, and a suppression of the antitumor immunity by inhibiting the secretion of IL-2 and IL-12, which represent the main anticancer cytokines in humans, respectively from T Helper-1 (TH1) lymphocytes, and by stimulating that of immunosuppressive cytokines, namely TGF-beta and IL-10, from regulatory T lymphocytes (T reg)[5,6]. On the other hand, both pineal and brain cannabinoid system play a natural anticancer activity[9-11]. The pineal gland has appeared to represent the main immunomodulating organ in the human body by modulating the cytokine network[11-14] through the light/dark circadian release of the indole Melatonin (MLT)[11], other less investigated indole, suchas the 5-Methoxy-tryptamine (5-MTT)[15], and beta-carbolines, namely the pinealine[16], all provided by anticancer activity, even though the mechanisms of action have been clarified for the only MLT. The pineal is the main anticancer organ in humans, and it counteracts tumor growth through several mechanisms, including a direct antiproliferative cytotoxic action, an anti-angiogenic activity, and an antitumor immunostimulatory effect, namely consisting of a direct stimulation of IL-2 and IL-12 secretions[11-14], while the cannabinoid agents would play an anticancer activity namely by a direct inhibition of cancer cell proliferation, whereas their effects on the antitumor immunity are still controversial[7,8]. The pineal gland may modulate the activity of both brain opioid and cannabinoid systems, but the pineal gland would namely consitute an unique fundamental functional axis with the cannabinoid system in mediating the perception of pleasure and the spiritual expansion of consciousness through a reciprocal stimulatory influence, since CB1 cannabinoid agonists may directly stimulate MLT release from the pineal gand[9], and on the other side MLT has been proven to contribute to the inhibition of Fatty Acid Amide Hydrolase (FAAH)[10], the enzyme responsible for cannabi-noid degradation[7,8], with a following increase in brain cannabinoid content. Then, the functionless of cannabinoid-pineal system constitutes a fundamental requirement for the status of health. The recent advances in Psycho Neuro Endocrino Immunology (PNEI) researches have shown than cancer progression is associated with a progressive unbalance between brain opioid and endocannabinoid systems, consisting of the association between hyperfunction of the opioid system and hypofunction of the cannabinoid system[7,8,17]. The cannabinoid hypofunction would be at least in part a consequence of the progressive decline in the pineal function with cancer progression, which constitutes the main cancer-related endocrine deficiency[11,18] because of the interactions occurring between pineal and cannabinoid system[9,10]. This unbalance would already explain cancer progression, because of the protumoral role of the opioid system and the anticancer one of the cannabinoid system. The existence of a cancer-related brain opioid system hyperactivity is documented by the evidence that the concomitant administration of the mu-opioid antagonist Naltrexone (NTX) may abolish the promoting effect of stress on cancer development[17]. On the other side, the occurrence of cancer-related brain cannabinoid system hypofunction would be suggested by the evidence of a progressive decline in pleasure perception, the so-called anaedonia, with tumor progression, because of the fundamental role of the cannabinoid system in the perception of pleasure, including appetite and sexual interest[7,8]. The endogenous cannabinoid system may be clinically investigated by measuring the blood or liquoralconcentrations of the two main endogenous cannabinoids, the Arachidonyl-Ethanol-Amide (AEA), the so-called anandamide because of its psychedelic effects, and the 2-Arachidonyl-Glycerol (2-AG), or in a more synthetic manner by the simple detection of the blood levels of FAAH, the main enzyme involved in cannabinoid metabolism and degradation[7,8,19], since it has been shown that high blood levels of FAAH are associated with abnormally low concentrations of both AEA and 2-AG, by reflecting a condition of cannabinoid hypofunction, whereas low FAAH levels allow increased cannabinoid concentrations[20], as an expression of cannabinoid hyperactivity. Moreover, it has been shown that the evidence of an enhanced FAAH synthesis or activity, which allows an endogenous cannabinoid deficiency, may induce a chronic inflammatory status[21], because of the anti-inflammatory activity of the cannabinoid system, mainly due to an inhibition of IL-17 secretion from TH17 lymphocytes[7,8]. Finally, it has been shown that the inflammatory response induced by the increased levels of FAAH may exert a negative prognostic significance in cancer, cardiovascular diseases, and neurodegenerative pathologies[21]. On the contrary, the inhibition of FAAH synthesis, with a following increase in the endogenous content of cannabinoids, has been proven to exert a therapeutic action in several human diseases by counteracting the inflammatory response[19-21]. Therefore, in addition to its importance in the perception of pleasure and consciousness status,the endocannabinoid system would play a fundamental role in maintaining the status of health, including the cardiovascular function and the immuno-inflammatory response. At present, one of the most simple FAAH inhibitors is represented by the same Cannabidiol (CBD), the non-psychotropic agent of Cannabis[7,8,22]. On the contrary, no study has been performed up to now in an attemp to evaluate the influence of the pineal gland and its main hormone

MLT on FAAH synthesis and activity. On the same way, no study has been carried to investigate the interactions occurring between FAAH activity and heart endocrine function, namely consisting of the secretion of atrial natriuretic peptide [ANP] and endothelin-1 [ET-1]. The anti-inflammatory action of ANP[23] and the pro-inflammatory one played by ET-1[24] could be due at least in part to a possible inhibitory effect of ANP and a possible stimulatory action of ET-1, respectively, on FAAH activity. Cancer-related opioid system hyperfunction may be simply blocked by the administration of the mu-opioid antagonist NTX, while the cannabinoid-pineal hypofunction may be corrected by the exogenous administration of pineal indoles and cannabinoid agonists. Therefore, the use of cannabinoids in cancer therapy could deserve not only palliative benefits, but it could also influence cancer progression itself because of the antitumor role of the cannabinoids agents[7,8]. FAAH inhibitors could be also successfully used to correct cancer-related cannabinoid system deficiency. On the contrary, there are controversial results concerning the use of the opioid antagonists, such as NTX, in Oncology, since either low-dose[25] or high-dose NTX[17,26], have been proposed, respectively in an attempt to modulate opioid receptor sensitivity, or to completely block the functionless of the opioid system and its immunosuppressive and protumoral activity. On the basis of the evidence of cancer-related opioid hyperactivity in association with cannabinoid failure, a study was performed to evaluate the impact of a correction of cannabinoid-pineal axis deficiency by pineal indoles and cannabinoid agents in association with a control of opioid system hyperfunction by the mu-opioid antagonist NTX on tumor growth and survival in advanced cancer patients, for whom no other standard anticancer therapies were available.

## Patients and Methods

The phase II study included 14 consecutive untreatable advanced solid tumor patients (M/F: 8/6; median age 62 years, range 54-81), for whom no other effective standard anticancer therapy was available, and who had already been under complementary medicine with the two main pineal antitumor hormones, consisting of Melatonin (MLT) and 5-Methoxytryptamine (5-MTT)[14,15], according to previous clinical experimental studies[27], both orally with MLT at a dose of 100 mg/day in the dark period of the day and 5-MTT at a dose of 10 mg during the light period of the day. Eligibility criteria were, as follows: histologically proven solid tumor, measurable lesions, no double tumor, no chronic therapy with opioids to avoid the possibile NTX-induced withdrawal syndrome, no availability olf other standard anticancer treatments, and progression under a previous therapy with the only pineal hormones. Tumor histotypes were, as follow: Glioblastoma (GBM): 7; malignant astrocytoma: 3; colon cancer: 1; gastric cancer: 1; pancreatic adenocarcinoma: 1; lung adenocarcinoma: 1. The clinical response was assessed by the more appropriate radiological examinations, and according to the WHO criteria. Under the previous therapy with the only pineal indoles, a disease control (DC) was obtained in 9/14 (64%) patients, consisting of partial response (PR) in 1 and stable disease (SD) in 8, whereas the remaining 5 patients had a Progressive Disease (PD). After disease progression, patients received the same doses of pineal indoles with the association of the mu-opioidantagonist

NTX at an oral dose of 20 mg/day in the evening, by evaluating the clinical response after 3 months of therapy. The experimental study was explained to each patient, and written consent was obtained. Moreover, on the basis of the well demonstrated negative prognostic significance of low values of lymphocyte-to-monocyte ratio (LMR)[29] because of the antitumor immunostimulatory and immunosuppressive role of lymphocytes and monocytes, respectively[30], LMR wase valuated at weekly intervals. Normal values of LMR obtained in our laboratory (95 % confidence limits) was greaterthan 2.1. Moreover, becuse of the possible hepatotoxicity of NTX, transaminase levels were also particularly monitored. Data were statistically analyzed by the chi-square t, and the Student's t test, as appropriate.

## Results

No complete response was observed in patients under therapy with pineal indoles plus NTX. A partial response (PR) was achieved in one patient with GBM. Seven other patients had a SD. Then, a DC (PR + SD) was achieved in 8/14 [57%], whereas the other 6 patients had a PD. The percentage of DC was higher in patients, who had already obtained a DC under the previous therapy with the only pineal indoles than in those who had a PD, event hough the difference was not statistically significant because of the low number of cases[6/9 (67%) VS 2/5 (40%)]. As far as the immune response is concerned, abnormally low pretreatment values of LMR were seen in 6/14 (43%) patients. The percentage of DC achieved in patients with low LMR values prior to therapy was lower than that achieved in patients with normal pretreatment LMR values, without, however, significant differences [5/8 (63%) vs 3/6 (50%)]. Moreover, as illustrated in Figure 1, LMR mean values increased in patients who achieved a DC, and decreased in those with PD with respect to the pretreatment values, even though the differences were not statistically significant. However, LMR mean values observed after 3 months of therapy in patients with DC were statistically significantly higher than those found in patients with PD (P < 0.05). No toxicity occurred on treatment, and in particular no important transaminase increase was observed under NTX administration.


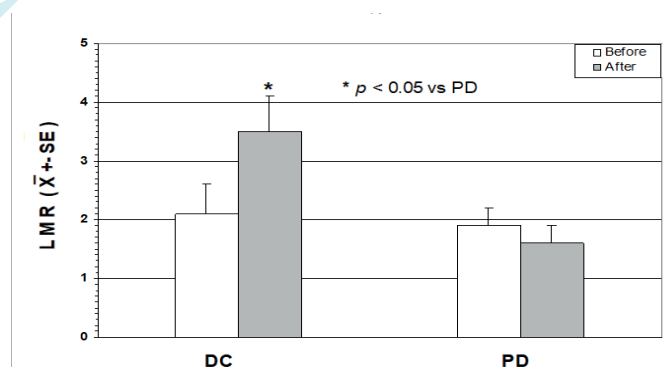
**Figure 1:** Lymphocyte-to-monocyte (LMR) in patients with disease control (DC) or progressive disease (PD) before and after 3 months of therapy

## Discussion

According to a possible psychoneuroendocrine of cancer, which

considers cancer-related immunosuppression of a consequence of an unbalancebe tweenopioid and cannabinoid-pineal systems, which represent the two main brain neuroimmunomodulatory systems, this preliminary phase II study seems to suggest that the association of a block of the opioid system through the administration of a mu-opioidantagonist, suchas NTX, may allow a further control of tumor growth in advanced cancer patients, for whom no other effective standard anticancer therapy was available, and who had already received some benefits from the previous therapy with the only most investigated pineal antitumor hormones, including MLT and 5-MTT, which may reactivate the functionless of cannabinoid-pinealaxis[7-10]. This finding is not surprising, since cancer-related neuroimmune alterations do not consist of the only endogenous cannabinoid system deficiency, but also on a concomitant hyperacitivy of the opioid system[17], which may be counteracted by the administration of the opioid antagonist NTX. This statement is particularly justified in the case of brain tumors, since their expression of opioid receptors has been proven to predict a greater biological malignancy and a worse prognosis[28]. Finally, the improvement in the efficiency of the antitumor immunity, as shown by the increase in LMR, observed in patients, who achieved a DC under NTX therapy, would suggest that NTX may stimulate the anticancer immunity by counteracting opioid system-mediated immunosuppression occurring in cancer. Obviousy, further studies will be required to better define the immunomodulating effects of NTX, particularly on regulatory T lymphocytes (T reg), which are the main suppressive regulator of the anticancer immunity, since in experimental conditions it has been shown that NTX may counteract T reg cell generation and activation[29]. However, since LMR represents a synthetic parameter reflecting the relation between antitumor immunostimulatory and protumoral immunosuppresive events, respectively exerted by lymphocyte and macrophage systems[30], LMR increase in patients, who obtained a control of tumor growth on NTX administration, would suggest that NTX-induced block of brain opioid system may contribute to cancer control by also improving the antitumor immunity. Further therapeutic results in terms of control of the clinical course of the neoplastic disease in cancer patients, for whom no other conventional treatment is available, could be achieved by the association with another antitumor pineal hormone of beta-carboline nature, the pinealine[16], as well as by the direct administration of cannabinoid agents, or cannabidiol to inhibit FAAH activity[7,8,22], with a following increase in brain cannabinoid content and function.

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Research Article

# A Study on the Influence of Spirituality on the Efficacy of Antitumor Therapies with Natural Anticancer Agents in Untreatable Metastatic Cancer Patients

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## Abstract

The recent discoveries of the existence of natural anticancer agents either from plants, such as Aloe, Myrrh and Magnolia, or from the human body, namely the pineal hormones, allowed the possibility to elaborate new therapeutic natural combinations as a link between the commonly used palliative and curative cancer therapies, which would have not considered in a separate manner. The present study was carried out to evaluate the influence of the spiritual status on the efficacy of a natural anticancer combination containing pineal anticancer hormones in association with Aloe, Myrrh and Magnolia extracts in a group of 70 untreatable metastatic solid tumor patients with life expectancy less than 1 year. The spiritual sensitivity was evaluated by an appropriate faith test for patients affected by an untreatable disease. The percentages of both objective tumor regressions and disease control obtained in patients with high faith score were significantly higher with respect to those found in patients with low faith score. On the same way, the 3- year percent of survival achieved in patients with high faith score was significantly longer than that found in the other group. This study would suggest the efficacy of an antitumor therapeutic strategies with natural anticancer agents also in metastatic cancer patients form whom no other standard antitumor treatment was available, with a greater efficacy in the presence of a real status of spiritual faith.

