NEO-ADJUVANT CHEMO/IMMUNOTHERAPY IN THE TREATMENT OF STAGE III (N2) NON-SMALL CELL LUNG CANCER: A PHASE I/II PILOT STUDY

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In a previous randomized study, we showed that adjuvant immunotherapy with tumor-infiltrating lymphocytes and recombinant interleukin-2 (rIL-2) significantly improved survival in resected N2-Non Small Cell Lung Cancer (NSCLC) patients. The present study assesses feasibility, safety and potential efficacy of combined neo-adjuvant chemotherapy and immunotherapy with peripheral blood mononuclear cells (PBMC) and rIL-2 in resectable N2-NSCLC patients. Eighty-two consecutive N2-NSCLC patients underwent neo-adjuvant chemotherapy with cisplatin and gemcitabine. Out of the 82 patients, 23 were also subjected to leukapheresis prior to neo-adjuvant chemotherapy while the remaining 59 did not. Collected PBMC were analyzed for viability and phenotype and then stored frozen in liquid nitrogen. Thawed PBMC were infused intravenously, 5 days before surgery. After the infusion, rIL-2 was administered subcutaneously until surgery. Only patients with a partial or complete response to neo-adjuvant chemotherapy underwent surgery: 13 patients in the experimental immunotherapy group (A) and 32 in the reference group (B). The two groups were homogeneous for all major prognostic factors. Median leukapheresis yield was 10 billion PBMC, (range 3-24 billions). Two to six billion PBMC were infused. The phenotypic analysis showed that similar proportions of CD4 and CD8 cells were present in leukapheresis products, and thawed PBMC, as well as in T lymphocytes isolated from the removed tumours. No severe adverse effects were observed following immunotherapy. No significant differences in overall survival (OS) and event-free survival (EFS) were seen between the two groups. However, the 5-year OS in group A was almost twice as much compared to group B (59% *vs* 32%). After adjustment for major prognostic factors, a statistically significant 66% reduction in the hazard of death was seen in patients receiving immunotherapy. The OS benefit was more evident in patients with adenocarcinoma than in those with squamous cell carcinoma. This study supports the favorable toxicity profile and potential efficacy of combining neo-adjuvant chemotherapy and immunotherapy with PBMC and rIL-2 in the treatment of N2-NSCLC patients.

Key words: non-small cell lung cancer, neo-adjuvant immunotherapy, neo-adjuvant chemotherapy, surgery, survival

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In spite of recent improvements in treatment, the majority of patients with N2-Non Small Cell Lung Cancer (NSCLC) ultimately will relapse and die from their disease. Thus, several novel strategies for the treatment of N2-NSCLC are currently under evaluation. We previously published the results of a randomized trial of adoptive immunotherapy with tumor-infiltrating lymphocytes (TIL) and recombinant interleukin-2 (r-IL-2) versus standard chemotherapy in the post-operative treatment of resected N2-NSCLC (1). Subsequently, in an attempt to improve the results of adjuvant immunotherapy with TIL and rIL-2, we combined chemotherapy, immunotherapy and radiotherapy in the post-operative treatment of patients with stage III-NSCLC (2). While the results of the former were promising, those of the latter were negative. Nevertheless, other groups studied different immunotherapeutic approaches. Following the pioneering studies on immunotherapy of lung cancer (3), Kimura et al. (4) conducted a prospective phase II study of chemo-immunotherapy using activated killer T cells and dendritic cells. In a multicenter phase IIB randomized trial, Butts et al. (5-6) used the liposomal MUC1 vaccine L-BLP25 following chemotherapy or chemo-radiotherapy in patients with non-progressive stage IIIB or IV NSCLC. In another multi-center, double-blind, randomized, placebo-controlled phase II study, Vansteenkiste et al. (7) tested the activity of MAGE-A3 as adjuvant therapy in completely resected stage IB or II NSCLC patients. Although suggesting a potential effect of combining chemotherapy with immunotherapy, all these studies did not produce clear and, most of all, definitive results.

