

## Clinical lessons from the first applications of BNCT on unresectable liver metastases.

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**Abstract.** After a long series of studies on the effects of neutron irradiation of <sup>10</sup>B loaded neoplastic cells both in culture and in animal experiments, we started the clinical application of BNCT on humans affected by liver metastases of a radically resected colon adenocarcinoma. The procedure we adopted includes a first surgical phase, with hepatectomy; a radiotherapeutic phase, in which the isolated liver, washed and chilled, is extracorporeally irradiated with thermal neutrons; and then a second surgical phase for the reconnection of the liver to the patient. Until now two patients have been subjected to the BNCT treatment. The first one survived 44 months with a good quality of life, and died because of diffuse recurrences of his intestinal tumour. The second patient had the same early perioperative course, but after 33 days a worsening of a dilatative cardiomyopathy, from which he was suffering, determined a cardiac failure and eventually death. This clinical experience, although limited, has shown that extracorporeal neutron irradiation of the liver is a feasible procedure, able to ensure the complete destruction of liver metastases and a possible long lasting survival. In our patients neutron irradiation caused massive cellular necrosis highly specific to tumour cells, whereas normal cells were mostly spared. Nevertheless, the impact of such a traumatic operation on the patient's organism must be taken into account. Finally, we have to be aware that the fight against tumour rarely leads to a complete victory. We now have an innovative weapon which is both powerful and partly unsettled: it must be refined and above all used.

### 1. Introduction

Almost 4 years ago, in December 2001, we performed the first in the world thermal neutron irradiation of a human isolated liver, which was affected by diffuse metastases of a colon carcinoma and had been previously loaded with a <sup>10</sup>B compound. 19 months later, in July 2003, the same procedure was applied again on another patient for the treatment of unresectable hepatic metastases of a carcinoma of the rectum. Both patients are dead at this time. Then it is now possible to draw some conclusions on the clinical aspects of this experience, on the positive and questionable facets of the procedure and the most important problems still to be solved.

The target of our project was the curative treatment of liver metastases from a colon adenocarcinoma. When diffuse to both lobes of the liver, they are a major oncological problem, being a frequent event in the natural history of a colon tumour and unresectable in 2/3 of the cases. The patients are therefore left without any real therapeutic possibility, as chemotherapy and radio frequency ablation obtain only palliative results. Moreover there was another reason for the choice of this target. In all traditional BNCT applications, typically in the treatment of cerebral glioblastoma, recurrences of the disease are frequent in the area which is peripheral to the main nodule and not treated by the neutron collimated beam. According to the concept first proposed by Pinelli *et al* [1,2,3], the ideal situation would be the one of an organ explanted after being loaded with a <sup>10</sup>B compound, dipped into an homogenous neutron field and then reconnected to the patient. This way,

not only a portion but the whole volume of the diseased organ would be exposed to the neutron flux, so that the dose of the absorbed radiation could be the same in any known or unknown neoplastic nodule, depending merely on  $^{10}\text{B}$  concentrations in their cells. In the case of the liver, from the surgical point of view, this project was feasible, and is named “*ex situ* liver autotransplantation”.

## 2. The experimental premises and preclinical validation

In 1984 a great multidisciplinary program of research started at the University of Pavia, with the following goals: 1) to define the conditions under which the neutron irradiation could obtain a therapeutic result on an explanted organ bearing areas of neoplastic populations; 2) to verify the possibility to achieve these conditions in the liver; 3) to apply the BNCT procedure both in cell cultures and in the experimental animal. The program involved, in a close cooperative relationship, the Pavia Section of the INFN (Istituto Nazionale di Fisica Nucleare) and the Department of Nuclear and Theoretical Physics, the Department of Animal Biology and the Department of Surgery. Due to the highly innovative idea which is at the base of the procedure (*i.e.* the extracorporeal irradiation of the whole liver), we had to choose a model for the experimental animal in which all the essential features of the human pathological situation could be created. Then the entire procedure of BNCT had to be performed on the animal, before an eventual human application. So a colon carcinoma was chemically induced in the syngeneic strain of rats BD-IX by ingestion of dimethylhydrazine. From its neoplastic cells a permanent line DHD/K12/TRb was obtained [4]. For *in vitro* experiments cultures of these cells were always used. *In vivo* researches were performed in rats bearing metastases obtained with the intrasplenic injection of neoplastic cells from the line mentioned above. As the carrier of B we used  $^{10}\text{BPA}$ , a substance that appears to be uptaken by cancer cells with an active transport mechanism so that  $^{10}\text{B}$  concentration in cells can be higher than the one in interstitial fluid and culture medium.

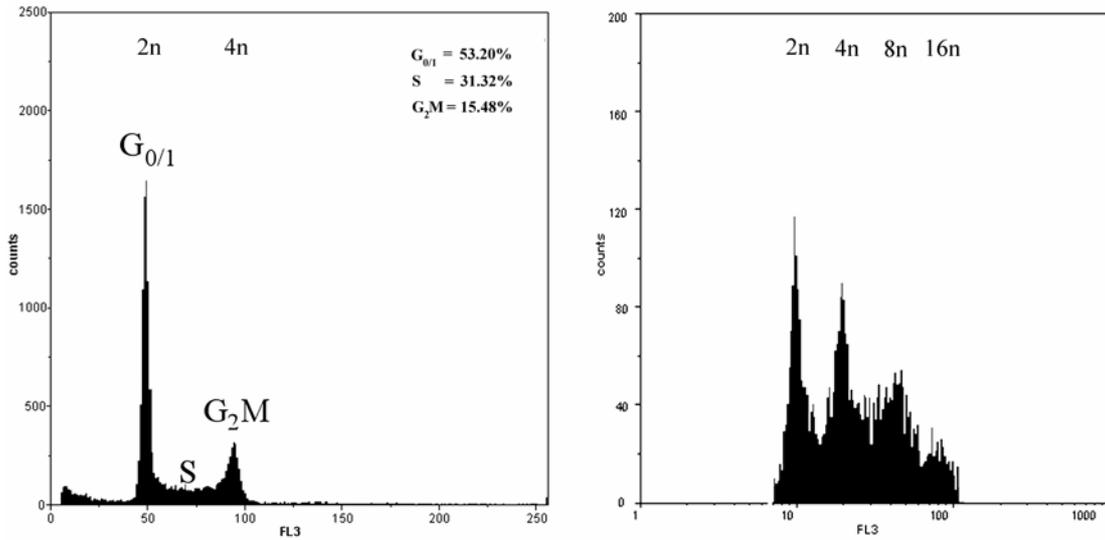
The *in vitro* effects of the neutron irradiation were studied on the coloncarcinoma cells following the modifications induced in their cell cycle phase distribution by means of the DNA flow cytofluorimetric analysis. Afterwards the cell proliferative capacity was evaluated by testing the plating efficiency.