*Keywords: spirituality, cancer disease, psychoneuroimmunology*

## Introduction

Being cancer a biological war between a human host and an apparently unconscious tumor mass, it is obvious that the prognosis of the neoplastic diseases may depend on both tumor characteristics and the psychobiological identity of cancer patients. Tumor characteristics regard histology, disease extension, biological grading and eventual genetic mutations of cancer cells. At the other side, the individual identity of the single cancer patients involves their consciousness status, psychological behaviour, life style, but also and mainly their endocrine, neuroendocrine and immune status in addition to their clinical conditions [1]. Until some years ago, the human diseases were considered to be due to organicistic or psychosomatic reasons. On the contrary, with the progressive advances in the area of Psychoneuroendocrinoimmunology (PNEI), it was understood that the psychospiritual status of patients may influence the biological body not only through the nervous system, but also through complex nervous, neuroendocrine and endocrine interactions with the immune cells, which after their activation may interact with the endocrine and nervous systems by releasing immunomodulating proteins, the so-called cytokines, which are also able to exert neuroendocrine effects by realising complex feed-back circuits between neuroendocrine and immune systems [2].

As far as the psychological and spiritual point of view is concerned, must be remarked that until few years ago and yet up to now by most researchers, the spirituality has been simply considered only as a part of the psychological status of humans, and only recently some preliminary clinical investigations have suggested that the spirituality is a different condition from both psychology and religion [3]. As far as the relation between psychology and spirituality is concerned, it is possible to affirm that the Psychology represents the analysis of the emotional life, which has its energetic matrix in the sexuality, whereas the Spirituality regards the reality of the different consciousness states. At the other side, the relation between Religion and Spirituality, according to a definition previously reported in the literature [4], the Spirituality is the research of the ultimate meaning of life, while Religion is only a set of beliefs and ritual practices within a well defined religious institution, then it would simply represents one of the possible ways to realize own self spirituality, even though more widely followed with respect to an individual manner to live and feel the spiritual dimension. Then, the individual spirituality may be realized through the same religion or other mysticai experiences, and it Is not a simpie set of emotions, but it constitutes a status of consciousness. Moreover, in agreement with PNEI discoveries [5], both emotions and consciousness states require a well defined psychoneuroendocrine mediation. Then, from a clinical

point of view, the two major problems concern the identification of adequate methods to clinically investigate not only the religious profile of patients, but also their spiritual sensitivity, as well as of possible eventual blood biochemical parameters able to reflect the psychological and spiritual status of patients and its influence on the clinical course of the neoplastic disease. However, most studies carried out up to now have been generally limited to the investigation of the influence of the personal religion rather than the real status of cancer patients. In any case, even though limited to the investigation of the influence of religion on the prognosis of cancer, preliminary clinical results seem to suggest that the religious support may allow an increase in the survival time of advanced cancer patients and to improve their clinical status, even though through still unknown mechanisms [3, 4]. The recent advances in PNEI knowledgements, by demonstrating that the immune responses in vivo are physiologically under a psychoneuroendocrine modulatory control [6,7], which represents the biochemical mediation of the spiritual and psychological status of the patients, may allow the hypothesis that the spiritual status may influence the clinical course of the neoplastic disease and the efficacy of the different antitumor therapies by stimulating the immune system and piloting it in an antitumor way through the activation of well-defined psychoneuroendocrine circuits [8]. Moreover, it has to be considered that until about 20 year ago, almost all scientific investigations in the oncological area were limited to the identification of possible carcinogens in the nature, either endogenous molecules, such as estrogens and androgens, or exogenous substances, capable of inducing the malignant transformation. On the contrary, more recent researches have demonstrated the existence of several antitumor plants containing well characterized anticancer molecules, in particular aloe hemodin from Aloe [9], guggulsterone from Myrrh [10] and honokiol from Magnolia [11], as well as more surprisingly the evidence of anticancer endogenous molecules, which would be responsible for the natural immunobiological resistance against cancer onset and growth, in particular some indole hormones released by the pineal gland, namely melatonin (MLT) [12] and 5-methoxytryptamine (5-MTT) [13], and the great group of beta-carbolines [14], which are mainly produced by pineal gland itself. All those natural anticancer agents has no important toxicity. Therefore, the existence of both endogenous and exogenous anticancer agents with a complete lack of biological toxicity but with well known antitumor properties, would justify their empioyment in the medical Oncology in an attempt to realize a link between the simple palliative and the curative therapies of cancer, since several anticancer natural agents, according to the PNEI knowledgements, may deserve both palliative and antitumor effects on cancer progression at least in terms of survival time. The present study was performed to investigate the influence of the spiritual status of consciousness on the antitumor efficacy of a psychoneuroendocrine regimen with antitumor pineal hormones in association with the most investigated anticancer plants in a group of metastatic solid tumor patients, for whom there is no other standard effective therapy of their tumor, by evaluating the spiritual status through a previously described clinical test to explore the spiritual faith in patients affected by an untreatable disease [15].

## Materials and Methods

The study included 70 untreatable metastatic solid tumor patients. Eligibility criteria were, as follows: histologically proven metastatic solid neoplasm, measurable lesions, no availability of standard antitumor therapies because of progression on previous chemotherapies, age or low performance status (PS), and life expectancy less than 1 year. Patients affected by metastatic breast cancer or prostate carcinoma were excluded from the study, because of the availability for those tumors of well tolerated hormonal therapies also by the standard medical Oncology. The faith test for patients affected by an untreatable disease employed in the study was performed by the observation of the clinicians in an attempt to exclude possible unconscious mental manipulations in their answers by the patients, and it consisted of the analysis of five major criteria [15], by assigning 20 points to each single criterion, with a maximum score of 100 points and by defining the presence of a real status of spiritual faith for a minimal score of at least 60 points or more. The five criteria were, as follows: 1) complete self-consciousness by the patients of the severity of their diagnosis and prognosis in terns of life expectancy: the absence of an adequate knowledge of the severe prognosis would transform the faith in a simple illusion; 2) lack of excessive anxiety: the anxiety would represent the opposite mental condition with respect to a real spiritual faith; 3) lack of an exaggerated attribution of value by the patients to the professional capacities of the single clinicians, being their disease as considered as untreatable on the basis of the standard medical therapies;4) lack of an excessive analytic tendency by the patients to understand the chemical mechanisms involved in the efficacy of treatments instead of their significance in terms of reactivation of an effective biological natural anticancer resistance; 5) perception of own neoplastic disease not only as a personal problem, despite pain and other intolerable symptoms, but also as an individual manifestation of a general universal suffering involving all humans. The clinical characteristics of patients are reported in Table 1. Lung cancer, pancreatic adenocarcinoma and colorectal cancer were the neoplasms most frequent in our patients. The PNEI strategy of cancer cure consisted of the oral administration of the two most investigated anticancer pineal hormones, MLT and 5-MTT, in association with a phyto-therapeutic regimen consisting of the administration of extracts of the most investigated antitumor plants, including Aloe arborescens, Myrrh and Magnolia. MLT was given at 100 mg/day during the dark period of the day, while 5-MTT was administered at 5 mg in the early afternoon. Magnolia cortex, with a honokiol content of at least 50%, was given at 500 mg twice/day. Finally, Aloe and Myrrh were given at a dose of 10 ml twice/day of a mixture of 60% Aloe and 40% Myrrh. Patients with brain metastases also received Boswellia at 1000 mg/day in the morning, because of its anti-oedema effect. The clinical response was assessed by the WHO criteria by repeating the radiological examinations at 3-month intervals. Data were statisticaliy analyzed by the chi-square test. The survival curves were calculated by the Kaplan-Meyer method and statisticaliy analyzed by the log-rank test.

**Table 1.** Clinical characteristics of 70 untreatable metastatic solid tumor patients.

| CHARACTERISTICS | |
|---|---|
| M/F: | 37 / 33 |
| Median age | 65 years (range 43 — 92) |
| Median PS (ECOG) | 1 (0—3) |
| RELIGIOUS FAITH | |
| - Specific religion: | 29/ 70 (41%) |
| - Catholic Christian religion: | 23 |
| - Protestant Christian religion: | 2 |
| - Oriental Christian religion: | 1 |
| - Buddhism: | 2 |
| - Islam: | 1 |
| - No religion or undefined religion: | 41/70 (59%) |
| *TUMOR HISTOTYPE* | |
| - Lung cancer: | 18 |
| - Nonsmall celi: | 15 |
| - Smail celi: | 3 |
| - Pancreatic adenocarcinoma: | 14 |
| - Colorectal cancer: | 13 |
| - Gastric adenocarcinoma: | 5 |
| - Biliary tract cancer: | 4 |
| - Hepatocarcinoma: | 3 |
| - Bladder carcinoma: | 3 |
| - Gynecoiogic tumors: | 4 |
| - Ovarian cancer: | 3 |
| - Endometrial adenocarcinoma: | 1 |
| - Melanoma: | 2 |
| - Soft tissue sarcoma: | 4 |
| *METASTASIS SITES* | |
| - Soft tissues: | 18 |
| - Bone: | 2 |
| - Lung: | 16 |
| - Liver: | 18 |
| - Liver + lung: | 6 |
| - Peritoneum: | 4 |
| - Brain: | 6 |
| PREVIOUS CHEMOTHERAPY: | 52/70(74%) |

## Results

The clinical response achieved in our patients is reported in Table 2. A complete response (CR) was obtained in 2/70 (3%) patients, who were affected the former by gastric cancer and the latter by lung adenocarcinoma. A partial response (PR) was achieved in other 9 patients (colon cancer: 2; melanoma: 2; lung cancer:1; pancreatic cancer:1; endometrial adenocarcinoma:1; biadder cancer:1; biliary tract carcinoma: 1). Then, an objective tumor regression was observed in 11/70 (16%) patients. A stable disease (SD) was found in other 41

patients. Therefore, a disease control (CR + PR + SD) was obtained in 52/70 (74%) patients, whereas the remaining 18 patients (26%) had a progressive disease (PD). A faith score of at least 60 points was found in 51/70 (73%) patients. By considering faith score in relation to the other individuai variables, no significant differences between males and females was observed in the percent of values of at least 60 points (28/37 (76%) vs 22/33 (67%)). On the same way, no difference in the percent of high faith score occurred in relation to the three most frequent neoplasms (lung: 12/18 (67%); colon: 9/13 (69%); pancreas: 9/14 (64%)). Moreover, more surprisingly there was no significant difference in the percent of faith score of at least 60 between patients who followed a specific religion and those who had no religion or no defined religion (22/29 (76%) vs 29/41 (71%). Finally, by considering the clinical response in relation to the faith score, the percent of objective tumor regressions (CR+PR) achieved in patients with faith score of 60 or more was significantly higher with respect to that found in patients with values iess than 60(11/51(19%) vs 1/19 (5%), P<0.05. On the same way, the percent of DC (CR+ PR+SD) achieved in patients with high faith score was significantly higher than that observed in those with low faith score (44/51(86%) vs 8/19 (42%), P< 0.01. Table 3 shows the clinical response in relation to the differeni values of faith score. A progressive increase in the percent of DC occurred concomitantly with the increase in faith score values. Finally, the 3-year survival curves observed in our patients are illustrated in Figure 1. The percentage of 3-year survival reached by patients with faith score of at least 60 was significantiy higher than that found in patients with low faith score (P<0.05).

**Table 2.** Clinical response (WHO criteria) in 70 untreatable cancer patients in relation to their faith score.

| CLINICAL RESPONSE + | | | | | | | |
|---|---|---|---|---|---|---|---|
| Patients | n | CR | PR | CR+PR | SD | DC | PD |
| Overall patients | 70 | 2 (3%) | 9 | 11 (16%) | 41 | 52 (74%) | 18 (26%) |
| Faith score > 60 | 51 | 2 | 8 | 10 (19%)* | 34 | 44(86%)** | 7 (14%) |
| Faith score < 60 | 19 | 0 | 1 | 1(5%) | 7 | 8(42%) | 11(58%) |

+ CR: complete response; PR: partial response; SD: stable disease; DC (CR + PR + SD): disease control; PD: progressive disease

* P< 0.05 vs low faith score; ** P< 0.01 vs low faith score

**Table 3.** Clinical response (WHO criteria) in 70 untreatable cancer patients in relation to the different values of faith score.

| CLINICAL RESPONSE | | | | | | | |
|---|---|---|---|---|---|---|---|
| FAITH SCORE (points) | n | CR | PR | CR + PR | SD | DC | PD |
| 20 | 5 | 0 | 0 | 0 | 1 | 1 (20%) | 4 (80%) |
| 40 | 14 | 0 | 2 | 2(14%) | 6 | 8 (57%) | 6 (43%) |
| 60 | 33 | 0 | 3 | 3(9%) | 18 | 21(64%) | 12 (36%) |
| 80 | 15 | 1 | 3 | 4(27%) | 9 | 13 (87%) | 2 (13%) |
| 100 | 3 | 1 | 0 | 1(33%) | 2 | 3 (100%) | 0 |

## Discussion

This study, ca rried out in a considerable number of untreatable metastatic cancer patients, would suggest that a neuroendocrine approach with endogenous anticancer molecules, such as the antitumor pineal hormones, and natural antitumor plants, may counteract cancer growth also in patients, who had been considered as untreatable according to the standard anticancer treatments. Moreover, the study shows that the efficacy of therapy is higher in cancer patients with a true spiritual faith, al least in the untreatable ones, even though it cannot be excluded that the reduced therapeutic efficacy observed in patients with low faith score may be simply due to an interruption or a discontinuation of therapy. In any case, even though we are only at the beginning of the possibility to understand the psychochemical mechanisms responsible for mediating the influence of the spiritual faith on the clinical course of the neoplastic diseases, the recent advances in PNEI knowledgements have demonstrated the possibility to modulate the immune system, including the anticancer immunity, by acting on its psychoneuroendocrine regulation [2, 16]. Then, in agreement with the PNEI discoveries, showing a stimulatory effect of both pleasure and spiritual sensitivity and an inhibitory one of stress and depression on the anticancer immunity, it is probable that the increased efficacy of cancer therapies with natural antitumor agents and the prolonged survival time achieved in patients with evidence of spiritual faith may mainly be due to an improvement in the potency of the immune reaction against cancer dissemination [17-19]. Moreover, the study would show that the presence of a real spiritual faith is relatively independent from the adhesion to a specific well defined religion, then it would represent an individual variable rather than to depend on external behaviours, such as the religious practices, by confirming the observations of previous authors, who had considered religion and spirituality as different human conditions [3, 4]. In more detail, since the anticancer action of the pineal hormones and of most antitumor piants is due to both antiproliferative and immunomodulating effects [20], at present, according to the PNEI discoveries, it is possible at present to identify two major functional psychoneuroendocrine systems involved in the mediation of the influence of emotions and spirituality on the anticancer immunity, consisting of the former brain opioid system-pituitary adrenal gland, which is related to stress, pain, anxiety and depression and which plays an inhibitory effect on the anticancer immunity by stimulating T regulatory (T reg) and inhibiting T helper-1 (TH1) lymphocyte functions [21], and the latter brain cannabinergic-mirror neuron-pineal gland functional axis, which on the contrary is related to both pleasure perce1ption and spiritual sensitivity, and which enhances the anticancer immunity by stimulating TH1 and inhibiting T reg activities [22-24]. In any case, both systems would be essential for the survival of the biological species, since the opioid system-pituitary-adrenal gland functional axis would play a fundamental role in the adoptive mechanisms to the environmental and social conditions, while at the other side the cannabinergic system-mirror neuron systems-pineal gland axis would be in relation to the both biological and mind evolution , as suggested by the appearance of cannabinoid receptors in a subsequent time with respect to that of the opioid ones [22], as well as by the evidence of the fundamental role of mirror neurons

in the processes of imitation, learning, language, memory and self-consciousness [23] and of the involvement of pineal molecules, such as the beta-carbolines, in mind expansion [25]. If successive studies will confirm the possibility to prolong the survival time and improve the clinical status of metastatic cancer patients, for whom no other standard therapy may be available, by the administration of natural endogenous and exogenous anticancer molecules, the application of the faith score could allow to predict the probability of efficacy of natural treatments themselves, as well as for the commonly used anticancer therapies in relation to the different tumor histotypes and disease extensions.