Notably, unpublished data from our laboratory showed that the proliferative and functional capacities of TIL isolated from NSCLC of patients who underwent neo-adjuvant chemotherapy were severely damaged, making it impossible to recruit and expand adequate numbers of functional TIL. Thus, the above-mentioned adjuvant immunotherapy (1) was unfeasible in neo-adjuvant chemotherapy treated patients. For this reason, we designed the present pilot study to assess the feasibility, safety and potential usefulness of combining neo-adjuvant chemotherapy and immunotherapy with peripheral blood mononuclear cells (PBMC) and rIL-2, in resectable N2-NSCLC patients. This approach was based on the hypothesis that lymphocytes collected before chemotherapy might preserve their proliferative and functional properties, maintaining, after infusion before the surgical treatment, the ability to restore the impaired immunological competence in patients treated with chemotherapy. Furthermore, leukapheresis, lymphocyte freezing and thawing, as well as the infusion of unmodified autologous cells are safe procedures, routinely used in hematological and oncological practices.

MATERIALS AND METHODS

Patient population

From 2002 to 2005, 82 consecutive patients with N2-NSCLC were enrolled in the study. The protocol was approved by the Ethics Committee of Regione Piemonte (Italy). All patients were informed of the aims and procedures of the study and signed a consent form.

Pre-operative assessment included: CT scan of the brain, chest, and upper abdomen; bronchoscopy; PET scan; and cervical mediastinoscopy. Inclusion criteria were: mediastinoscopic demonstration of N2-NSCLC; absence of distant metastases; disease considered completely resectable; cardiopulmonary, hematologic, renal and hepatic functions adequate for the planned treatment; performance status (PS) 0-1; no prior therapy with biological response modifiers, anti-neoplastic agents or steroids.

Study design, treatment and follow-up

The process of selection of the study population is summarized in Fig. 1. All 82 patients received 3 cycles of induction chemotherapy with cisplatin and gemcitabine. Among these patients, 23 agreed to undergo subsequent neo-adjuvant immunotherapy and underwent leukapheresis before starting neo-adjuvant chemotherapy. Among the other 59 patients, 6 refused the experimental immunotherapy and 53 could not undergo leukapheresis because of laboratory unavailability. These 59 patients were included in the study as reference to allow comparison of overall survival (OS) and event-free survival (EFS) for merely exploratory purposes.

Only patients who showed either a partial or a complete response to the mediastinum and no tumor progression after neo-adjuvant chemotherapy (as documented by CT scan and PET scan) underwent surgery and represent the study population: 13 out of the 23 (57.0%) patients who underwent leukapheresis and subsequent immunotherapy (experimental group A) and 32 out of the 59 (54.3%)

immunologically untreated patients (reference group B). Of the remaining patients, 10 in group A and 27 in group B, who showed either no response to neo-adjuvant chemotherapy or disease progression were not surgically treated.

In the 13 responders of the experimental group A, thawed PBMC (2 to 6 billion cells) were infused intravenously five days before surgery. This period was defined on the basis of the capacity of thawed lymphocytes to remain in lung tissues (8).

Thereafter, rIL-2 was administered subcutaneously (3 million IU per m^2/day) for the next 4 days. The same oncological and technical principles were applied for each surgery. Tumor resection was classified as complete (R0), microscopically incomplete (R1), or macroscopically incomplete (R2). Complete mediastinal lymph node dissection was carried out in all cases, as previously described (9-10). A small fragment of tumor was also processed in the laboratory as previously described (11) in order to establish whether a loco-regional immune response had been induced by neo-adjuvant immunotherapy. Lymphocyte populations and subpopulations were analyzed using immunophenotyping and flow cytometry, as described below. Follow-up included physical examination, routine blood tests and tumor marker evaluation (CEA, CYFRA and NSE) every 4 months, chest X-ray every 6 months, and CT scan of the chest and upper abdomen every year unless a relapse was suspected earlier. PET scan was used to document a tumor relapse suspected on the basis of CT images.