Flow cytometry is a technique of flowing cell analysis able to measure physical or chemical characteristics, like cell size and shape or quantities of any cell component or fraction that can be detected by a fluorescent probe. One of the major applications of flow cytometry is the analysis of the DNA content that can provide information on both the proportion of cells in each phase of the cell cycle and the ploidy status (*i.e.* the ratio of the DNA content of a population to that of the diploid one) [5]. Plating assay is one of the commonest way to assess cell survival after radiation. Assessing survival depends on the demonstration that cells retain the proliferative capacity to form colonies containing more than fifty cells [6].

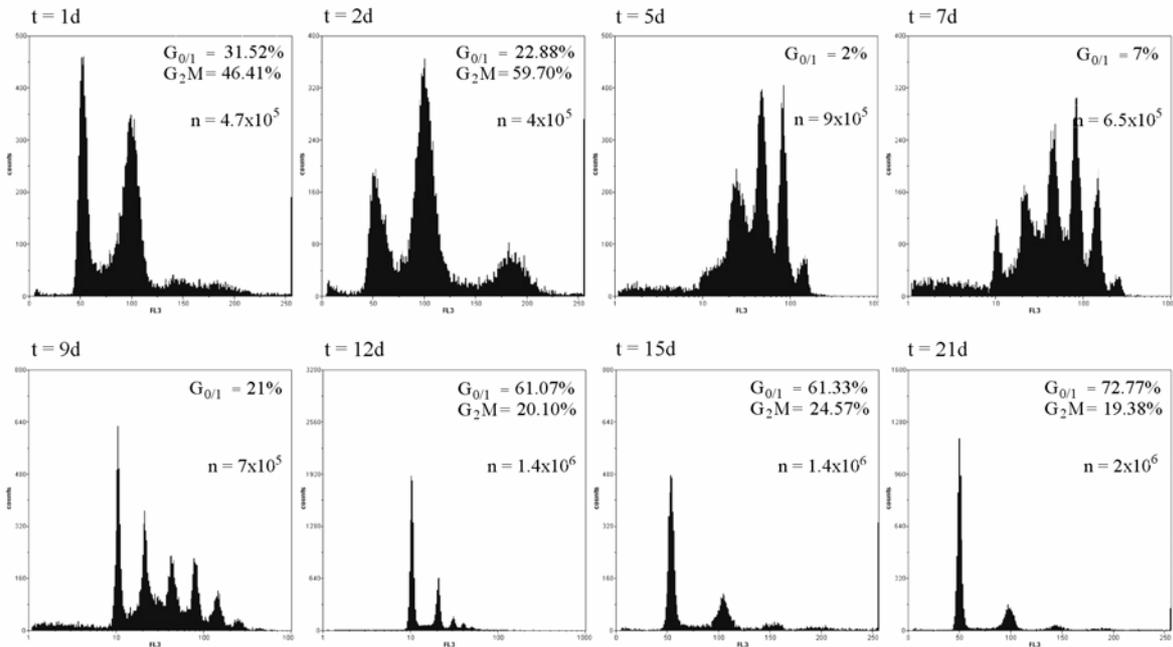
Experiments for the *in vitro* evaluation of radiation effects were performed on cells maintained for 18 hours in 20 ppm  $^{10}\text{BPA}$  enriched medium and then, after washing, exposed to neutron irradiation ( $7 \times 10^{12} \text{ cm}^{-2}$ ,  $10'$ ). Immediately at the end of treatment cells were seeded in both culture flasks, one for each observation time (1,2,5,7,9,12,15,21 days), and Petri dishes: the first seeding was intended for cell counting and DNA analysis, the second for plating efficiency test. Controls of boron enriched non irradiated cells ( $\text{B}^+\text{R}^-$ ), boron lacking irradiated cells ( $\text{B}^-\text{R}^+$ ) and boron lacking non irradiated cells ( $\text{B}^-\text{R}^-$ ) were treated as the irradiated boron enriched cells ( $\text{B}^+\text{R}^+$ ). For DNA analysis cells were stained with propidium iodide, a red emitting fluorescence dye that stoichiometrically binds to double stranded DNA molecules.

Plating efficiency test was performed by seeding cells in five replicate Petri dishes for each selected concentration which ranged from 50 to 250 cells for control samples and from  $1 \times 10^5$  to  $5 \times 10^5$  for treated cells.