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# A Study on the Endocrine Function of Pineal Gland with Regard To Immune Alterations in Cancer Patients

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## Abstract

Several experimental studies have demonstrated that pineal gland plays a key anticancer role through the release of several hormones provided both by cytotoxic and immunostimulatory effects; the best-researched of all is melatonin (MLT). Despite the well documented anticancer role of the pineal gland, few clinical studies have been performed up to now in order to explore the pineal function in cancer patients. However, the results agree with the evidence of a progressive decline in the pineal function during the clinical course of neoplastic disease. Moreover, it is known that cancer progression is associated with a progressive decline in the effectiveness of anticancer immunity, which is mainly activated by lymphocytes and suppressed by monocyte-macrophage system, and which may be clinically investigated by evaluating the simple lymphocyte-to-monocyte ratio (LMR). So far, however, very few data about the possible relation between cancer-related immune and pineal alterations are available. The present study was carried out to evaluate the pineal function in a group of non-metastatic and metastatic cancer patients in relation to the LMR values. The pineal function was investigated by measuring the light and dark urinary excretion of the main MLT metabolite, the 6-sulfatoxymelatonin (6-MTS). A normal light/dark rhythm in the circadian excretion of 6-MTS was present in the non-metastatic patients, whereas a loss of pineal rhythm occurred in the metastatic group. In the same way, a normal light/dark rhythm was present in the patients with normal LMR values, whereas no rhythm was observed in those with abnormally low LMR values. According to the results previously reported by other authors, this study confirms that the metastatic neoplastic disease is characterized by a loss of the light/dark rhythm in the pineal function. Moreover, by showing a relation between the loss of light/dark pineal rhythm and low LMR values, this study would suggest that cancer-related immune alterations may depend at least in part on the altered pineal function,
because of the fundamental immunomodulatory role of pineal itself.

## Introduction

Despite the well demonstrated anticancer role of the pineal gland in experimental conditions [1-3], very few clinical studies have been performed up to now to evaluate the pineal endocrine function in cancer patients [4,5]. In any case, either in animals or in humans, cancer progression has appeared to be constantly associated with a progressive deficiency in the pineal endocrine function, as shown by the progressive decline in the production of the most investigated pineal hormone, the indole hormone melatonin (MLT), which has been proven to play a physiological anticancer activity through either a direct cytotoxic action, or an immunostimulatory effect on the anticancer immunity [6,7]. In more detail, MLT antitumor cytotoxic action is due to several mechanisms, the most important of them are consisting of the induction of tumor cell apoptosis and the inhibition of the action of several tumor growth factors. At the other side, the stimulatory effect of MLT on the anticancer immunity is mainly depending on a stimulation of the main antitumor cytokines in humans, consisting of IL-2 and IL-12. In normal conditions, MLT is mainly secreted during the dark period of the day [8], with a following well defined light/dark circadian rhythm in MLT secretion. MLT, however, is not the only hormone responsible for the anticancer activity of the pineal gland, since at least two other pineal hormones have appeared to exert a direct anticancer cytotoxic action, consisting of the indole 5-methoxytryptamine [9] and the beta-carboline pinoline [10]. Moreover, histological damages of the pineal gland have been demonstrated in patients died from cancer [11]. Therefore, the progressive decline in MLT secretion would not represent the only pineal endocrine deficiency with cancer progression. Therefore, the pineal endocrine deficiency would constitute one of the main biological alterations responsible for cancer

onset and development. The pineal endocrine deficiency could either precede or promote cancer development, or be induced by cancer dissemination. Pineal deficiency may predispose to tumor onset, since it has been demonstrated that a pineal deficiency induced by surgical or pharmacological pinealectomy has been proven to promote cancer development, whereas the administration of pharmacological doses of MLT has appeared to reduce the incidence of both spontaneous and chemically-induced tumors [12]. In fact, all psychological conditions predisposing to cancer, including stress, depression, pleasure repression and changes in light/dark rhythm, are constantly characterized by potential alterations in the pineal endocrine function [8-12]. On the other hand, tumor cells may directly produce the enzyme 2, 3-indole-dioxygenase (IDO) [13], which is able to induce tryptophan depletion and a consequent pineal endocrine deficiency, since all pineal hormones originate from tryptophan itself. In addition to the pineal endocrine deficiency, another fundamental biological alteration stimulating cancer growth is the lack of an effective anticancer immune reaction. Despite its complexity, it is currently known that the anticancer immune response in humans is substantially due to T helper-1 (TH1) lymphocytes through the release of IL-2 and to dendritic cells by the secretion of IL-12 [14,15]. IL-2 plays an anticancer activity by inducing the evolution of NK cells into LAK cells, which are able to destroy fresh human cancer cells irrespectively of their antigenicity [16]; IL-12 instead is able to counter cancer cell proliferation by stimulating cytotoxic T lymphocytes, which exercises an antigen-dependent cytotoxicity, by promoting T lymphocyte differentiation into TH1 cells, by counteracting the generation of regulatory T lymphocytes (T reg) [17], which in contrast suppress the antitumor immunity [18], and by playing an anti-angiogenic activity. MLT stimulates the anticancer immunity through several mechanisms, which may be synthetized into three essential effects: stimulation of IL-2 release from TH1 lymphocytes, stimulation of IL-12 secretion by dendritic cells [7] and inhibition of T reg cell generation [19]. Therefore, due to the stimulatory effect of MLT on the anticancer immunity, cancer-progression related immunodeficiency could depend at least in part on the pineal endocrine failure. Up to now, however, there are no data available on the possible relationship between cancer-related pineal deficiency and immune alterations, which may characterize the advanced neoplastic diseases. Until a few years ago, the clinical evaluation of the immune status of cancer patients required several and expensive immune detections, including the measurement of lymphocyte subsets and cytokine blood concentrations. Recent studies, however, have demonstrated that the simple lymphocyte-to-monocyte ratio (LMR) may reflect the interaction between the anticancer immune reaction, which is mainly mediated by lymphocytes, and the chronic inflammatory status, which is mediated by the monocyte-macrophage system and which suppresses the anticancer immunity by allowing the generation of T reg lymphocytes. Therefore, abnormally low LMR values represent a sign of immunosuppression of anticancer immunity. For this reason LMR may be a simple and inexpensive biomarker for the clinical follow-up of the of anticancer immunity status in cancer patients in relation to the response to the various antitumor therapies and to the clinical course of the neoplastic disease. The present study was performed to investigate which relation may exist between the pineal endocrine function, evaluated by detecting MLT secretion, and the immune status, as synthetized by evaluating LMR, in a group of cancer patients with locally limited or metastatic disease.

## Patients and Methods

The study included 30 consecutive cancer patients affected by the most common neoplasms, 16 of whom showed a metastatic disease. Eligibility criteria were, as follows: histologically proven solid tumor, measurable lesions, and no therapy with drugs potentially influencing MLT secretion from at least 1 week prior to study, including corticosteroids, opioids, beta-blockers and alpha-2 agonists. The clinical characteristics of patients are reported in **Table 1**. MLT secretion was assessed by measuring the urinary daily excretion of its main metabolite, the 6-sulphatoxy melatonin (6-MTS), by comparing the values observed during the light (8.0 AM-8.0 PM) and the dark (8.0 PM-8.0 AM) period of the day. 6-MTS was detected by a commercially available enzyme immunoassay kits (Melatonin-Sulfate Urine – ELISA, IBL INTERNATIONAL GMBH / Tecan Group Company) and the values were reported as mcg/ml. The circadian rhythm of MLT was considered to be within the normal range when 6-MTS values of the dark urinary sample were at least two times greater than those of the light sample. Finally, LMR values were considered to be normal when they were greater than 2.1 (95% confidence limits). Data were reported as mean ± SE, and statistically analyzed by the chi-square test, and the Student's t test, as appropriate.

**Table 1** Clinical characteristics of 30 solid tumor patients.

| Characteristics | n |
|---|---|
| Sex (M/F) | 13/17 |
| Median age (years) | 62 (37-81) |
| **Tumor histotype** | |
| Breast cancer | 10 |
| Lung cancer | 4 |
| Colorectal cancer | 4 |
| Gastric cancer | 3 |
| Pancreatic cancer | 3 |
| Ovarian cancer | 3 |
| Sarcoma | 3 |
| **Disease extension** | |
| Locally limited disease | 14 |
| Metastatic disease (Dominant sites) | 16 |
| Soft tissues | 3 |
| Bone | 2 |

2

| Lung | 2 |
|------|---|
| Liver | 3 |
| Peritoneum | 3 |
| Brain | 3 |

## Results

A normal 6-MTS rhythm, with night values at least greater two times than the light ones, was present in only 14/30 (47%) patients. Moreover, the percentage of patients with normal pineal rhythm observed in the non-metastatic group was significantly higher with respect to that found in the metastatic group (10/14 (71%) vs. 4/16 (25%), P<0.01). Abnormally low values of LMR were seen in 11/30 (37%) patients, and the percentage of patients with low LMR values was significantly higher in the metastatic group than in the non-metastatic one (3/14 (21%) vs. 8/16 (50%), P<0.01). Moreover, the percentage of patients with normal pineal rhythm was significantly higher in the group of patients with normal LMR values than in those with abnormally low LMR values (11/19 (59%) vs. 3/11 (27%), P<0.05). On the other side, the percentage of normal LMR values observed in the group of patients with normal 6-MTS rhythm was significantly higher than that found in patients, who had no rhythm (12/14 (86%) vs. 7/16 (44%), P<0.01). Furthermore, patients with normal 6-MTS rhythm showed significantly higher mean values of LMR than those found in patients, in whom the rhythm was absent (4.7 ± 0.4 vs. 2.3 ± 0.3, P<0.01). Day and night 6-MTS urinary values observed in non-metastatic and in metastatic patients, as well as in patients with normal or abnormally low LMR values are illustrated in **Figure 1**. In patients with locally limited tumor, a light/dark rhythm was still present, with night mean values of 6-MTS significantly higher with respect to those found during the light period of the day (P<0.025). Conversely, no circadian rhythm in 6- MTS production was seen in metastatic patients, since there was no significant difference between day and night 6-MTS mean values. Night mean values of 6-MTS observed in non-metastatic patients were significantly higher than those found in metastatic patients (P<0.05). Likewise, night mean values of 6-MTS observed in patients with normal LMR values were significantly higher than those found in patients with low LMR values (P<0.05), while no significant difference occurred in 6-MTS mean values during the light period of the day.
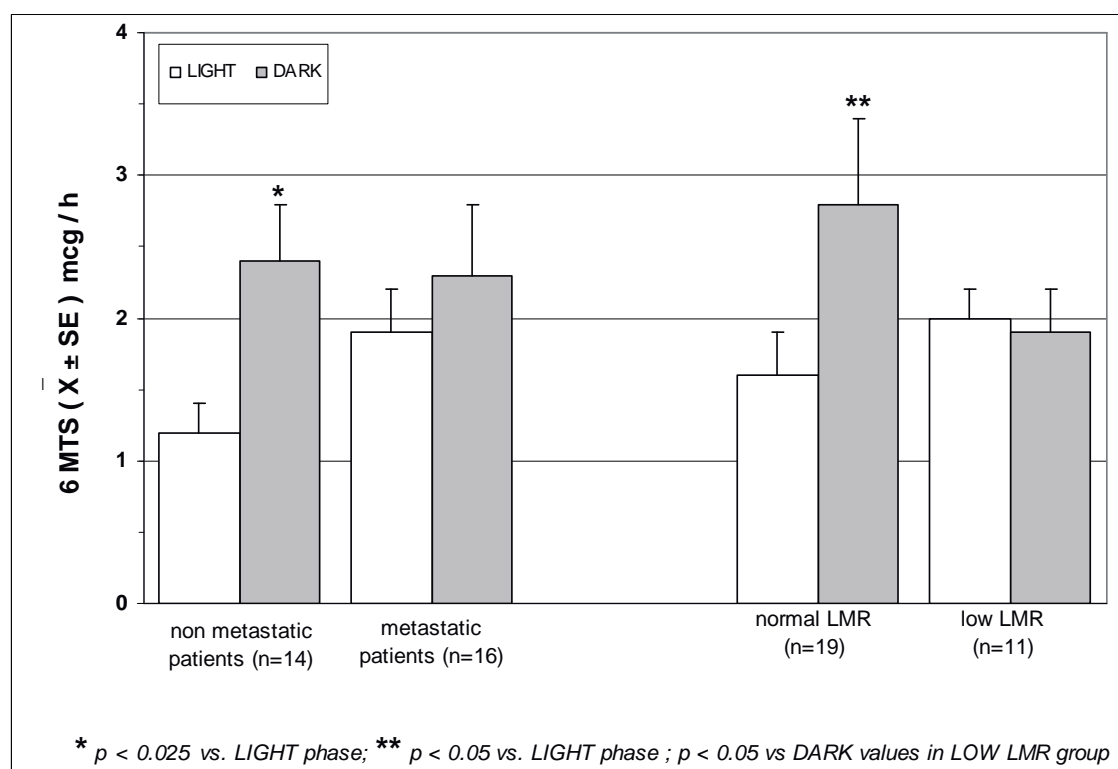


**Figure 1** 6 MTS light and dark mean values in non-metastatic and metastatic cancer patients and in those with normal or low lymphocyte-to-monocyte ratio (LMR).