Leukapheresis and processing of peripheral blood mononuclear cells

Leukapheresis was performed using the standard procedure. Blood was drawn from peripheral veins. Leukocytes were collected through a continuous-flow blood cell separator (Cobe Spectra; Gambro BCT Europe, Zaventem, Belgium), operating a software version 6.1 Auto PBSC. Acid citrate dextrose (ACD-A) was used as anticoagulant for whole blood (anticoagulant ratio 13.5-14:1). Isolated cells were analyzed and differential cell counts were computed using a Bayer Advia 120 blood cell analyzer. PBMC were also evaluated for the presence of lymphocyte populations and subpopulations using flow cytometry (Becton Dickinson FACSaria) and a panel of fluorochrome-labeled monoclonal antibodies specific for lymphoid antigens. These included CD45, CD2, CD3, CD4, CD8, CD16, CD19, CD20, CD25, CD56, CD69 and HLA-DR (Coulter-Beckman).

Mononuclear cells were stored frozen in liquid nitrogen in cryovials at the median concentration of 64 million cell/ml in saline solution supplemented with human serum albumin (30% v/v) and DMSO (10% v/v). Before infusing cells, cryovials were thawed at 37°C and DMSO was removed by centrifugation. Dead cells were also removed using a discontinuous Ficoll-Paque gradient. The cells were then harvested from the interface, counted, and diluted in 500 ml buffered saline solution supplemented with 20% human serum albumin. The washing procedure was required because the clinical unit where the infusions were performed, was several kilometers away from the laboratory. Nevertheless, the procedure had been previously validated in a similar clinical setting (12). Quality controls of the frozen product included bio-safety (absence of bacteria, fungi, mycoplasmas and endotoxins), and cell viability. A phenotypic analysis was also performed on thawed cells as described (12).

Statistical analysis

All eligible patients who underwent surgery were included in the analysis. The primary aim of the study was the assessment of the feasibility and safety of the experimental treatment. Secondary aims were the evaluations of overall survival (OS) and event-free survival (EFS). These additional aims were included merely for exploratory purposes. Overall survival was estimated from the date of surgery to the date of last contact or death from any cause. Event-free survival was estimated from the date of surgery to the date of occurrence of any event including local relapse, controlateral primary tumor, distant relapse or death from any cause, whichever came first. Differences between the two groups in baseline prognostic factors were assessed by the Pearson *chi*-square test. OS and EFS were obtained from Kaplan-Meier analyses, and the primary comparison between the two groups was carried out using the log rank test. For each prognostic factor, stratified univariate analyses and log rank tests were also used to assess statistical differences between the two treatment groups. Cox's model was used for multivariate analyses to assess the independent prognostic role of each prognostic factor, while adjusting for the effect of the other factors. The variables included in the models as covariates were: patient's age (≤60 years, 61-65 years, >65 years), gender, tumor histology (squamous carcinoma, adenocarcinoma), pathological T (pT0, pT1, >pT1), tumor grade (GI or G2, G3), nodal status (N0, N2) and immunotherapy. Hazard ratios (HRs) for each variable were obtained by exponentiating the coefficients estimated by the Cox's models. Modifications of the relative effect of neo-adjuvant chemo/immunotherapy as compared to standard neo-adjuvant chemotherapy across the strata of each covariate were assessed by introducing the appropriate interaction terms in the model. These covariates by treatment interaction terms were introduced in the model one at a time. The likelihood ratio test was used to evaluate