Results of flow cytofluorimetric DNA analyses of  $\text{B}^-\text{R}^-$  cells (on the left) and of  $\text{B}^+\text{R}^+$  cells (on the right) are reported in figure 1. The variations of the cytometric profiles in  $\text{B}^+\text{R}^+$  cells *vs* time after irradiation (in lin or log mode) are illustrated in figure 2. Histograms correspond to the different observation times after irradiation. The percent values refer to the proportion of cells in the  $\text{G}_{0/1}$  and  $\text{G}_2\text{M}$  phases to the whole of the cells in culture while the n values indicate the number of cells recollected from each flask at the studied times. The remarkable  $\text{G}_2\text{M}$  block ( $t=1\text{d}$  and  $2\text{d}$ ) and the



**Figure 1.** Examples of flow cytometric DNA histograms. On the left, a typical histogram (linear scale) of the exponentially growing DHD/K12/TRb cell line. Cells are distributed in three cell cycle fractions:  $G_{0/1}$ , S and  $G_2M$  respectively corresponding to resting or presintetic diploid cells (2n), to DNA sintetizing and to the dividing ones (4n). On the right, a multiclonal DNA profile obtained after BNCT treatment (log scale) showing diploid and aneuploid cell fractions: 2n, 4n, 8n and 16n. FL3= red fluorescence channel number proportional to DNA content; counts= number of counted events

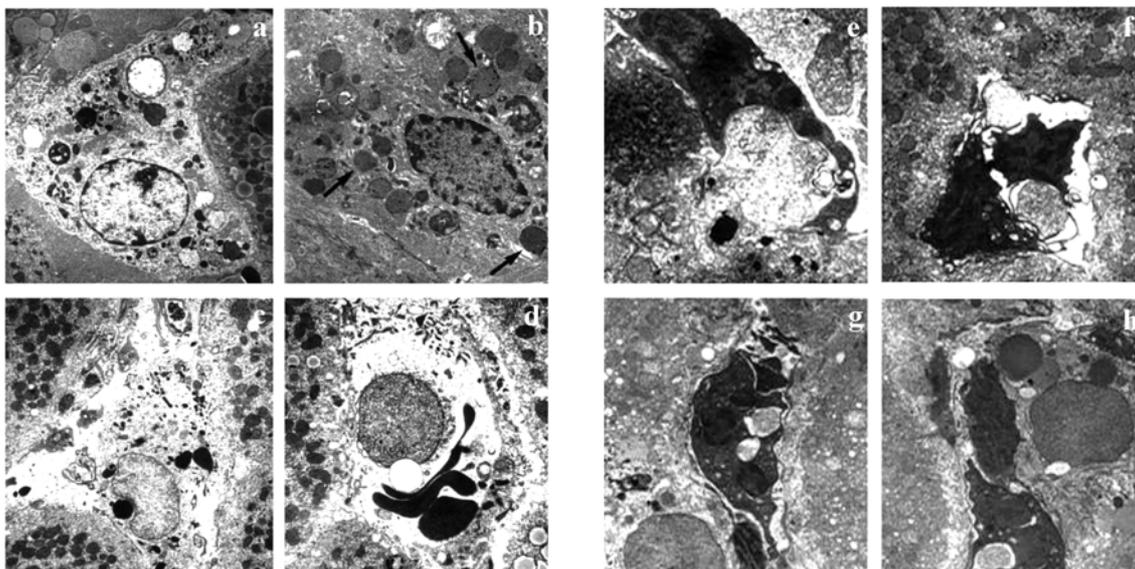


**Figure 2.** DNA content analyses performed on the DHD/K12/TRb cell line at subsequent times after BNCT treatment. Histograms at  $t=1,2,15,21$  days are acquired in linear mode while those at  $t=5,7,9,12$  days in log mode to compact in the scale even the very high fluorescent signals (poliiploid cells)

multiclonal profile, with progressive appearance of higher DNA content subpopulations in subsequent times, are expression of severe cellular damages that progressively result in cell death. As a consequence there is a dramatic fall in the number of cells that can be recovered in the flasks as

compared to controls. In effect, despite the equal number ( $1 \times 10^6$ ) of re-cultured cells, the concentrations reached, for instance after five days, in the control flasks, ( $1,4 \times 10^6$  ( $B^+R^+$ ),  $4,5 \times 10^6$  ( $B^+R^-$ ) and  $6 \times 10^6$  ( $B^-R^-$ )), are significantly higher than that of  $B^+R^+$  cells, that are only  $9 \times 10^5$ . What we see in cell cultures can be thought of as an accelerated model of what may happen *in vivo*. Due to radiation effects, damaged cells can die, not only with the modalities of necrosis and apoptosis, which are microscopically evident. Another mechanism of cell deletion is correlated with mitotic delay and then with a fall in  $G_{0/1}$  and the corresponding increase of  $G_2M$  cells. It results in a slowing down in proliferation and gradual disappearance of this cell population (self-extinction), which in the sequence of figure 2 peaks at about 7 days. Plating efficiency confirms the effectiveness of the treatment but evidences the existence of a resistant cell subpopulation equal to about 0.05% of the treated cells. As shown in DNA histograms of figure 2 ( $t=5d \rightarrow 21d$ ), the resistant fraction progressively grows up, restoring a pattern which recalls the original cell population. The resistant cell fraction very likely belongs to cells, which are quiescent at the moment of irradiation and then unable to reach adequate  $^{10}B$  levels.

The preclinical validation tests *in vivo* consisted in performing the entire BNCT procedure in the rat with hepatic metastases of a colon carcinoma. That was accomplished overcoming important technical issues [3,7,8,9], obtaining the proof that 1) the procedure was compatible with the survival of the animal; 2) necrosis and apoptosis were easily recognizable and largely represented in the neoplastic nodule in consequence of treatment (figure 3 a-d); 3) normal hepatic tissue was preserved; 4) Kupffer cells with important phagocytic activity were ubiquitous (figure 3 e,f); 5) leucocytes appear to be involved too in the process of removal of cellular debris (figure 3 g,h).



**Figure 3.** Ultrastructure of the liver of a rat after induction of hepatic metastases, BNCT and reimplantation (x4000). (a, b, c, d): metastatic colon carcinoma cells (DHD/K12/TRb). Electron-dense bodies in cytoplasm are clear signs of apoptotic damage; (e, f): activated Kupffer cells in intense phagocytosis; (g, h): scavenger activity in leucocytes.

### 3. The clinical applications

In late 2001, having obtained the approval of Ethics Committee of our Hospital and of Ministry of Health, we started the program of clinical applications of BNCT on an isolated liver [10].