## Discussion

As in previous preliminary experimental and clinical investigations, this study confirms that cancer progression is associated with a progressive decline in the nocturnal pineal production of MLT, with a consequent progressive loss of the natural resistance against cancer growth, which is mainly mediated by the pineal gland and the immune system. Moreover, this study also confirms that cancer dissemination is constantly characterized by profound immune alterations, as

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shown by the evidence of a decline in LMR values in metastatic patients, confirming that cancer progression is associated with a progressive decline in lymphocyte functions and with a concomitant increase in monocyte-macrophage system activation, that mediates the suppression of the anticancer immunity by allowing the generation of T reg cells [20].

## Conclusion

Finally, this study shows that there is an association between pineal and immune alterations, because of the evidence of a greater percentage of immune alterations in those patients, who had no light/dark circadian rhythm in the pineal endocrine function. Therefore, cancer progression-related alterations in the antitumor immunity would depend, at least in part, on the progressive decline in the pineal endocrine function. This finding is not surprising considering the stimulatory role of MLT on the generation of an effective anticancer immune reaction through the activation of TH1 lymphocyte functions and the inhibition of the monocyte-macrophage system [21]. Therefore, cancer therapy with MLT could represent a new antitumor treatment, capable of acting at the same time either as an endocrine therapy, or as an immunotherapy by inducing an effective anticancer immune reaction through a modulation of the same mechanisms responsible for the central psycho-neuro endocrine regulation of the immune system, including the anticancer immunity [22]. Obviously, further clinical studies will be required to *in vivo* confirm the great number of immune effects induced by MLT, namely by monitoring changes in lymphocyte subsets and in the blood levels of those cytokines mainly involved in the control of the inflammatory response and the antitumor immunity, including the antitumor cytokines IL-2 and IL-12, and the immunosuppressive ones IL-10 and TGF-beta. If further studies will confirm the capacity of MLT to pilot the cytokine network in an antitumor way, MLT could be successfully used in association with the recent anti-checkpoint inhibitor immunotherapies of cancer.

## Acknowledgments

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# Five Year-Survival with High-Dose Melatonin and Other Antitumor Pineal Hormones in Advanced Cancer Patients Eligible for the Only Palliative Therapy

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## Abstract

Cancer progression has appeared at least in part to be due to a deficiency of the mechanisms responsible for the natural antitumor immune response. Moreover, more recent studies have demonstrated that cancer-related immunosuppression does not depend only of alterations of immune cells themselves, but also on an altered neuroendocrine regulation of the antitumor immune response, which is mainly inhibited by the mu-opioid agonists, such as beta-endorphin, and stimulated by the pineal gland through at least three immunostimulating molecules, able to exert a direct antiproliferative anticancer activity without any important biological toxicity, consisting of the indole hormones melatonin (MLT) and the 5-methoxytryptamine (5-MTT), and of the beta-carboline pinealine (PNL). Finally, cancer progression has been shown to be constantly associated with a progressive decline in the endocrine function of the pineal gland, which could be involved in cancer dissemination itself. Then, the simple endocrine oncostatic pineal replacement therapy could counteract cancer growth and enhance the survival time, as suggested by preliminary clinical studies. On the basis, a pineal endocrine regimen was proposed in a group of untreatable advanced solid tumor patients, for whom no other effective standard anticancer therapy was available. The study included 212 patients, suffering from the most common tumor histotypes and eligible for the only best supportive care and with life expectancy less than 1 year. All pineal indoles were given orally at the time corresponding to that of their maximal circadian secretion, every day without interruption until disease progression. MLT was given at pharmacological doses (100 mg/day in the night period), while 5-MTT during the light period and PNL at the onset of the evening were administered at mild-pharmacological doses (5-MTT: 10 mg/day; PNL: 1 mg/day). A disease control (DC) was achieved in 111/212 (52%) patients, with an objective tumor regression in 16/212 (8%), irrespectively of tumor histotype. A 1-year and 5-year percentages of survival were achieved in 46% and 11%, respectively, and there were significantly higher in patients with DC than in the progressed ones. Finally, the evidence of normal pretreatment values of lymphocyte-to-monocyte ratio (LMR) and/or their normalization on therapy have appeared to be associated with most favorable clinical results. No biological toxicity occurred on pineal endocrine oncostatic treatment. This study shows that an endocrine substitutive therapy with the most known antitumor pineal hormones may represent a new non-toxic inexpensive anticancer therapy, which can improve the survival and control cancer growth also in patients for whom no other effective therapy is available, at least to improve their life. By concluding according to their results the palliative therapy of untreatable cancer patients for whom no other standard therapy available could be associated with a concomitant therapy with natural anticancer agents, namely the same pineal hormone.

**Keywords:** Lymphocyte-to-monocyte ratio; Melatonin; Metoxytryptamine; Pineal gland

## Introduction

Each living organisms may generate both pro-tumoral and anti-tumoral events, from whose equilibrium depends the physiological growth of the normal cells until their apoptosis-induced death. The antitumor biological response, which is responsible for the natural resistance against cancer growth, would depend not only on immune factors, but also on the physiological psychoneuroendocrine regulation of the immune system, which may act by either stimulating or suppressing the antitumor immunity, as shown by the great number of researches in the area of the Psycho-neuro-endocrino-immunology (PNEI) [1-3]. In particular, it has been shown that the opioid system may inhibit the anticancer immunity [4] by promoting the generation of regulatory T lymphocytes (T reg), which may suppress the antitumor immune response through the secretion of immunosuppressive cytokines, such as TGF-beta and IL-10 [5], and by inhibiting T helper-1 lymphocyte (TH1) and dendritic cells functions [6], with a following decline

in the production of IL-2 and IL-12, respectively, that represent the main antitumor cytokines in humans [7,8]. On the contrary, the anticancer immunity has been proven to be stimulated by the pineal gland through the release of several indole hormones [9] and beta-carbolines [10], whose activity is connected with the brain cannabinergic system, by constituting a fundamental neuroendocrine functional axis [11]. More in detail, stress-induced promoting effect on cancer onset and development has appeared to be mediated by the opioid system, mainly through the release of mu-opioid agonists, such as the beta-endorphin, since it may be blocked by the concomitant administration of the mu-opoid antagonist naltrexone [4]. On the other hand, pleasure and spiritual expansion of mind may counteract tumor dissemination by activating the pineal-cannabinergic functional axis [12]. As far as the pineal activity is concerned, the main anticancer molecules are consisting of the indoles melatonin (MLT) and 5-methoxytryptamine (5-MTT) [9], and the beta-carboline pinealine [10], which exert their anticancer action by either directly inhibiting cancer cell proliferation, or stimulating the anticancer immunity, namely through the activation of TH1 lymphocytes and dendritic cells, with a following enhanced production of IL-2 and IL-12 [13,14]. The antitumor immunomodulating effects of MLT are mainly due to the stimulation of lymphocyte functions [15], whereas those played by 5-MTT, pinealine, as well as by cannabinoids, would mainly depend on an inhibition of macrophage-mediated immuno-inflammatory response [9,10], which has been proven to suppress the anticancer immunity [16,17]. Therefore, from a neuroimmune point is concerned, cancer growth may be considered as the consequence of an altered balance involving the main structures responsible for the neuroimmunomodulation of the immune responses, consisting of an enhanced brain opioid sistem activity in association with a concomitant diminished function of the pineal-cannabinergic system axis [18]. In fact, the progressive decline in the pineal function, namely consisting of a progressive lack of the nocturnal increase in MLT levels with a consequent disappearance of its physiological light/dark circadian rhythm [19], would represent the main cancer progression-related endocrine deficiency either in animals, or in humans [20,21]. Cancer-related pineal endocrine deficiency woul regard not only MLT, but probably the whole pineal endocrine activity, since pineal histological damages have been described in patients died from cancer [22]. However, despite it is known since more than 50 years that the pineal gland plays a fundamental role in the maintenance of the natural anticancer immunobiological resistance [9-11] and the complete absence of any biological toxicity exerted by the pineal indole and beta-carboline hormones [19], few clinical studies have been performed up to now with MLT alone or MLT in association with other antitumor pineal molecules to evaluate their efficacy in the treatment of advanced cancer patients, who failed to respond to the conventional chemotherapies and target therapies, at least in terms of palliative therapy. In any case, preliminary clinical studies have already shown that high-dose MLT alone may induced a stabilization of the neoplastic disease in a clinically relevant percentage of cancer patients, for whom no other standard

anticancer therapy was available, and with life expectancy less than 6 months-1 year [23]. Moreover, it has been shown that the anticancer activity of MLT is a dose-dependent phenomenon, and may be further amplified by the concomitant administration of other antitumor pineal molecules, namely 5-MTT and pinealine [23-25]. However, many others natural anticancer strategies have been elaborated in the last year [26-28]. The present study reports the 5-year survival achieved by the pineal endocrine therapy with high-dose MLT plus 5-MTT plus pinealine in advanced cancer patients, for whom no other standard antitumor therapy was available, and its relation with the clinical response and the immune status by determining the lymphocyte-to-monocyte ratio (LMR), which has been proven to reflect and to synthetize the complex interaction between immunosuppressive and immunostimulatory events involved in the antitumor immunity [29].

## Materials and Methods

### Patient enrollment

The study included 212 advanced cancer patients, for whom no other standard anticancer therapy was available, then eligible for the only palliative treatment, who had a follow up of at least five years. Eligibility criteria were, as follows: histologically proven solid tumor, measurable lesions, metastatic or advanced neoplastic disease, no availability of conventional anticancer therapy because of lack of response to the previous standard treatments or poor clinical conditions unable to sustain a chemotherapeutic approach, no double tumor, and life expectancy less than 1 year.

### Study plan

All pineal hormones were given orally. MLT was administered at 100 mg/day during the dark period of the day, according to its physiological circadian rhythm, generally half-hour before sleeping. 5-MTT was given at 10 mg/day during the light phase of the day, generally at 1.00 P.M. Finally, pinealine was administered at 1 mg/day in the evening, generally 3 hours prior to sleep. Moreover, the supportive care was planned according to a phythotherapeutic approach, by using plants, which have been proven to give some subjective benefits in previous clinical studies [30], namely Aloe, Myrrh, and Magnolia. The treatment with pineal hormones was continued without interruption until disease progression. In the presence of a clear subjective clinical benefit, pineal hormone therapy was still continued despite the progression of the neoplastic disease. The clinical characteristics of patients are reported in **Table 1**. The clinical response was evaluated according to WHO criteria by repeating the radiological investigations, including CT scan and NMR, before the onset of treatments and at 3-month intervals until disease progression. Moreover, the clinical response was correlated with LMR values, which were detected prior to therapy and at 1-month intervals. Normal values of LMR obtained in our laboratory (95% confidence limits) were greater than 2.1. Data were statistically analyzed by the chi-square test, the Student's

t test. Finally, the survival curves were made according to the Kaplan-Meyer method, and statistically assessed by the log-rank test.

**Table 1** Characteristics of 212 untreatable advanced cancer patients treated with pineal endocrine therapy (PET).

| Characteristics | n |
|---|---|
| M/F | 118/94 |
| Median age (years) | 63 (22- 92) |
| Median performance status 1 | ( 0 – 3) |
| Previous chemotherapy | 178/212 (84%) |

# Results

## Clinical response to therapy

The clinical response and the 5-year percentages of survival observed in the overall patients and in relation to the single tumor histotypes are reported in **Table 2**. A complete response (CR) was achieved in 2/212 (1%) patients (non-small cell lung cancer: 1; gastric cancer: 1). Moreover, a partial response (PR) was obtained in other 14/212 (7%) patients (non-small cell lung cancer (NSCLC): 2; colorectal cancer: 2; pancreatic adenocarcinoma: 1; hepatocarcinoma: 1; biliary tract cancer: 2; ovarian cancer: 2; bladder cancer: 1; triple negative breast cancer (TNBC): 1; melanoma: 2). Then, an objective tumor regression was achieved in 16/212 (8%) patients. A stable disease (SD) was observed in 95/212 (45%). Therefore, a disease control (DC) (CR+PR+SD) was achieved in 111/212 (52%) patients, whereas the remaining 101 patients (48%) had a progressive disease (PD). As shown, the 5-year survival observed in the overall patients and in relation to their clinical response is illustrated in **Figure 1**. The 1-year, 3-year and 5-year survival percentages were 46%, 18%, and 11%, respectively. Moreover, the survival time obtained in patients, who achieved an objective tumor regression (CR+PR), was significantly longer with respect to that found in those, who had no tumor regression (P<0.01). Finally, the survival time found in patients with SD was also significantly longer than that observed in patients with PD (P<0.05).