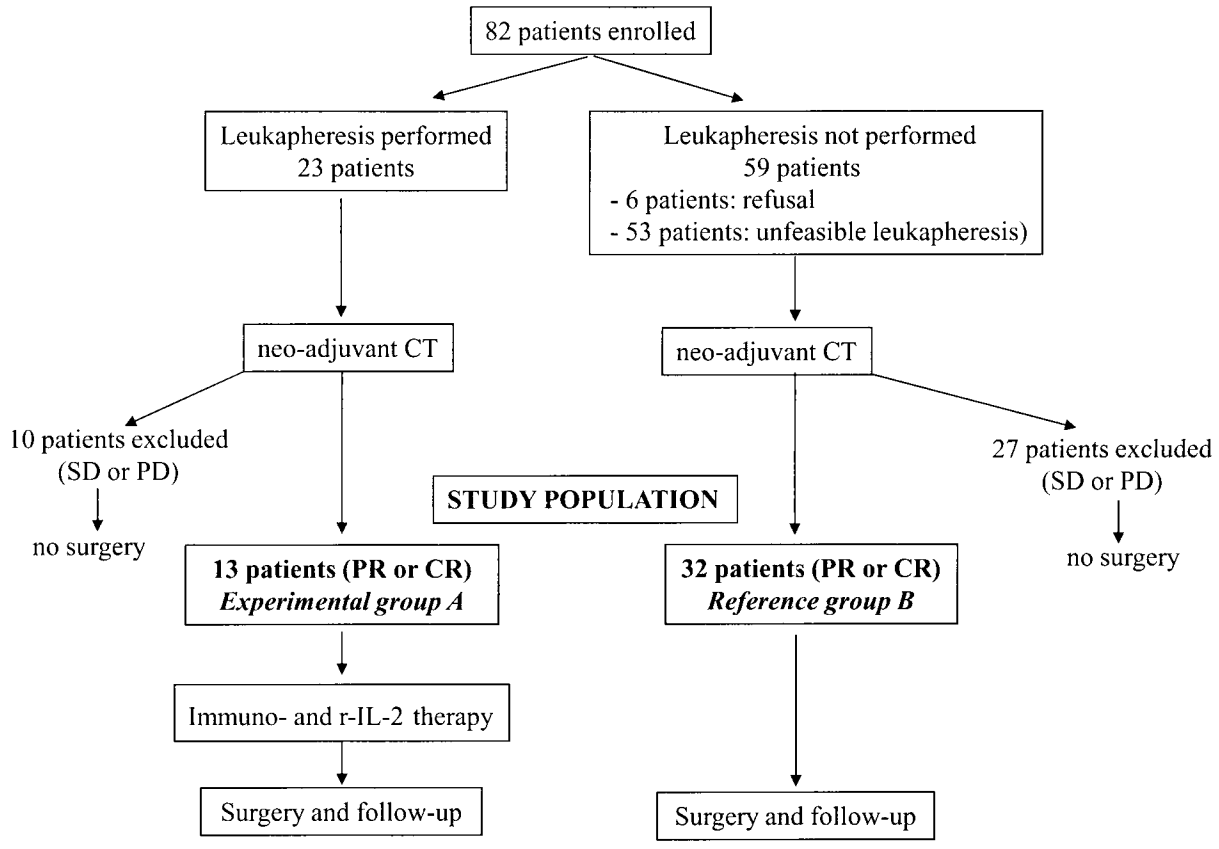


Fig. 1. *Algorithm for the selection of the patient population. CT = chemotherapy; SD = stable disease; PD = progressive disease; PR = partial response to neo-adjuvant CT; CR = complete response to neo-adjuvant CT; r-IL-2 = recombinant interleukin 2.*

the statistical significance of each interaction term. All statistical analyses were two-sided and were performed using the SPSS package (version 16.0 for Windows).