According to the guide lines approved for the clinical protocol the patients should be young (< 55 y), with liver-only metastases of a colon adenocarcinoma already radically excised and have all vital organs in good health without serious impairment of liver function. The procedure we adopted consisted basically of three phases. The *first surgical phase* includes: liver perfusion with a solution of  $^{10}BPA$ ; biopsies of metastatic and normal hepatic tissue in order to verify that a concentration ratio

of  $^{10}\text{B}$  greater than 4:1 exists between the two samples; and then, hepatectomy with contemporary starting of a veno-venous extracorporeal circulation. In the *radiotherapeutic phase* the isolated liver is washed and chilled, transferred to the thermal chamber of Triga Mark II nuclear reactor and irradiated with the neutrons fluence of  $4 \times 10^{12} \text{ cm}^{-2}$ . The irradiation time averages 10 min; it varies according to the histopathological characteristics of tissues, and in particular to the degree of liver neoplastic involvement. Then, in the *second surgical phase*, the liver is reconnected to the patient, the extracorporeal by-pass is abolished and the abdomen is closed.

The preoperative status and some peculiarities of the surgical phases of the first patient are summarized in table 1. The relevant data of radiotherapeutic phase were favourable with a ratio between the  $^{10}\text{B}$  concentrations in tumour and normal tissues near to 6:1.

**Table 1.** BNCT Pavia Project. From the clinical record of the 1<sup>st</sup> patient

Sex and age	male, 48 y old
Primary tumour	sigmoid adenocarcinoma pT3 G2 N1 M1 <sup>a</sup>
Vital organ state	good
Hepatic involvement	14 small bilobar synchronous metastases
Residual liver function <sup>b</sup>	63% <sup>c</sup>
Vascular abnormalities	none
Dose of $^{10}\text{BPA}$	300 mg/Kg b.w. through a colic vein
Duration of the radio surgical procedure	21 h
Duration of the anhepatic time	5 h 30'

<sup>a</sup> According to the TNM classification of tumours

<sup>b</sup> Assessed by the galactose elimination capacity (GEC) test

<sup>c</sup> Normal values > 70%

After the success of the first performance on humans of our BNCT procedure, around the middle of 2003, we proceeded to a new clinical application. In table 2 the salient pathological features of the patient are indicated. In his clinical picture three negative aspects were noteworthy: the residual liver function was poor, the vascular anatomy of the liver was abnormal, and the cardiac function was deficient due to a dilatative cardiomyopathy with a stroke volume of 40%.

Considering the  $^{10}\text{B}$  concentrations in tumour and normal tissue, and therefore the doses of absorbed radiations, the figures in this patient were as good as for the first one; the duration of the procedure was shorter, in spite of the difficulties raised by the surgical correction of the above-mentioned vascular anomaly.

**Table 2.** BNCT Pavia Project. From the clinical record of the 2<sup>nd</sup> patient

Sex and age	male, 39 y old
Primary tumour	rectal adenocarcinoma pT3 G2 N1 M1 <sup>a</sup>
Vital organ state	dilatative cardiomyopathy
Hepatic involvement	11 small and large synchronous metastases
Residual liver function <sup>b</sup>	58 % <sup>c</sup>
Vascular abnormalities	right hepatic artery from superior mesenteric artery
Dose of $^{10}\text{BPA}$	300 mg/Kg b.w. through a colic vein
Duration of the radio surgical procedure	18 h 40'
Duration of the anhepatic time	6 h 10'

<sup>a</sup> According to the TNM classification of tumours

<sup>b</sup> Assessed by the galactose elimination capacity (GEC) test

<sup>c</sup> Normal values > 70%

### 3. Post-operative follow-up

Both patients underwent a second operation during the post-operative course. We performed a peritoneal toilet of a large blood collection at the 7<sup>th</sup> post-operative day (p.o.d.) in the first patient and an unsuccessful attempt to surgically correct an hepatic artery thrombosis in the second patient at the 30<sup>th</sup> p.o.d..

The early peri-operative period (*i.e.* the first three weeks after BNCT) was very similar in both patients. They showed symptoms of hepatic and renal insufficiency and an early rhabdomyolysis with profound asthenia, great tissue imbibition and signs of cerebral impairment. These clinical manifestations, which were present in both patients, appeared in a definite sequence and showed a similar evolution, apparently depicting a syndrome. We may tentatively attribute it to a great amount of humoral mediators, probably cytokines, produced as a consequence of a large volume of cellular lysis.

In figure 4 a-e the trend of some laboratory values of clinical importance is indicated, during the first month after BNCT. Normal levels are given as straight horizontal interrupted lines. The enzyme creatin kinase (CK) and myoglobin are two chemical components of striated muscle fibers. Their raise much above the normal levels in the first 10 p.o.d. (figure 4 a) is the demonstration of the severe rhabdomyolysis we mentioned above. The values for the microsomal liver enzymes glutamic oxalacetic transaminase GOT (figure 4 b) and glutamic pyruvic transaminase GPT (figure 4 c) show a significant early increase in both patients with return to normal values after about 10 days.

Bilirubin clearing capacity (figure 4 d) takes much more time to get back to normal. Protrombin level (*i.e.* the index of the coagulative capacity of blood, also directly dependent on the liver function) is obviously low soon after the end of the operation (figure 4 e), but it raises to normal level after the first few days and then remains within acceptable values, with large fluctuations.

The diagram of figure 5 shows the impact of BNCT procedure on renal function. Spontaneous diuresis was suppressed in the first 2 weeks with the obvious need for an extracorporeal dialysis; afterwards we had a complete recovery of renal function.