**Table 2** Clinical response (WHO citeria) and survival time to pineal endocrine therapy (P.E.T.) in 212 untreatable advanced cancer patients, and their relation to tumor histotype.

| Patients + | Clinical Response ++ | | | | | | | Survival Time (Year) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | CR | PR | CR + PR (%) | SD | DC (%) | PD | 1 | 2 | 3 | 4 | 5 |
| Overall Patients | 212 | 2 | 14 | 16 (-8%) | 95 | 111 (-52%) | 101 | 98 (-46%) | 56 | 38 | 27 | 23 (-11%) |
| **Tumor Histotype** | | | | | | | | | | | | |
| Lung cancer | 36 | 1 | 2 | 3 | 16 | 19 | 17 | 17 | 9 | 7 | 5 | 5 |
| -NSCLC | 29 | 1 | 2 | 3 | 14 | 17 | 12 | 15 | 7 | 6 | 4 | 4 |
| -SCLC | 7 | 0 | 0 | 0 | 2 | 2 | 5 | 2 | 2 | 1 | 1 | 1 |
| Colorectal cancer | 25 | 0 | 2 | 2 | 13 | 15 | 10 | 13 | 8 | 5 | 4 | 4 |
| Pancreatic cancer | 22 | 0 | 1 | 1 | 10 | 11 | 11 | 12 | 4 | 3 | 2 | 1 |
| Gastric cancer | 12 | 1 | 0 | 1 | 3 | 4 | 8 | 3 | 3 | 3 | 2 | 1 |
| Biliary tract cancer | 11 | 0 | 2 | 2 | 2 | 4 | 7 | 6 | 3 | 2 | 2 | 1 |
| Hepatocarcinoma | 6 | 0 | 1 | 1 | 3 | 4 | 2 | 3 | 2 | 1 | 0 | 0 |
| Ovarian cancer | 14 | 0 | 2 | 2 | 7 | 9 | 5 | 9 | 6 | 3 | 2 | 2 |
| Bladder cancer | 5 | 0 | 1 | 1 | 3 | 4 | 1 | 3 | 2 | 1 | 1 | 1 |
| Prostate cancer | 4 | 0 | 0 | 0 | 3 | 3 | 1 | 3 | 3 | 2 | 2 | 2 |
| TNBC | 6 | 0 | 1 | 1 | 2 | 3 | 3 | 3 | 2 | 2 | 1 | 1 |
| Soft tissue sarcoma | 15 | 0 | 0 | 0 | 8 | 8 | 7 | 5 | 3 | 3 | 2 | 2 |
| Melanoma | 10 | 0 | 2 | 2 | 4 | 6 | 4 | 4 | 2 | 2 | 1 | 1 |
| Glioblastoma | 46 | 0 | 0 | 0 | 21 | 21 | 25 | 19 | 9 | 4 | 3 | 2 |

NSCLC: non-small cell lung cancer; SCLC: small cell lung cancer; TNBC: triple negative breast cancer, CR: complete response; PR: partial response; SD: stable disease; DC: disease control; PD: progressive disease
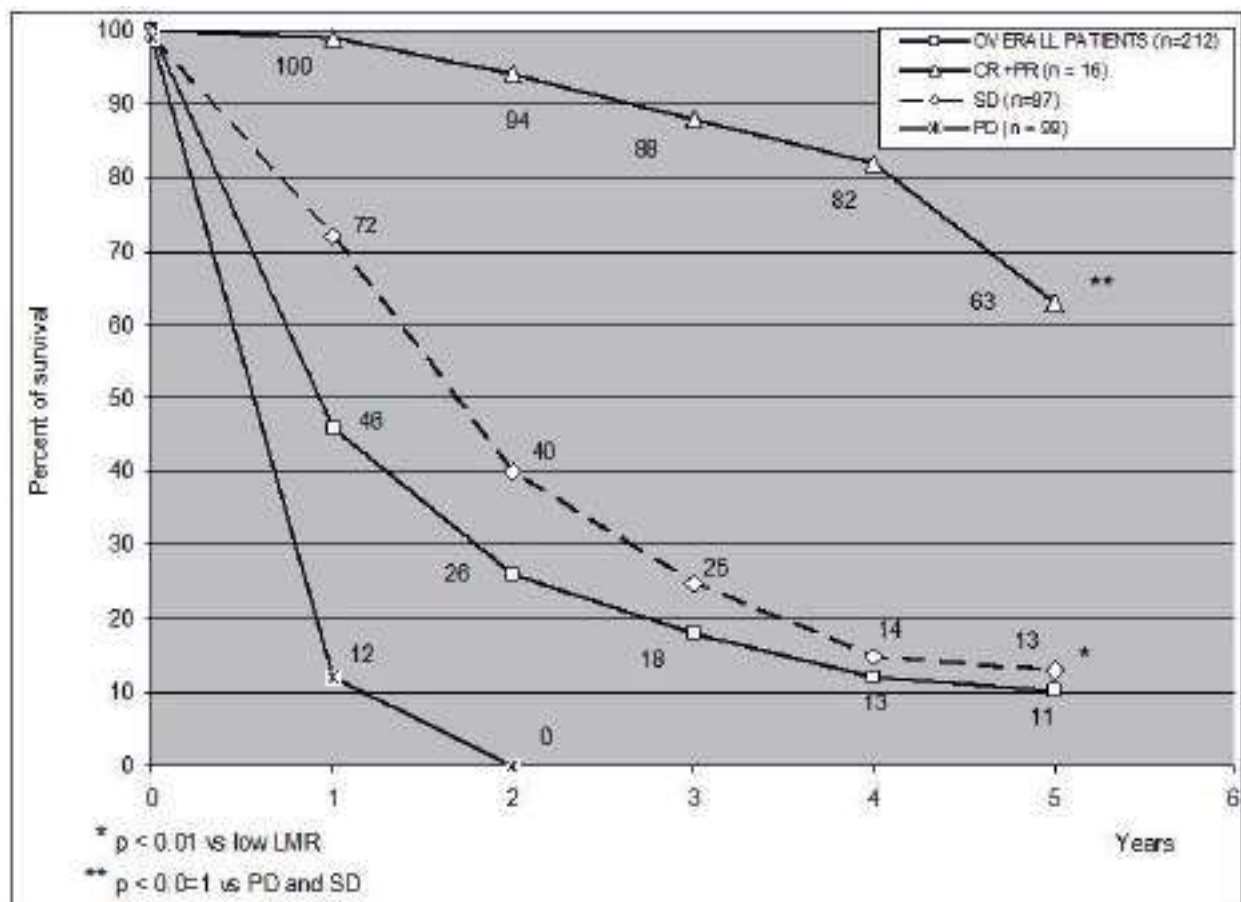
3

**Figure 1** Five-year survival in relation to the clinical response.

## Immune effect of therapy

From the point of view of the immunological status is concerned, abnormally low pretreatments values of LMR were seen in 131/212 (62%) patients. The clinical response in relation to LMR pretreatment values are shown in **Table 3**. As reported, both objective tumor regression and DC percentages observed in patients with normal pretreatment values of LMR were significantly higher than those found in patients with abnormally low LMR values prior to therapy (P<0.01 and P<0.05, respectively). In addition, as illustrated in **Figure 2**, the 5-year percentage of survival observed in patients with normal LMR values prior to therapy was significantly longer than that achieved in patients with low pretreatment LMR values (P<0.01). Finally, as far as patients with PD are concerned, 44/101 (44%) patients, who had a PD, continued the pineal therapy despite the progression of their disease, because their improved clinical status. After 6 months and 1 year, only 34/101 (34%) and 2/101 (2%) were still alive. Both patients still alive at 1 year had continued the pineal therapy, whereas no

patient, who interrupted the treatment, was alive. Moreover, the percentage of 9-month survival achieved in progressed patients, who continued the pineal therapy, was significantly longer than that found in those, who interrupted the endocrine treatment (14/44 (32%) vs. 0/57, P<0.05). Finally, abnormally low LMR values prior to therapy were seen in 70/101 (69%) patients with PD. The 9-month survival percentage observed in patients with PD but normal pretreatment values of LMR was significantly longer than that found in progressed patients with abnormally low values of LMR prior to therapy (9/31 (29%) vs. 5/70 (7%), P<0.05). The treatment was well tolerated, and most patients experienced a clear subjective benefit in mood, anxiety, sleep quality and asthenia. No biological toxicity occurred under pineal therapy, and some transient undesirable effects, such as headache, increase in anxiety, and sleep disturbances, occurred for few days in only 23/212 (11%) patients, without the need to interrupt the treatment.

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**Table 3** Clinical response (WHO criteria) in relation to LMR pretreatment values to pineal endocrine therapy (P.E.T.) in 212 untreatable advanced cancer patients.

| Lmr Pretreatment Values + | Clinical Response ++ | | | | | | |
|---|---|---|---|---|---|---|---|
| | n | CR | PR | CR+PR (%) | SD | DC (%) | PD (%) |
| Normal Values | 81 | 2 | 9 | 11 (14%) * | 52 | 63 (78%)** | 18 (22%) |
| Low Values | 131 | 0 | 5 | 5 ( 4%) | 43 | 48 (37%) | 83 (63%) |

+ LMR: lymphocyte-to-monocyte ratio; normal values more than 2.1; ++ CR: complete response; PR: partial response; SD: stable disease; DC: disease control; PD: progressive disease

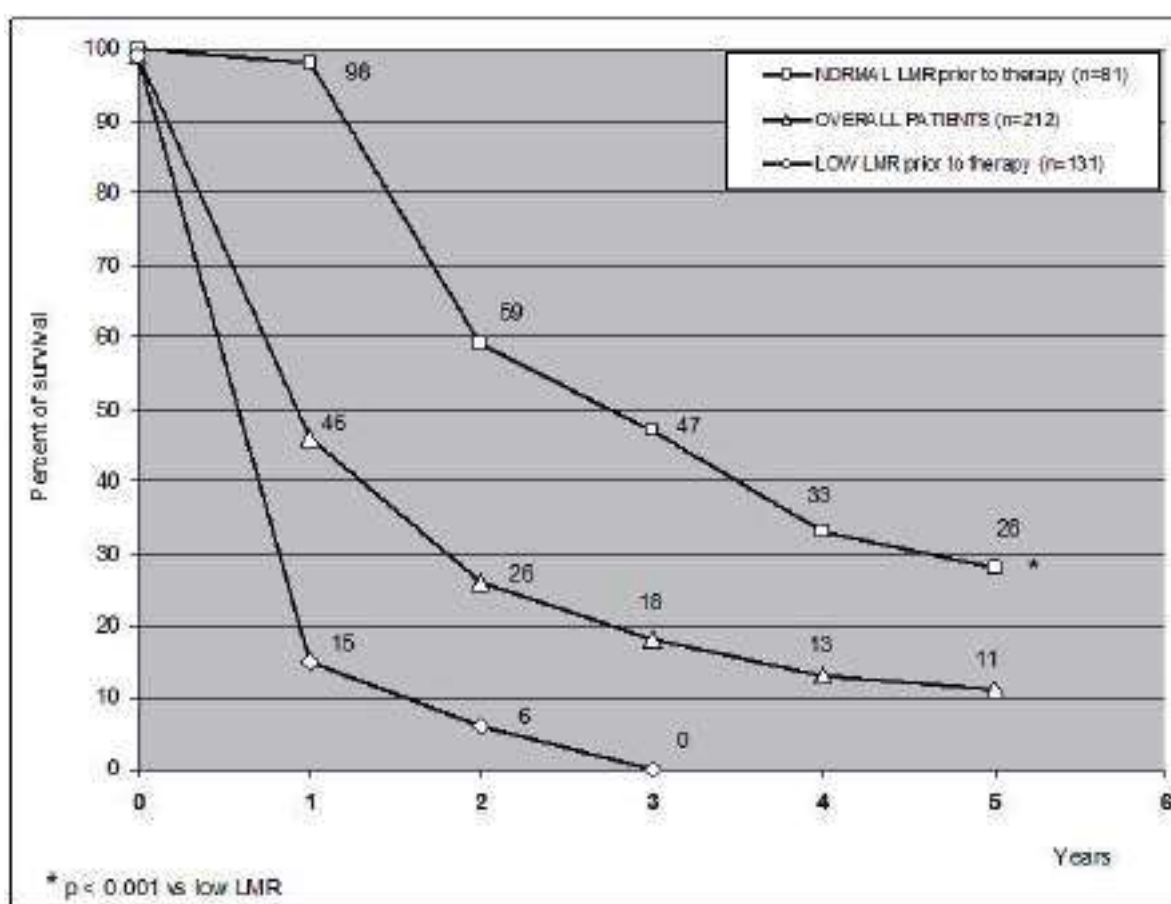*P<0.01 vs. low LMR values; P<0.05 vs. low LMR values



**Figure 2** Five-year survival in relation to LMR pretreatment values.

## Discussion

According to previous preliminary clinical results [23-25], this study confirms in a greater number of untreatable advanced cancer patients and for a longer period of follow-up that the endocrine therapy with high-dose of MLT in association with the administration of the other two main anticancer molecules of the pineal gland, including 5-MTT and pinealine, may induce some tumor regression and prolong the survival time in patients eligible for the only palliative therapy because of the lack of response to the previous antitumor therapies, and life expectancy lower than 1 year. Moreover, the pineal endocrine therapy-induced prolongation of the survival time has appeared to be greater in patients, who achieved an objective tumor regression or disease stabilization, by suggesting that pineal endocrine-induced control of cancer growth is not a simple epiphenomenon, since it has been proven to predict a longer survival. This finding is not surprising since the only MLT has been already observed to represent the only molecule capable of counteracting the

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whole six main mechanisms responsible for cancer dissemination [23], including stress-induced immunosuppression, cancer cell transformation, intercellular joint alterations, stimulation of the neoangiogenic processes, tumor cell production of immunosuppressive factors and tumor expression of FAS-L, which allows the apoptosis of T lymphocytes after their interaction with the cancer cells [30]. In addition, the antitumor activity of MLT may be enhanced by the concomitant association with other pineal anticancer molecules, by justifying the possible evidence of tumor regressions or tumor stabilization also in very advanced cancer patients, for whom no other standard anticancer therapy may be available. Moreover, this study would suggest that the efficacy of a pineal endocrine antitumor therapy is greater in patients with normal pretreatment values of LMR, which may synthetize the whole status of the anticancer immunity in the single cancer patient [29]. The different efficacy of therapy may be influenced by the previous therapies, namely radiotherapy, because of the influence on lymphocyte count. Then, the evidence of abnormally low LMR values would reflect an immunosuppressive status of the anticancer immunity, with a consequent lower efficacy of the various anticancer treatments. Finally, previous studies had already shown a greater efficacy of the anticancer therapies in the presence of a real spiritual faith condition, as assessed by an adequate clinical test [31]. Some recent biomarkers, such as LMR, could be use full to clinically monitor the immune status of cancer patients [32,33]. Then, in the presence of a clinical response consisting of objective tumor regression or neoplastic disease stabilization, of a normal LMR values prior to therapy and an adequate spiritual faith score, it is probable that the pineal endocrine antitumor therapy may contribute to the control of the neoplastic growth and modify the prognosis of an untreatable advanced neoplastic disease also in patients, for whom no other conventional anticancer therapy may be available. On the contrary, tumor histotype does not seem to influence the efficacy of the pineal anticancer therapy in a relevant manner, even though glioblastoma and pancreatic adenocarcinoma would seem to represent the less responsible neoplasms to the treatment. However, by considering their low life expectancy after failure of the various therapies, glioblastoma and cancer of pancreas would be also influenced by the pineal therapy, at least in terms of survival time with respect to the expected one. Obviously, further randomized studies with the only best supportive care (BSC) or with BSC plus the pineal endocrine anticancer therapy will be required to confirm that the administration of the main anticancer molecules produced by the pineal gland may prolong the survival time also in patients with advanced cancer, eligible for the only palliative therapy and with life expectancy less than 1 year, since the survival of untreatable cancer patients, for whom no other standard anticancer therapy is available, is constantly generally less than 1 year or 6 months.

## Conclusion

This preliminary study, by showing a possible increase in the survival time in patients with untreatable tumors and life expectancy less than 1 year, then suitable for the only supportive care by the simple administration of the immunostimulating pineal hormones would suggest that the separation between palliative and curative has to be abrogated by the existence in the nature of several non-toxic anticancer agents, namely within the same human body, which could be administered to untreatable cancer patients with respect to the only palliative therapy. Moreover, further studies by evaluating other immune parameters, such as tumor infiltrating lymphocytes, will be required to better define the immunomodulating effects of pineal therapy.