RESULTS

Patients characteristics

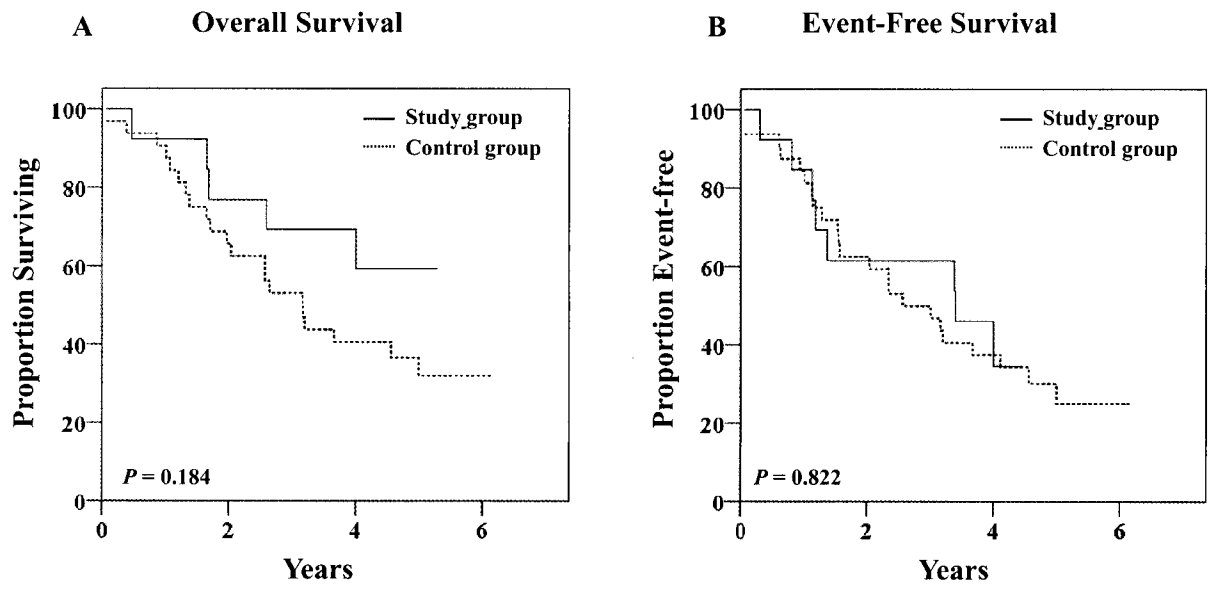
Of the 45 patients selected for statistical analyses, 13 underwent neo-adjuvant chemotherapy and immunotherapy. The other 32 patients received chemotherapy alone and were used as a reference.

Overall, the median observation time between surgery and death or censoring was 3.7 years. Nineteen patients (42%) were alive at the end of the study with a median follow-up of 4.8 years (4.3 years for group A and 5.2 years for group B). Among these patients, 14 (74%) were free of disease and 5 (26%) were not. Other 17 (38%) patients went in progression and died thereafter. Overall, an event (relapse or death) was recorded for 31 patients (69%).

Patient and tumor characteristics are summarized in Table I. For each prognostic factor, patient distribution between subgroups was rather homogeneous, except for tumor grade: G3 disease was more often found in group A (p=0.012). Noteworthy, more than half of the patients (n=25, 55.6%) benefited from the induction chemotherapy, down-staging from pre-induction N2 to post-induction N0: 7 in group A and 18 in group B.

Leukapheresis results and immunological evaluation

Leukapheresis processed 6500 (range 5550–



	Study group			Control group		
Years	N	OS	95%CI	N	OS	95%CI
2	10	77	(74-79)	21	66	(63-68)
5	6	59	(56-62)	7	32	(29-35)

	Study group			Control group		
Years	N	EFS	95%CI	N	EFS	95%CI
2	8	62	(59-65)	20	63	(59-65)
5	3	35	(32-38)	5	25	(23-28)

Fig. 2. *Kaplan-Meier survival curves for all selected patients included in the two groups. A) Overall survival. B) Event-free survival. Study group = neo-adjuvant immunotherapy subsequent to induction chemotherapy; control group = only induction chemotherapy; N = number of patients at risk; OS = overall survival and EFS = event-free survival, with 95% confidence interval (CI) in parentheses. p values from log rank tests (two-sided) = 0.184 (A) and 0.822 (B).*

6866) ml of blood from patients (median weight 72 kg ; range 51–84 kg). The median peripheral blood leucocyte count was 104 ×109 cells /L (range 40–220), with a percentage of peripheral blood mononuclear cells (PBMC) of 28 (range 14-42). Median leukapheresis time was 130 minutes (range 120-185) at a flow rate of 53 ml/min (range 50-56)