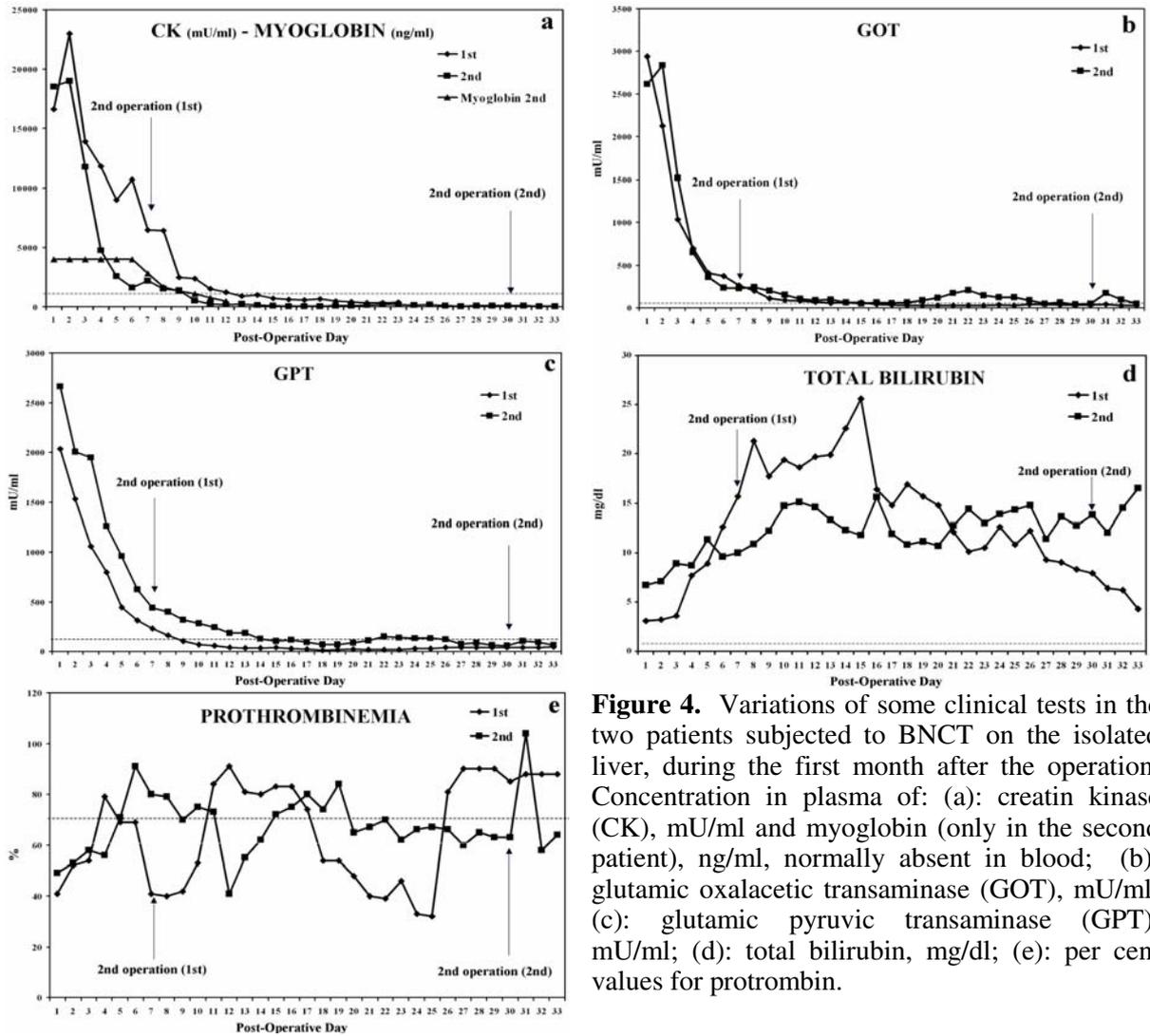
The late peri-operative period (*i.e.* the fourth week after BNCT) was different in the two patients. In the first one we had a quick improvement in health with a progressive return to normal of laboratory values. In particular all neoplastic markers were negative. There was also an increase in residual liver function up to a value of 73% for GEC. In the same patient the evolution of CT images of the liver lesions in corresponding radiograms is shown in figure 6 a,b (above and below) from pre-operative situation to the one at the 7<sup>th</sup> p.o.d.. After BNCT in the site of the neoplastic nodules we observe the presence of areas of tissue necrosis, which are larger than the previous metastases.

In the second patient we had the same evolution of the metastatic lesions to necrosis (figure 7 a-c).

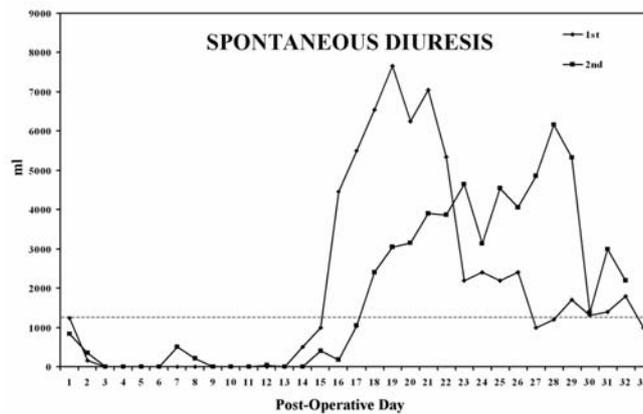
Smaller signs of necrotic damage appeared inside the liver even where pre-operatively we could not detect metastases (figure 7, above). We are led to believe that "occult" neoplastic spreading was treated too. In effect in this patient, before BNCT, a CT scan had shown 5 diffuse metastases, but in the intraoperative US control we found 11. Likewise the first patient seemed to be affected by 6 metastases that during the operation turned out to be 14.

Towards the end of the first month after BNCT the clinical situation of the second patient worsened: he indeed had a vascular complication consisting in a thrombosis of the hepatic artery and then a cardiac failure with fluid imbalance, pulmonary edema and death in the 33<sup>th</sup> p.o.d.. An autopsy was performed and, besides disclosing the signs of the cardiocirculatory failure, it confirmed the massive necrosis of the liver metastases. Macroscopically, on the surface of the liver (figure 8 a) and also in the depth of the organ (figure 8 b) several whitish masses of various size and shape, quite similar to cotton flocks, were present; corresponding to them microscopic evidence of massive necrosis of coagulative type was dominant with deletion of any appearance of a histological structure (figure 9). A widespread hyperplasia of Kupffer cells with clear signs of functional activation was also noticeable. On the periphery of several nodules, focal areas of seemingly vital neoplastic cells with few mitotic images could be observed (figure 10). Normal hepatic tissue was affected by a diffuse fibrosis at the 3<sup>th</sup> stage in this second patient as well as in the first one, who got a biopsy taken in August 2003 from an apparently preserved area of the liver (see next).