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## Mini Review

# The modulation of the endocannabinoid system in the treatment of cancer and other systemic human diseases

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## Abstract

Despite cancer is at present considered as a systemic disease, in the clinical management of patients the neoplastic disease is often up to now generally considered and treated as a loco-regional pathology. The systemic nature of cancer is documented by the evidence of the fundamental role of the immune system in the control of tumor onset and dissemination. However, it must be taken into consideration that the in-vivo immune responses are under a physiological neuroendocrine control, namely played by brain opioid and cannabinoid systems. The endogenous cannabinoid system has been proven to exert a fundamental anti-inflammatory action. Then, since the chronic inflammatory status has appeared to promote cancer growth and dissemination, it could be clinically important to evaluate the functional status of the endogenous cannabinoid system in cancer patients. The endogenous cannabinoids, the most important of them are anandamide and 2-arachidonyl glycerol, are destroyed by the fatty acid amide hydrolase (FAAH) enzyme, whose levels are inversely correlated to those of the endogenous cannabinoids. Moreover, it has been observed that the evidence of abnormally high blood concentrations of FAAH, which reflect low levels of cannabinoids, has appeared to predict a poor prognosis in cancer patients. Therefore, the determination of FAAH blood levels would have to be included within the laboratory analyses of cancer patients in an attempt to synthetically evaluate their neuroimmunomodulatory status. Finally, the inhibition of FAAH synthesis and activity could represent a new possible approach in the bio-immunotherapy of human tumors.

## Introduction

It is known that the endocannabinoid system exerts a fundamental role in the regulation of most biological functions by inducing metabolic, immunomodulatory and psychochemical effects, and in particular it has been shown that brain cannabinoid system plays an essential role in both pleasure perception and pain control [1]. Moreover, the recent discoveries in the area of the Psycho-neuro-endocrino-immunology (PNEI) have demonstrated that immune system-mediated systemic human diseases, including cancer and autoimmunity, would be due to an altered neuroendocrine regulation of the immune responses rather than to a primary alteration of immune cell functions themselves [2]. Despite its great complexity, the psycho-neuroendocrine regulation of the immunity has appeared to mainly depend on two major brain interneuronal systems, consisting of the cannabinoid [1] and the opioid system [3,4]. There are three essential opioid receptors, mu-, delta- and kappa receptors, and two main cannabinoid receptors, CB1 and CB2. The psychedelic psychotropic effect of cannabinoids is mediated by the activation of the CB1 receptor, whereas CB2 receptor, which is namely expressed by the immune cells, is involved in the modulatory effects of cannabinoids on the immuno-inflammatory biological response [1]. The two main endocannabinoids are represented by the arachidonyl-ethanol-amide (AEA), the so-called anandamide, and the 2-arachidonyl-glycerol (2-AG) [1]. The main enzyme involved in cannabinoid degradation is the fatty acid amide hydrolase (FAAH) [1]. The importance of the neuroimmunomodulatory processes exerted by brain opioid and cannabinoid systems is confirmed by the evidence of a possible enhanced or diminished activity of both brain opioid and cannabinoid systems in the pathogenesis of cancer, as well as other systemic immune-mediated diseases, including autoimmunopathologies and cardiovascular diseases. In particular, it has been shown that stress-induced promoting effect on tumor development has appeared to be mediated by an enhanced opioid system activity, since the administration of mu-receptor opioid antagonists may abrogate the influence of stress on tumor progression [4]. Moreover, it is known that the progressive lack of pleasure perception, the so-called anaedonia, represents one of the main and most frequent cancer progression-related symptoms. Then, because of the fundamental role of endocannabinoids in the perception of pleasure [1], the evidence of cancer-related anaedonia would suggest that tumor diffusion may be characterized by a progressive failure of brain cannabinoid function, with consequent alterations in immune system function. On the contrary, other diseases, such as the acute schizofrenia, has been proven to be associated with an enhanced brain cannabinoid system activation [5]. Obviously, before analyzing the modulatory effects of the endocannabinoid system and most in general of the neuroendocrine system, it has to be synthetically considered the functionless of the immune system and the cytokine network, since the influence of the neuroendocrine system on the immunity is mainly mediated by its influence on the cytokine network.

## The functional structure of the immune system and the anticancer immunity

Despite its great complexity, the immune system is mainly constituted by two major cell systems, the old or innate immunity, mainly mediated by the granulo-monocyte system and NK cells, and the new or aquired immunity, mainly mediated by the lymphocyte system. The immune status is the end results of two major dynamics, represented by the immunostimulation and the immunosuppression, which are respectively mainly exerted by the lymphocyte and the macrophage systems. Within the lymphocyte system, the only regulatory T lymphocytes (T reg) (CD4+CD25+) exert an immunosuppressive anti-inflammatory action, and their generation has appeared to be under a macrophage stimulatory regulation. The connection between innate and acquired immunity is namely represented by the dendritic cells (DC), mainly through the release of IL-12 [6], which would represent the main link between old and new immunity, then between macrophage and lymphocyte systems, whose interactions are responsible for the overall types of immune response. The immune response is mainly activated by T helper-1 (TH1) (CD4+) lymphocytes through the release of IL-2 and gamma-IFN. TH1-induced immune activation allows two different types of cytotoxic response, antigen-dependent and antigen-independent cytotoxicity, respectively mediated by cytotoxic T lymphocytes (CD8+) after IL-12 stimulation and NK cells after their IL-2-induced evolution into LAK cells [7]. Cancer would be characterized by a decline in TH1 and DC count and activity in association with an enhanced macrophage and T reg cell function. On the other side, the autoimmune diseases are characterized by a decline in T reg cell system activity, with following low levels of IL-10 and TGF-beta, in association with an enhanced activation of TH17 lymphocytes (CD4+CD17+) and a consequent enhanced secretion of IL-17, which would constitute the main inflammatory cytokine involved in the pathogenesis of the autoimmune disorders. From a PNEI point of view, the cytokine alterations occurring in cancer, autoimmunity, cardiovascular diseases, and neurodegenerative pathologies could be due at least at the beginning of the pathology to an altered neuroendocrine control of cytokine network itself [2]. Then, the cytokine network could be influenced by acting on its psychoneuroendocrine regulation, which is mainly exerted by the opioid and cannabinoid systems, rather than to directly act on the various cytokine secretions. IL-12, in addition to its importance in the relations between innate and acquired immunity, would also play an essential role in the interactions between cannabinoid system and immunity [8], since IL-12 has been proven to inhibit FAAH activity, with a consequent increase in cannabinoid endogenous content, whereas IL-10 may stimulate FAAH, with a consequent diminished cannabinoid concentration. Then, the immune system may modulate the function of brain cannabinoid system by simply influencing FAAH synthesis and activity, by enhancing the activity of the cannabinoid system through the release of IL-12, and by decreasing its function through that of IL-10, respectively responsible for the immunoactivation or the immunosuppression. The secretion of IL-10, which exerts an anti-inflammatory immunosuppressive activity [2], is stimulated by the opioid system [3], which is active in stress, pain and depressive conditions, whereas the cannabinoid system is involved in pleasure and spiritual sensitivity conditions [1]. Then, these evidences would explain the immunosuppressive effects of stress and the immunostimulatory one of the pleasure, and the spiritual expansion of mind.

## The neuroendocrine regulation of the immune system

The central nervous and the neuroendocrine systems influence the immune system by acting on the cytokine network and modulating cytokine secretions [1-5]. The neuroimmunomodulation is namely exerted by the two major brain interneuronal systems, represented by the opioid and cannabinoid systems. The mu-opioid agonists, such as morphine and beta-endorphin, play an immunosuppressive activity [3,4] by stimulating the secretion of IL-10 and TGF-beta, which inhibit the antitumor immunity [9], as well that of IL-17 [10], and by inhibiting that of the two main anticancer cytokines in humans, IL-2 and IL-12. More controversial are the immunomodulating effects of delta-and kappa-opioid agonists. On the same way, contradictory results have been reported about the immune effects of cannabinoids, since both stimulatory and inhibitory effects on IL-2 , IL-12, TGF-beta and IL-10 secretions have been observed, whereas most studies have confirmed the inhibitory action of cannabinoids on TNF-alpha and IL-17 secretions, which would explain their anti-cachectic and anti-inflammatory activities, respectively. The controversial results concerning the immune effects of cannabinoids would depend by the fact that they are mediated by the interactions between brain cannabinoid system and pineal gland [11], whose essential role in the modulation of the immune system has been well proven.

## Clinical applications of the knowledge of the cannabinoid system

### Clinical investigation of the endocannabinoid system

The endocannabinoid system may be clinically investigated by measuring the blood and liquoral concentrations of the two major endocannabinoid agents, consisting of AEA and 2-AG, or probably in a more simple and synthetic manner by determining the blood concentrations of the enzyme responsible for cannabinoid degradation and metabolism, the FAAH, whose enhanced production may allow a decline in the endogenous cannabinoid content [1]. On the contrary, a diminished synthesis of FAAH would allow an increased cannabinod system activation. The acute phase of schizophrenia would be associated with an enhanced cannabinoid activity, as confirmed by the evidence of abnormally high levels of AEA in association with low concentrations of FAAH [5]. Because of the well demonstrated anticancer properties of cannabinoid agonists [1], due to several mechanisms, including direct anti-proliferative cytotoxic effect, anti-angiogenic activity, and inhibitory action on macrophage-mediated immunosuppressive inflammatory events, the evidence of a schizophrenia-related cannabinoid system hyperactivation could explain the low frequency of cancer in schizophrenic patients [5]. On the contrary, an enhanced production of FAAH, with a consequent decline in cannabinoid system activity, could constitute a risk factor for cancer onset and development, because of the fundamental role of the endocannabinoid system in the natural biological resistance against cancer in the optimal psycho-neuroendocrino-immune status of health [1,11]. In fact, it has been demonstrated that tumor expression of FAAH is associated with a more biological malignancy and a poor prognosis in some tumor histotypes, including prostate cancer [12]. Then, the inhibition of FAAH synthesis, with a consequent increase in cannabinoid concentrations, could constitute a new possible biological approach in the treatment of tumors. Moreover, by considering that FAAH activation may induce an enhanced inflammatory response by suppressing the anti-inflammatory action of the endogenous cannabinoids and a consequent enhanced production of inflammatory cytokines, such as IL-1beta, IL-6, TNF-alpha and IL-17, the inhibition of FAAH synthesis and activity could exert therapeutic benefits also in the cure of cardiovascular diseases [13], and neurodegenerative disorders [14], which are also determined at least in part by an enhanced inflammatory response. Psychiatric diseases themselves are

also characterized by the evidence of an enhanced inflammatory status, as suggested by the possible evidence of high levels of inflammatory cytokines, namely IL-6 [15]. By summarizing, FAAH inhibitors or stimulators could be successfully employed in the treatment of human diseases, respectively characterized by an abnormally low or abnormally high function of the endocannabinoid system. As far as the cardiovascular system is concerned, the endocannabinoid system may influence heart and endothelium functions by the simple regulation of FAAH synthesis, whose increase would allow an enhanced production of inflammatory cytokines, which negatively influence both cardiac and nervous activities. Then, from a therapeutic point of view, the inhibition of FAAH activity to enhance brain cannabinoid functionis more important than its eventual stimulation to reduce brain cannabinoid content. Another important enzyme in the control of the cardiovascular system, which is connected to FAAH through several neuroendocrine interactions, is neprilysin (NEP), also called enkephalinase, a zinc-dependent membrane peptidase involved in the metabolism and degradation of several vasoactive peptides, including atrial natriuretic peptide (ANP), endothelin-1 (ET-1), and enkephalins [15]. NEP synthesis and activity may influence the cardiovascular functions by affecting the inflammatory response, whose end-result would depend on its major degradation of molecules, suchas ANP [16] or ET-1 [17], which are provided by anti-inflammatory immunostimulatory or inflammatory and immunosuppressive effects, respectively. The inhibition of NEP activity has appeared to enhance the vasodilator effect of the angiotensin II-type 1 receptor antagonists, such as valsartan, as well as their inhibitory action on ET-1 secretion [17].

### Therapeutic implications

At present, the most simple manner to modulate the functionless of the endogenous cannabinoid system, whose alterations have been proven to be involved in cancer, autoimmunity, neuropsychiatric and cardiovascular diseases, is represented by the control of FAAH synthesis and activity. Several FAAH inhibitors have been elaborated [18], and the pineal hormone MLT has appeared to cooperate with FAAH inhibitors to counteract FAAH activity with a following further increase in brain cannabinoid content [19]. On the contrary, leptin, a chemokine produced by the adipocytes provided by a stimulatory effect on inflammatory cytokine production, may stimulate FAAH activity, with a following decline in brain endocannabinoid content, which allows a decline in appetite and food intake [20]. Most of FAAH inhibitors are profen or phenyl-alkyl-sulfonyl-fluoride derivatives, and preliminary clinical studies seem to suggest the potential therapeutic efficacy of FAAH inhibitors in several human diseases, including cancer, depression, and cardiovascular pathologies, all characterized by a chronic enhanced inflammatory status, due at least in part to an endocannabinoid system deficiency. By summarizing, the measurement of FAAH blood concentrations is already sufficient to investigate the inflammatory status of patients, since the evidence of abnormally high levels of FAAH reflects and allows an enhanced inflammatory response because of the decline in the endogenous cannabinoid content and function induced by the high levels of FAAH. Then, it is possible to modulate the inflammatory status of patients by simply acting on FAAH synthesis and activity. Moreover, since the hyperactivation of the inflammatory response may be considered as the common pathological mechanism involved in the main human systemic diseases, including cancer and autoimmune diseases, the control of the inflammatory status by cannabinoids agents provided by anti-inflammatory effects would represent a fundamental point in the treatment of the already now untreatable human systemic pathologies.

## Conclusions

It is already known that the cannabinoid agents may play a fundamental role in the treatment of most advanced cancer-related symptoms, including cachexia, anorexia, anaedonia, vomiting and pain. In addition to these therapeutic properties, at present it has to be also taken into consideration the important role of the cannabinoid system in the systemic control of the inflammatory response, and the functional status of the cannabinoid system may be clinically established by the simple determination of FAAH levels, whose concentrations are inversely correlated to those of the endogenous cannabinoids. Then, the determination of FAAH levels would have to be included within the routinary laboratory analyses of cancer patients, since the evidence of high FAH levels has been proven to be associated with an enhanced inflammatory response and with a suppression of the anticancer immunity [2], then with a worse prognosis in terms of both response to therapy and survival time.

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# A phase-2 study of high-dose pineal antitumor hormone melatonin as an adjuvant therapy in triple negative breast cancer.