In the surviving patient the subsequent post-operative course was uneventful until August 2003.



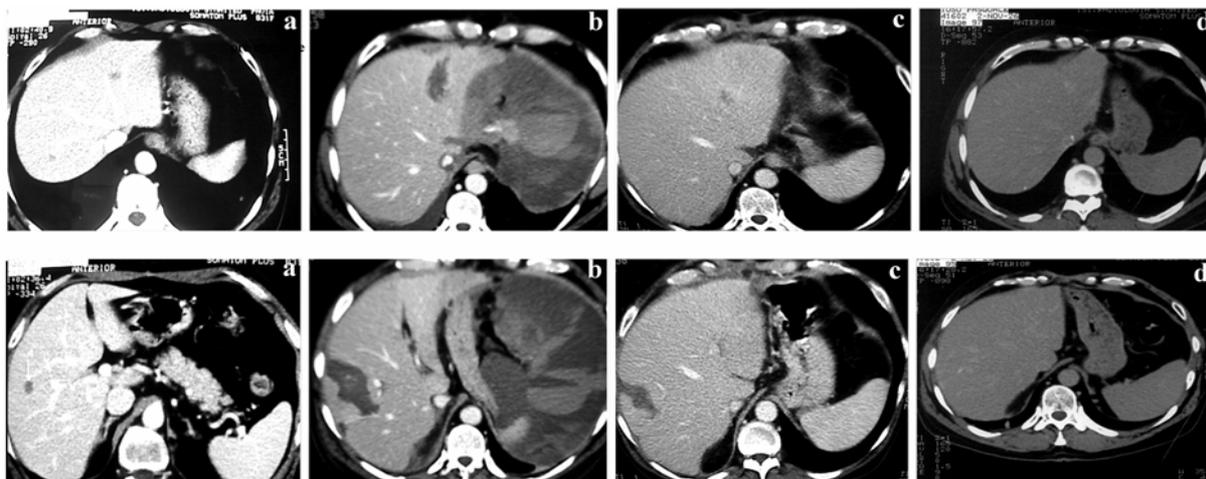
**Figure 4.** Variations of some clinical tests in the two patients subjected to BNCT on the isolated liver, during the first month after the operation. Concentration in plasma of: (a): creatin kinase (CK), mU/ml and myoglobin (only in the second patient), ng/ml, normally absent in blood; (b): glutamic oxalacetic transaminase (GOT), mU/ml; (c): glutamic pyruvic transaminase (GPT), mU/ml; (d): total bilirubin, mg/dl; (e): per cent values for protrombin.



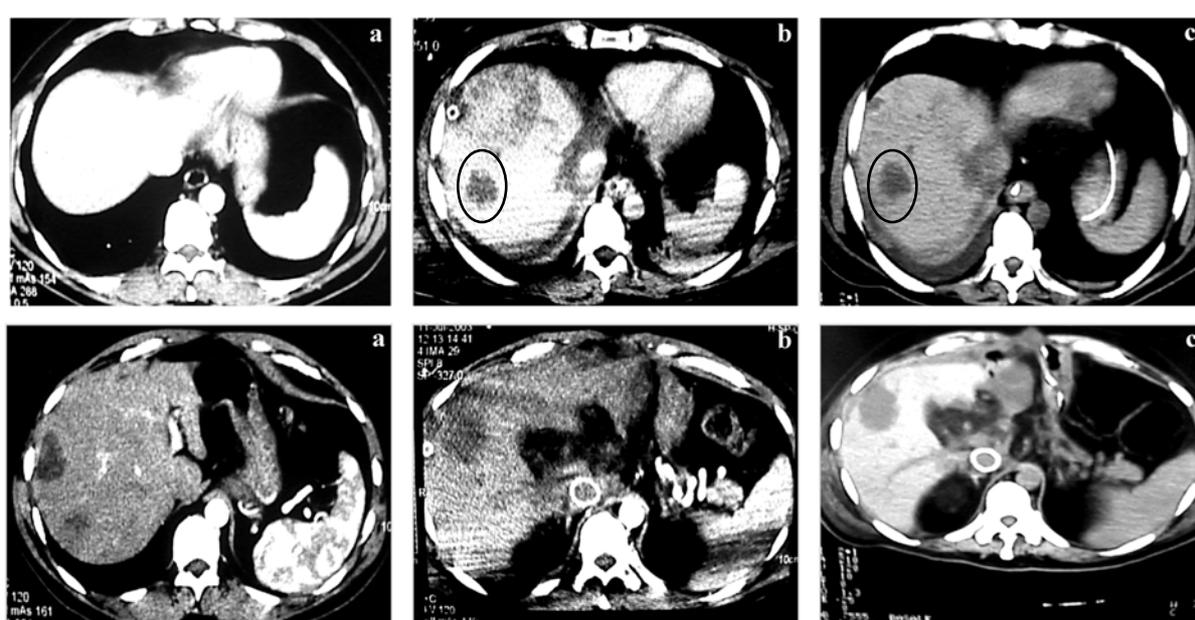
**Figure 5.** Volume of spontaneous diuresis after BNCT, ml/24 h

The areas of necrosis in the liver structure were gradually substituted by normal hepatic tissue: this process was already advanced at the time of a CT control scan after 6 months and it was complete at 12 months (figure 6 c and d).

20 months after the BNCT procedure there was an increase of the value for the neoplastic marker carcinoembryonic antigen (CEA), and in a CT scan a nodule appeared, just external to the left lobe of the liver. The patient was reoperated and the nodule (maximum diameter = 13 mm) was removed. Inside it and in a diaphragmatic scar adherent to the liver (2 mm wide), focal infiltrations of colon carcinoma cells were detected. Normal hepatic tissue was biopsied. No intrahepatic metastases were found out even with an intraoperative US scan. After excision of the neoplastic foci the CEA value



**Figure 6.** Sequence of CT images of the liver on a cranial (above) and a caudal (below) level in the first patient subjected to BNCT. Evolution at different times of the metastases towards necrosis with final substitution by normal hepatic tissue. (a): pre-operatively; (b): at 7 days, (c): at 6 months; (d): at 12 months after the procedure.



**Figure 7.** Modification of CT images of the liver on a cranial (above) and a caudal (below) level in the second patient from (a) the pre-operative aspect up to the situation (b) at 10 days and (c) at 21 days during the peri-operative follow-up after BNCT. In the (b) and (c) images of the upper row, an area of necrosis is encircled, not corresponding in the preoperative scan (a) to any known metastasis

tapered to normal level. The patient, who had always refused any antineoplastic treatment after BNCT, was convinced at this point to follow an adjuvant chemotherapy regimen.

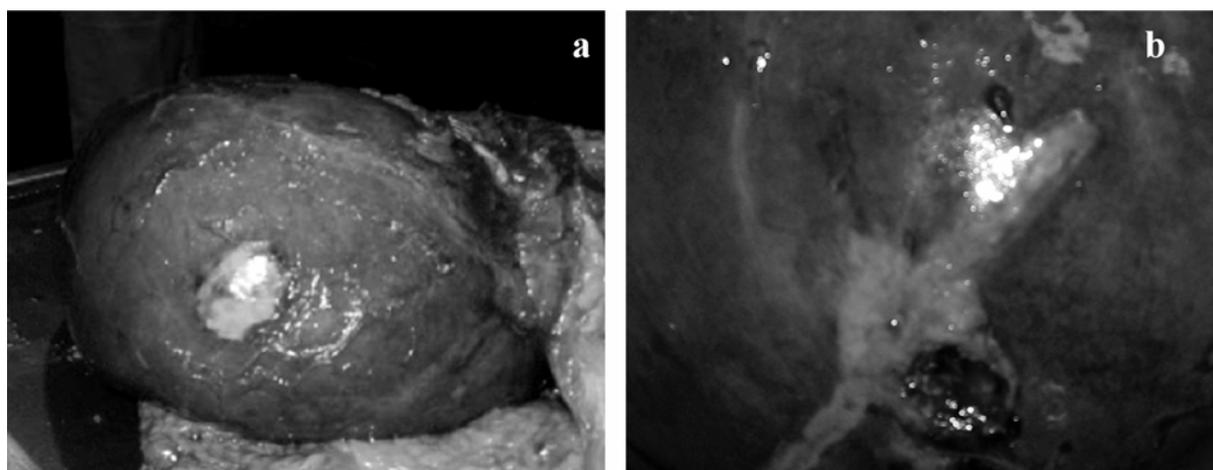
In August 2004, 33 months after BNCT, a new increase of neoplastic markers anticipated the appearance of images of hepatic and extrahepatic recurrences at a CT scan. A new chemotherapeutic regimen started, but with poor results. In February 2005 a further surgical revision of the abdomen showed an only partial neoplastic involvement of the liver, together with a peritoneal carcinosis with multiple nodules, some of which were unresectable. In April 2005 (40 months after BNCT) a new protocol of immunochemotherapy began. In July 2005 the clinical state declined and the patient died on August 4<sup>th</sup> (44 months after BNCT).

#### 4. Discussion and conclusions

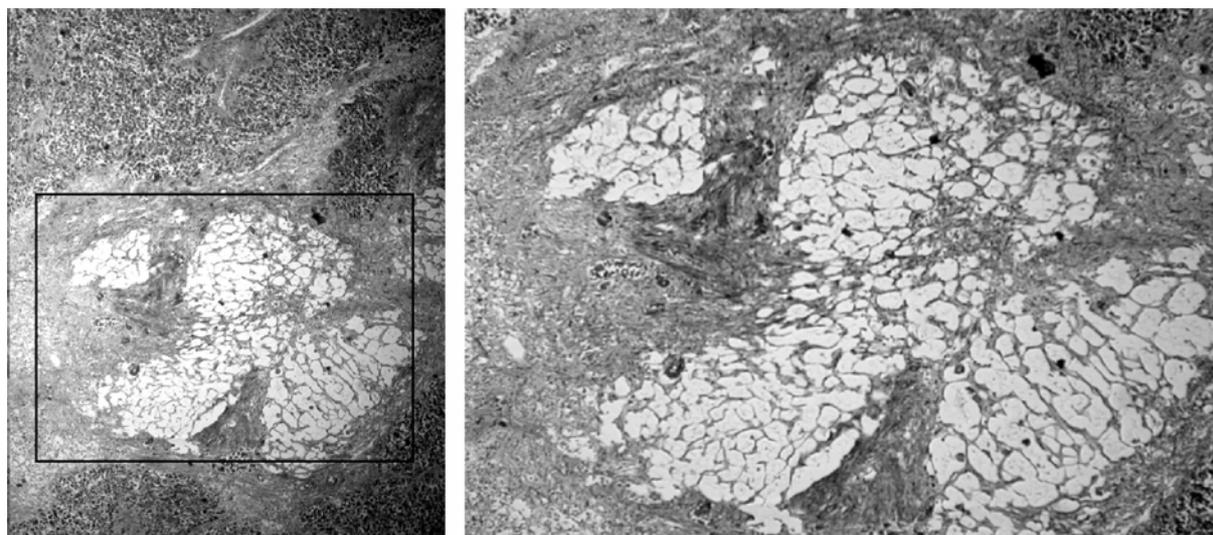
Our final considerations are based on the results obtained with the application of an entirely new procedure on only two patients. If the number of clinical cases was greater, we could be much more detailed and firm in our conclusions. Regrettably, indifference and a lack of will from the part of the Italian health authorities caused a suspension of financial support for the program, which is now stopped.

The exciting experience we have above recorded has some aspects which are clear, objective, undeniable. They are also very positive in terms of oncological efficacy. Other aspects are less favourable, questionable and require improvements. Each one of these issues would deserve a separate discussion. Owing to a shortage of space we can give here no more than a simple enumeration of them.