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## Abstract

**It is known that triple negative breast cancer (TNBC) is the most prognostically negative mammary tumor, because of its lack of sensitivity to the main growth factors for breast cancer, including estrogens and EGF. However, at least three other hormones would have to be considered, consisting of prolactin (PRL), oxytocin (OT), and the pineal hormone melatonin (MLT). PRL would stimulate TNBC growth, whereas MLT and OT would play an inhibitory action in several tumor histotypes, including TNBC, even though at present only clinical studies with MLT have been performed, by demonstrating that it's in human anticancer activity is a dose-dependent phenomenon. On these bases, a study was planned to evaluate the effects of high-dose MLT chronic administration as an adjuvant therapy on the percent of 3-year progression-free period (PSF) in TNBC after adjuvant chemotherapy. The study included 14 consecutive TNBC patients, who were treated with MLT at 40 mg/day orally in the evening every day without interruption, by comparing the results to those observed in a control group of 16 TNBC patients with comparable clinical characteristics. The 3-year PFS percentage achieved in MLT group was significantly higher than that found in the control group, either in patients with or without node involvement. No MLT-related biological toxicity occurred. On the contrary, most patients referred a mood improvement. These preliminary results justify further randomized study with or without high-dose MLT in TNBC patients, in an attempt to prolong their survival.**

## Introduction

Until few years ago, the endocrine oncological researches have been mainly performed in an attempt to identify possible hormones and growth factors involved in the stimulation of tumor growth, including estrogens for breast cancer and endometrial adenocarcinoma, androgens for prostate cancer, prolactin (PRL) PRL for breast and prostate tumors, and EGF and GH for several tumor histotypes. However, in the last years it has been identified also the existence of endogenous hormones provided by antitumor activity, namely oxytocin (OT) [1] and the pineal hormone melatonin (MLT) [2]. Moreover, it is known that the classical endocrine therapy of breast cancer has no efficacy in triple negative breast cancer (TNBC) because of its lack of sensitivity to hormonal stimulation. On the same way, anti-HER-2 monoclonal antibodies have no therapeutic activity in TNBC [3]. However, it has to be remarked that at least three other hormones would have to be taken into consideration because of their involvement in the control of breast growth, including PRL, OT and the pineal hormone MLT, which have been proven to exert opposite effects, consisting of a stimulatory effect of PRL [4] and an inhibitory action of OT and MLT on breast cancer cell proliferation, including that of TNBC [5]. In more detail, the effects of PRL on TNBC growth are yet controversial, since some authors have also reported an inhibitory action of PRL on TNBC growth [6]. On the same way, the biological and prognostic significance of PRL receptor (PRL-R) expression in

TNBC is still unclear, even though most studies have shown that PRL-R expression may be associated with a more biological malignancy [4,6]. Cannabinoid agents have also appeared to inhibit the growth of TNBC expressing cannabinoid receptors [7]. The anticancer effect of OT is still only experimental evidence. On the contrary, all experimental and clinical studies performed up to now have constantly demonstrated the inhibitory activity of the pineal hormone MLT on several tumor histotypes, including breast tumors, including the TNBC. Moreover, it has been shown that tumor expression of MLT receptor (MT-R) may predict a less malignancy and a more favourable prognosis in terms of both response to therapy and survival times [5], even though the antitumor action of MLT is at least in part independent from MT-R expression [8]. The antitumor mechanisms of MLT are multiple and complex [9,10], and however, they include a direct cytotoxic antiproliferative action, a cell differentiating effect, an anti-angiogenic activity, an immuno stimulatory action on the anticancer immunity, namely consisting of stimulation of TH1 lymphocytes (TH1) and dendritic cells, with a consequent enhanced production of the two main antitumor cytokines in humans, consisting of IL-2 and IL-12, respectively [11,12]. Then, MLT would constitute at present the only natural molecule potentially able to counteract the overall phases responsible for cancer progression. Moreover, MLT is the only molecule, which has shown no lethal dose, because of the down-regulation of MT-R exerted by the normal cells, whereas tumor cells are unable to modulate MT-R expression,

then there are exposed to the cytotoxic action of MLT in a dose-dependent manner [13]. On these biological bases, as well as by considering the complete lack of toxicity by MLT, an experimental clinical study was performed in an attempt to evaluate the influence of an adjuvant endocrine therapy with high-dose MLT on 3-year progression-free survival (PFS) in a group of non-metastatic TNBC women after the classical adjuvant chemotherapy.

## Patients and Methods

The phase-2 study included 14 consecutive non-metastatic TNBC women (median age 53 years, range 28-68). Eligibility criteria were, as follows: histologically proven TNBC other than the apocrine tumor, measurable lesions, no metastatic location, no double tumor, and previous adjuvant chemotherapy. The experimental protocol after approvation of the Ethical Committee was explained to each patient, and written consent was obtained. Depending on the different oncological Institutions, the adjuvant chemotherapy was consisted of carboplatin plus gemcitabine in 8, carboplatin plus taxol in 4, and 5-fluorouracil, epirubicin and cyclophosphamide in the remaining 2 patients. MLT was given orally at a dose of 40 mg/day during the dark period of the day according to its physiological light/dark circadian rhythm [6]. If we consider that the physiological daily endogenous production of MLT is less than 2 mg, a dosage of 40 mg/day may be retained as a mild pharmacological schedule. MLT was administered every day without interruption until disease recurrence. Patients were monitored for a minimum follow of 3 years. The results were compared with those observed in a control group of 16 non-metastatic TNBC women, who had also received the adjuvant chemotherapy. Data were statistically analyzed by the chi-square test. Moreover, the PFS curves were calculated according to Kaplan Meir method, and analyzed by the log-rank method.

## Result

Table 1 shows the clinical characteristics of TNBC women and the 3-year PFS percentage in MLT group and in controls. The two groups of patients were well comparable for the main biological characteristics, including age, menopause status, node involvement and type of adjuvant chemotherapy. The 3-year percentage of PFS achieved in MLT group was significantly higher than that found in the control group, who did not received MLT (10/14 (71%) *vs.* 6/16 (37%), P<0.05). The percentage of relapse found in MLT group was significantly lower than that occurring in the control group (4/14 (29%) *vs.* 10/16 (63%), P<0.05). The percentage of recurrence was lower in MLT group than in controls also in relation to node involvement (node involvement: 1/6 (17%) *vs.* 3/7 (43%); node involvement: 3/8 (38%) *vs.* 7/9 (78%), P<0.05). On the contrary, no significant difference occurred between visceral and non-visceral sites of relapse (visceral recurrence: 3/4 (75%) *vs.* 7/10 (70%). However, the percentage of brain recurrence observed in MLT group was lower than that found in controls (1/14 (7%) *vs.* 3/16 (19%), even though the difference was not statistically significant. Finally the 3-year

PFS achieved in MLT group was significantly longer that that found in the control groups (P<0.05). No MLT-related toxicity occurred. On the contrary, most patients referred a mood improvement and a more regular sleep quality.

***Table 1.*** *Clinical characteristics of TNBC patients and 3-year progression-free survival (PFS) in MLT group and in controls.*

| Characteristics | MLT Group(n=14) | Control Group (n=16) |
|---|---|---|
| Median age (years) | 53 (28-68) | 55 (34-70) |
| Node involvement | 8/14 (57%) | 9/16 (56%) |
| Adjuvant chemotherapy Carboplatin-Gemcitabine Carboplatintaxol FEC | 8 4 2 | 8 5 3 |
| Recurrence ratio Sites of relapse Node Bone Lung Liver Brain | 4/14 (29%) -------------------- 1 0 1 1 1 | 10/16 (63%) *P<0.05 ------------------------------------ 2 1 1 3 3 |

## Discussion

The results of this preliminary study would seem to *in vivo* confirm the antitumor properties of the pineal hormone MLT also against the TNBC, as suggested by the lower percentage of recurrence in patients chronically treated by MLT as a potential endocrine adjuvant therapy of TNBC. Obviously, further studies in a greater number of patients and with a longer follow up period will be required to confirm the potential efficacy of MLT as an adjuvant endocrine therapy of TNBC. In any case, by also considering the complete lack of MLT toxicity, the results of this study would be already sufficiently promising to justify a randomized study with or without MLT, either alone or in association to the classical adjuvant chemotherapy in the treatment of TNBC women. The typical cancer endocrine therapies on the basis of their action mechanisms are in the reality anti-endocrine treatments, since their action consists of blocking the activity of potential protumoral hormones, such as estrogens for breast cancer and androgens for prostate cancer. On the contrary, the endocrine therapy of MLT, as well that with somatostatin for somatostatin receptor expressing neuroendocrine tumors [14], would represent a direct antiproliferative endocrine therapy of cancer. More predictive clinical information concerning the possible efficacy of MLT as a possible adjuvant endocrine therapy for TNBC may be drawn from the immunochemistry detection of MT-R expression on TNBC cells, since MT-R tumor expression would predict a greater efficacy of MLT itself. Finally, because of the dose-dependency of the antitumor activity of MLT [13], more promising results in reducing the percentage of recurrence in TNBC women could be achieved by a greater dosage of MLT, which has been proven to have no lethal dose [9-12].

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# The Antitumor Endocrine Molecules of Human Body

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## Abstract

Several potential antitumor plants have been proposed and employed in the complementary medicine of tumors, but paradoxically only few attemption has been spent to investigate possible anticancer endocrine-like molecules within human body itself. In fact, at least ten antitumor hormones, provided by antiproliferative, antiangiogenic and immune-modulating effects, have been already identified up to now within the human body, including the pineal hormones melatonin [MLT], 5-methoxytryptamine and pinealine, the neurohypophyseal hormone oxytocin, the endocannabinoids anandamide and 2-arachidonyl-glycerol, the thymic hormones thymosin-alpha 1 and thymulin, somatostatin and the cardiac hormone atrial natriuretic peptide. At present, the only sufficiently investigated hormone from a clinical point of view is the pineal indole MLT, which has appeared to be effective in the treatment of several cancer-related symptoms, namely piastrinopenia, cachexia, mood disorder and asthenia's and to prolong the survival time in patients with disseminated cancer and life expectancy less than 1 year. Therefore, at least MLT would have to be included within the commonly used drugs in the medical Oncology.

**Keywords:** Anticancer agents; Anticancer resistance; Atrial natriuretic peptide; Beta-Carbolines; Cannabinoids; Metatonin; Oxytocin; Pineal indoles

## Introduction

It is known that most antitumor molecules commonly used in the treatment of human neoplasms are drawn from plants and mushrooms, or chemically synthetized, whereas the human body has been namely investigated up to now to identify possible endogenous tumor growth factors and hormones provided by potential pro-tumoral activity rather than the antitumor ones, such as estrogens for breast cancer, androgens for prostate cancer, beta-endorphin for brain tumors [1], FSH for ovarian and endometrial tumors [2,3], PRL for breast and prostate carcinomas [4], and GH and IGF-1 [5] for several tumor histotypes. On the contrary, only few attemption has been spent to identify possible antitumor endogenous endocrine hormones, which could be involved in maintaining the natural anticancer resistance of the human body, and to clinically evaluate their potential efficacy in the therapy in human tumors. However, even though their clinical application is still at the beginning and very few clinical studies are available, at present at least ten endogenous anticancer endocrine molecules, whose action mechanisms have been already well documented in a great number of experimental studies, have been identified, including the pineal indoles melatonin [MLT] [6], corresponding to the N-acetyl-5-methoxytryptamine, andt he 5-methoxytryptamine [5-MTT] [7], the pineal beta-carboline pinealine [PNL] [8], the neurohypophyseal hormone oxytocin [OT] [9, 10], the endogenous cannabinoids arachidonyl-ethanol-amide [AEA], the so-called anandamide, and 2-arachidonyl-glycerol [2-AG] [11], the cardiac hormone atrial natriuretic peptide [ANP] [12], the thymic hormones thymosin-alpha 1 and thymulin [13], as well as somatostatin, at least for somatostatin receptor expressing neuroendocrine tumors [14]. Within the group of the endogenous molecules potentially provided by antitumor activity, in addition to the endocrine-like or neuro active molecules, cytokines have also to be considered, and at present the most important anticancer cytokines in humans are represented by IL-2 [15] and IL-12 [16], because of their capacity of activating the antigen-independent and the antigen-dependent anticancer cytotoxicity, respectively. Then, the human body may potentially control tumor development and growth through either the immune response, namely by the secretion of IL-2 and IL-12, or the production of endocrine molecules capable of exerting cytotoxic or antiproliferative activity, namely through the pineal gland [17-20], which represents the main source of anticancer hormones of the human body, and which constitutes with brain endocannabinoid system a fundamental functional axis involved in the perception of pleasure and in the spiritual expansion of mind [11, 21]. Moreover, it has to be remarked that cytokine network and neuroendocrine system are linked by complex and reciprocal interactions, which influence the _in vivo_ immune functional status. Therefore, the natural anticancer resistance of the human body substantially would depend on the functionless of the psycho-neuro-endocrino-immune [PNEI] system [22,23], that may be considered as the synthesis among immune function, neuroendocrine activity and psycho-spiritual life.