Beginning with the positive aspects, our experience confirms the tremendous efficacy of BNCT in destroying the tumour cell populations and the high selectivity of its killing effect. No other kind of antineoplastic therapy stands comparison with this one. This statement is based first of all on the results of macroscopic inspection and of microscopic evaluation of the liver in the second patient. The presence of perifocal neoplastic cells in irradiated nodules, in our view, must not be regarded as evidence of failure of the treatment, instead it is a witness of the process that leads to the gradual disappearance of cellular populations, in which a slowed proliferative capacity results from the radiation damage (self-extinction). The location of these cells on the periphery of the neoplastic necrotic masses may be easily explained with the absence of a vascular supply in the depth of the nodule. The strong activation of phagocytic cells which occurred both in the rat irradiated liver and in



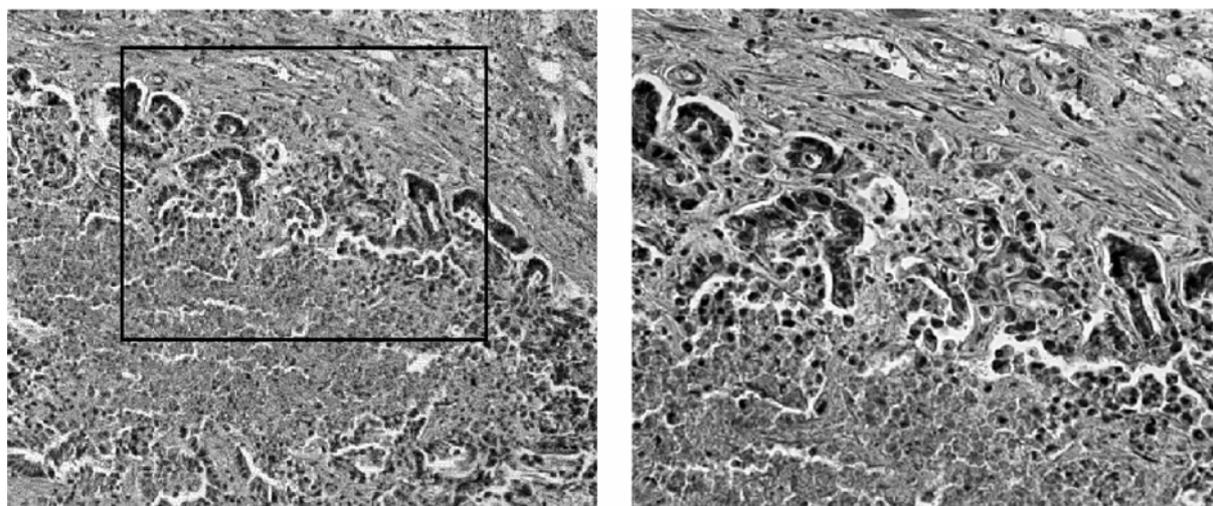
**Figure 8.** Macroscopic appearance of the liver at the autopsy of the second patient 33 days after BNCT. (a): external view of the convexity and (b): coronal section through the right lobe. Multiple whitish necrotic areas are widespread all over the capsular surface and in the depth of the organ.



**Figure 9.** Light microscopy study at two different magnifications of a massively necrotic metastasis. Autoptical finding from the liver of the second patient (33 days after BNCT). Haematoxylin and eosin stain (x10, left; x20, right)

the clinical application is usually defective in the natural evolution of metastases. So this is one of the most interesting and stimulating aspects of our experience.

Also in terms of survival time the obtained result is exceptional because we got 44 months of high quality life for our patient, compared to a median survival time of 4-10 months expected according to the medical literature [11,12]. Another very important information we collected is the proof of the low impact of neutron irradiation on normal liver tissue. In spite of a diffuse hepatic fibrosis, which developed in both patients, the liver function, as assessed by GEC, improved in the first patient after BNCT (73% vs 63% pre-operatively) and was also better (81%, and then completely normal) in a further control in March 2005 (39 months after BNCT and during chemotherapy).



**Figure 10.** Viable adenocarcinoma cells are still recognizable on the periphery of some severely necrotic nodules in the liver of the second patient. Haematoxylin and eosin stain (x20, left; x40, right)

About the questionable aspects, as already said, we suspect that the clinical and biochemical derangements observed in both patients during the first 3 weeks of the peri-operative period are an expression of a “post-irradiation syndrome”, which could be mediated by the release of a great amount

of cytokines. If it should be confirmed we would have several pharmacological ways to control it, and we could even try to remove these powerful mediators through an early extracorporeal dialysis.

Liver autotransplant is a hard task both for patient and surgeon. An *in situ* irradiation of the liver, with the method of hypothermic exsanguineous perfusion (the so named “*in situ* liver autotransplantation”), should reduce the surgical trauma and the time required for the procedure; but in this case a proper shielding of the patient’s body and a bed-side source of neutrons would be needed.

The appearance of recurrences is obviously a very disappointing event in the post-operative course of our long-term surviving patient; it was a late yet fatal accident. Owing to the time elapsed from the date of BNCT to their first appearance (20 months), we can suspect that they were not due to the islets of apparently vital neoplastic cells seen in the histopathological specimens from the second patient, about one month after neutron irradiation. In effect, clinical experience as well as theoretical considerations about the replication time of these tumours (which averages 13 days in our previous evaluations), suggest that this span between the two events is too long. As already mentioned, these same cells could be suffering from a mitotic delay, so, in spite of normal morphological appearance, on the way of a complete extinction. Therefore recurrences could be thought of as having a different starting point and coming from a double source: extrahepatic (from the peritoneal mantle) and intrahepatic (from cells refractory to BNCT, maybe mingled with the apparently vital tumoral cells of our histopathological specimens). From our experiences of irradiation of cell cultures we had the proof that such cells do exist, albeit in a minimal fraction (the proportion is 1 out of 2000 cells). Researches are in progress to define the functional characteristics of these cells, we suppose they are quiescent cells. The best way to kill them would be therefore by stimulating them to proliferate before perfusing the B compound. Of course much experimental work is needed before such hypothesis may become a therapeutic tool.

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