## The Neuroendocrine Control of the Antitumor Immunity

The influence of the psychological life, consisting of all possible emotions and sexual fancies, on the immune system would be mainly mediated by brain opioid system, namely through the mu-opioid receptor. In fact, brain opioid system has been proven to be involved in most cancer-promoting conditions, including stress and depression [24]. On the contrary, brain endo cannabinoid system with its connections with the pineal gland plays a fundamental role in mediating the perception of pleasure and the spiritual sensitivity [11]. Brain opioid hyper activation may predispose to cancer by both a direct stimulation of cancer cell proliferation, and a suppression of the anticancer immunity through an inhibition of IL-2 and IL-12 secretion and a stimulation of immunosuppressive cytokines, namely IL-10 and TGF-beta [25]. On the contrary, the pineal-cannabinoid system axis may play an anticancer role by stimulating IL-2 and IL-12 production, with a following activation of the antitumor immunity, and inhibiting cancer cell proliferation [11,21], since either the pineal hormone MLT, or the cannabinoid agents have been proven to inhibit cancer growth through a direct cytotoxic effect by inducing the apoptotic process by acting on a specific cell receptor, consisting of MLT receptor [MT-R] for MLT and cannabinoid receptor [CB-R] for the cannabinoid agonists. In contrast to the anticancer role of the pineal gland, which represents the main anticancer organ in the human body [17-20], it is known since many years that the hypophysis gland plays a major tumor promoting effect, since PRL may be a tumor growth factor at least for mammary and prostate carcinomas [4], FSH and LH may indirectly promoting breast and prostate tumors by stimulating the production of sexual hormones, and at least FSH could be a direct tumor growth factor for gynecologic neoplasms [2,3], beta-endorphin may be a growth factor for glioblastoma [1], and finally GH and IGF-1 may be potentially involved in the stimulation of several tumor histotypes through complex interactions with the various endogenous tumor growth factors, namely EGF itself, even though the relation between GH and EGF and other tumor growth factors has still to be better investigated and defined [5]. Finally, from the point of view of its effects on tumor growth, the neurohypophysis with respect to the anticancer activity of the pineal gland and the tumor promoting activity of the adeno hypophysis, the neuro hypophysis may exert both activities, since OT has been proven to exert antiproliferative effects against several tumor histotypes [9,10], whereas the other neurohypophyseal hormone, the vasopressin [ADH], would exert a preferential protumoral activity, namely by situating the angiogenic processes [26]. Moreover, in addition to the opposite preferential influence of pineal-cannabinoid axis and hypophysis-opioid system unity on tumor growth, heart itself may influence tumor growth in an opposite way by modulating both anticancer immunity and cancer cell proliferation through its endocrine activity, by stimulating the anticancer immunity [27] and inhibit cancer cell proliferation by the secretion of ANP [12], or in an opposite way by that of endothelin-1 [ET-1], which may inhibit the anticancer immunity by counteracting lymphocyte activation, and stimulate tumor growth by acting as a direct tumor growth factor, enhancing the potency of other tumor growth factors, and playing an angiogenic activity, due to a direct stimulation of VEGF secretion, which in turns stimulates ET-1 production, then VEGF and ET-1 are reciprocally linked by a positive feedback circuit [28]. Therefore it is possible to identify into the human body, two opposite ways with respect to tumor onset and growth, a protumoral way, which is linked to the psych emotional life, and which is chemically mediated by brain opioid system, adenohypophysis, ADH and ET-1, and on the other side an antitumor way, which is the expression of both pleasure, including the sexual one, and spirituality, and which is biochemically mediated by brain cannabinoid system, pineal, OT and ANP. ADH and ET-1 are linked by a reciprocal stimulatory action [29], as well the secretion of OT and ANP [30]. Finally, MLT and ANP secretions are connected by a positive feedback mechanism, because of their reciprocal stimulation [31,32]. The evidence of the antitumor activity of the pineal gland, which is connected with both spiritual life and pleasure perception through its relation with brain end cannabinoid system, as well as the evidence, even though in an opposite manner, of the preferential photomural role of the hypophysis, which is involved in the regulation of the biological life in normal and stress conditions, would have to be already sufficient for both Physicians and Tologists to recognize that human body, and most in general the Biology, is structured for both pleasure and spiritual expansion of self-consciousness, whose repression negatively influences the functionless of the physiological natural anticancer resistance, and predispose to tumor development or recurrence. As far as the importance of each single potential anticancer molecule of the human body in the treatment of human tumors is concerned, the promising studies on the anticancer and immune-modulating properties of the thyme hormones have been interrupted since may years ago, then at present no define conclusion may be proposed about the possible use of thyme hormones in the treatment of human tumors. The anticancer properties of somatostatin and its analogues are well known in the clinical oncology. Therefore, the major attempt concerning the natural anticancer agents of the human body has to namely concern the pineal hormones, OT, the endogenous cannabinoid agents, and the cardiac hormone ANP.

## The Main Anticancer Agents of Human Body

Most clinical studies are limited to the only pineal indole MLT [33]. MLT is namely produced during the dark period of the day. However, MLT is not the only anticancer hormone produced by the pineal gland, since at least another indole hormone has to be considered for its documented anticancer properties, the 5-MTT, which is mainly produced during the light period of the day. All pineal indoles are originated from tryptophan and serotonin. Moreover, the pineal indoles may be transformed into beta-carbolines, which originate from the condensation of indole-ethyl-amines and aldehydes. Beta-carbolines are provided by both anticancer and psychedelic effects, by playing a fundamental role in self-consciousness processes and in the chemical mediation of the spiritual life. At present, more than 20 beta-carbolines have been identified, the most known of them is the 6-methoxy-1,2,3,4-tetrahydro-beta-carboline, the so-called pineline [PNL] [8], and the main endogenous source of beta-carbolines is represented by pineal itself. At present, the main mechanisms involved in the control of tumor growth are consisting of possible direct cytotoxic action, induction of cancer cell apoptosis, inhibition of tumor growth factor production or growth factor receptor activation, inhibition of the action of protumoral hormones, inhibition of tumor neo-angiogenesis, induction of an effective anticancer immune reaction, and resolution of cancer-related chronic inflammatory status [34], which is mainly mediated by the macrophage system. In fact, cancer related-chronic inflammatory status has been proven to constitute the main mechanism responsible for suppression of the anticancer immunity, due to the production of immunosuppressive inflammatory cytokines, such as IL-1-beta, IL-6, and TNF-alpha, or to the secretion of anti-inflammatory cytokines, but also provided by immunosuppressive

activity, namely IL-10 and TGF-beta, because of their stimulation of T regulatory [T reg] lymphocyte generation [35], which would represent the main immune cells responsible for cancer-related immunosuppression. By considering these possible anticancer mechanisms of action, it has been demonstrated that the anticancer molecules of the human body, as well as the anticancer principles of the most investigated antitumor plants, including Aloe, Myrrh, Boswellia, Magnolia, Graviola Curcuma and Cannabis, may exert their anticancer activity through several biological mechanisms, which included both antiproliferative cytotoxic and immune stimulatory effects.

## The Pineal Hormone Melatonin

MLT is the main natural anticancer molecule clinically investigated in the curative or palliative treatment of human neoplasms [33]. From a palliative point of view, MLT has appeared to be effective in the treatment of cancer-related thrombocytopenia, neoplastic cachexia by inhibiting TNF-alpha secretion, mood depression, anxiety, including the anticipatory vomiting on chemotherapy, asthenia, sleep disorders, and to partially prevent cardio-toxicity and neuro-toxicity of the various chemotherapeutic agents, while no relevant effect has been observed in the treatment of neutropenia, anemia, and alopecia. On the other hand from a curative point of view, MLT would represent up to now the only existing molecule potentially able to counteract the overall biological mechanisms responsible for tumor onset, growth and dissemination, including both spontaneous and chemically-induced malignant transformation, intercellular joint alteration with the following change in intracellular matrix characteristics, which allows the neo-angiogenic process, and abrogation of the mechanisms responsible for cancer-related immunosuppression, including cancer cell Fas-L expression, which is responsible for T cytotoxic lymphocyte apoptosis in the case of its cell contact with the neoplastic cell. The great variety of biological effects played by MLT may be explained on the basis of its capacity of controlling DNA gene expression. Then, the anticancer activity of MLT is due to the overall three main mechanisms involved in the control of tumor growth, including cytotoxic-antiproliferative, anti-antigenic and imuno-stimulatory effects. MLT may exert several immune-modulatory effects [36], but from an oncologic point of view, the most important immune-modulating properties of MLT are represented by the stimulation of IL-2 and IL-12 secretion from T helper-1 [TH1] lymphocytes and dendritic cells, respectively, as well as by counteracting macrophage-mediated immunosuppression. MLT may act in an antitumor way by either acting on specific MT-R expressed by tumor cells, or in independent receptor manner as a free-radical scavenger [37]. Then, with respect to the overall common palliative therapies of cancer, which are limited to the treatment of each single cancer-related symptom, MLT medical therapy may not only contribute to the relief of cancer-related symptoms, but also prolong the survival time of untreatable disseminated cancer patients, in association with an acceptable quality of life, by abrogating the paradoxical separation between cure and palliative therapy of cancer. *In vivo*, MLT has been proven to exert an evident anticancer action only at pharmacological doses and with an administration once day during the dark period of the day, corresponding the daily phase, during which its production is physiologically maximal [38]. In humans, the anticancer efficacy of MLT has appeared to be a dose-dependent phenomenon [39], and at present no dose-limiting toxicity has been observed until at

a dosage greater than 500 mg/day. MLT may be administered orally, intramuscularly or intravenously. At present, within the great variety of cancer complementary medicines, the treatment with high-dose MLT, either alone or in association with other pineal in doles and antitumor plants, is the only non-standard medical oncological therapy, which has been proven to prolong the 5-year survival in patients with metastatic cancer, for whom no conventional antitumor therapy was available and with life expectancy less than 1 year [40]. Most human tumors may potentially respond to MLT, since promising results have been observed in melanoma, non-small cell lung cancer, gastric cancer, prostate cancer, triple negative breast cancer, pancreatic adenocarcinoma, sarcoma, brain tumors and brain metastases due to solid tumors. The antitumor properties of MLT are not surprising, since the pineal failure represent the main cancer-related endocrine deficiency [41], and it may involve the whole pinel gland, because of the evidence of pineal histological damage in patients died from cancer [42], even though at present the evidence of abnormally low blood levels has been documented for the only MLT [41]. The possible importance of the deficiency of pineal antitumor hormones other than MLT is also suggested by the evidence that the association of 5-MTT or PNL to MLT may improve the clinical efficacy of MLT itself [43]. By synthetizing, the significance of MLT cancer therapy is justified by either its anticancer properties at pharmacological doses, or as a substitute treatment to correct cancer-related pineal deficiency.

## The Pineal Indole 5-methoxytryptamine and the Beta-Carboline Pinealine

Both 5-MTT and PNL may play a direct anticancer anti proliferative activity. *In vitro*, 5-MTT has been proven to exert an anticancer action superior to that of MLT itself [7], while its immune-modulating effects need to be further investigated and defined. The same anti-inflammatory activity of both 5-MTT and PNL needs to be better analyzed. Moreover, at present is still unknown whether 5-MTT and PNL may act on specific receptors, or by modulating MT-R or neurotransmitter and benzodiazepine receptors [BNZ-R], as well as demonstrated for the beta-carboline molecules. Finally, PNL has appeared to exert antidepressant effects by inhibiting MAO activity, but paradoxically in association with anxiety, due to block of BNZ-R [44].

The Endocannabinoid Anandamide and 2-Arachidonyl-Glycerol The endogenous cannabinoids AEA and 2-AG are synthetized at cell surface levels starting from the arachydonic acid and by the anandamide synthase, and they were catabolized by the fatty acid amide hydrolase [FAAH], whose increased levels are associated with a reduced cannabinoid content [11].The cannabinoid agonists may exert a direct cytotoxic effect, which is mediated by the CB1 receptor, on most cancer cell histo types by inducing the apoptosis or inhibiting growth factor receptor activation. On the contrary, the immune-modulating effects of cannabinoids are namely mediated by the CB2 receptor. The psychdelic effect is only a CB1 receptor-dependent event. In contrast to MLT, whose main target immune cell would be represented by the TH1 lymphocyte, cannabinod immune-inflammatory activity is namely due to the inhibition of macrophage production of inflammatory immunosuppressive cytokines, such as IL-1-beta, IL-6 and TNF-alpha, as well that of IL-17 from TH17 lymphocytes [11]. At present, the only documented efficacy of cannabinoid agents in the care of human tumors is that in brain glioblastoma [45]. In any case, cannabinoids agents hav been proven to be effective at least in the treatment of the following cancer-related symptoms:

anaedonia, neoplastic cachexia, anorexia, vomiting, and pain, including the neuropathic one [11]. No clinical study has been performed up to now with AEA or 2-AG in the treatment of cancer. However, it is probable that their effects may be similar to that of the exogenous cannabinoids.

## The Neurohypophyseal Hormone Oxytocin

Until few years ago, OT was considered only for its importance in partum and lactation. OT has also appeared to play an important role in behavioral functions, including affective life, maternal profile and social relationships. Finally, OT may exert antalgic and memory inhibition activities. In addition to these endocrine and psychological effects, OT has appeared to act as a growth regulator and to exert an proliferative effect on several tumor histotypes [9,10,46], including breast cancer, prostate cancer, gynecologic tumors, and brain neoplasms in experimental conditions by acting on a specific OT receptor, whereas it could potentially stimulate the growth of trophoblast-derived tumors. OT may also exert anti-angiogenic [10] and anti-inflammatory effects, while its influence on the immune system has still to be investigated. Finally, in experimental studies OT has appeared to be effective in preventing chemotherapy-induced neurotoxicity at a dose of about 100 micrograms/kg b.w. [47]. At present, however, no clinical study of OT has been performed in cancer treatment.

## The Cardiac Hormone Atrial Natriuretic Peptide

ANP is produced by cardiac myocytes. In addition to its vasodilator, natriuretic and cardiac protective effects, ANP would also play an important anticancer activity, due to several mechanisms[48], including the inhibition of cancer cell proliferation of ANP-receptor expressing tumors and tumor growth factor-induced tumor cell proliferation, the inhibition of angiogenesis by blocking VEGF secretion, and the stimulation of the anticancer immunity by counteracting cancer-related chronic inflammation and activating T lymphocyte system. Unfortunately, no clinical study of ANP in cancer cure has been performed. In any case, because of its short half-life, long acting ANP analogues will be required in the clinical practices.

## The Anti-Mullerian Hormone

Within the endogenous endocrine-like molecules potentially provided by anticancer activity, the anti-mullerian hormone [AMH] would have also to be included, a glycoprotein of TGF-beta family. AMH is produced by fetal Sertoli cells, and it is responsible for the regression of Mullerian ducts in males, with the following inhibition of the development of uterus and Fallopian tubes. AMH may be also produced by ovarian granulosa cells, and it would play an important role in ovarian folliculogenesis. From a clinical point of view [49], the evidence of abnormally low blood levels of AMH is the more precocious and adequate marker of menopausal status with respect to FSH itself. AMH decrease also during pregnancy. On the other side, high levels of AMH have been reported in polycystic ovarian syndrome, and namely in granulosa cell ovarian tumors. AMH has also appeared to inhibit aromatase activity, then, because of its importance in influencing testosteron metabolism, AMH could influence the sexual differentiation and identity also during the adult life. Preliminary studies would also suggest an antiproliferative activity of AMH, namely in endometrial cancer and other gynecologic tumors, by inhibiting epidermal growth factor-induced cancer cell proliferation [50].

## Conclusion

The well documented existence of several natural anticancer nontoxic molecules, either in the human body, or from plants, would have to interrupt the separation between palliative and curative therapies of human neoplasms, since most natural anticancer agents may exert both palliative and curative effects by counteracting tumor growth, with a consequent improvement not only in cancer-related symptomatology and quality of life, but also in the survival time. In particular, on the basis of the great number of experimental and clinical studies confirming its anticancer properties, the refusal of MLT use in the clinical therapy of cancer patients would have to be considered as an incredible medical failure of the medical sciences.

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