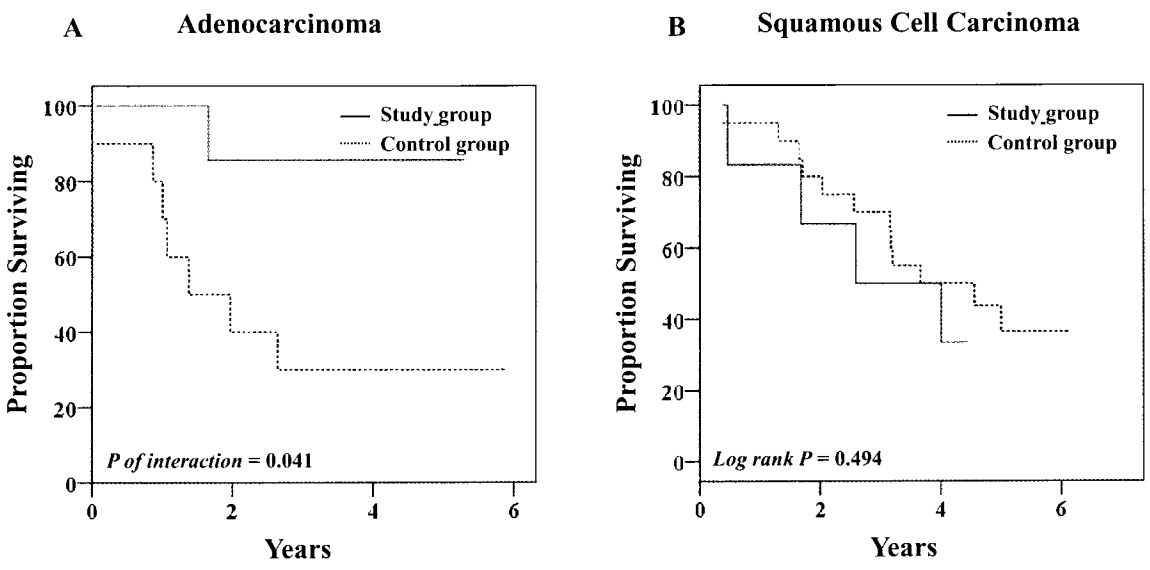
Leukaphereses had a good yield (median 10 billion PBMC; range 3-24 billions). The percentage of lymphocytes was high (72%, range 61-95%). After thawing, a median of 4 billion PBMC (range 2-6 billions) was infused. In these samples, the percentage of lymphoid cells was also high (85%, range 79-100%). The phenotypic analysis showed that a variable proportion of CD4 and CD8, as well as activated lymphocytes, was present. A similar proportion was observed in T lymphocytes isolated from the removed tumor. Notably, both B and NK cells were virtually absent in samples collected from the tumor site, while T cells expressing either the "helper" or the cytotoxic phenotype were constantly detected (Table II). In tumor samples obtained from patients treated with neo-adjuvant chemotherapy alone, T cells were absent.

Feasibility and safety of the experimental therapy

The planned treatment was completed without any significant side effect in the 13 patients of the group A. No adverse reaction was observed following PBMC infusion. Minor symptoms, such as fever, related to rIL-2 administration were easily controlled with appropriate drugs

Overall survival and event-free survival

By August 1st 2008, 26 deaths had been recorded,



	Study group			Control group		
Years	N	OS	95%CI	N	OS	95%CI
2	6	86	(83-88)	4	40	(38-44)
5	6	86	(83-88)	3	31	(29-35)

	Study group			Control group		
Years	N	OS	95%CI	N	OS	95%CI
2	4	67	(64-70)	16	80	(77-82)
5	2	33	(30-36)	5	37	(34-40)

Fig. 3. *Kaplan-Meier survival curves according to tumor histology. A) Adenocarcinoma. B) Squamous cell carcinoma. Study group = neo-adjuvant immunotherapy subsequent to induction chemotherapy; control group = only induction chemotherapy; N = number of patients at risk; OS = overall survival, with 95% confidence interval (CI) in parentheses. p values from tests (two-sided) = 0.041 (A, interaction test) and 0.494 (B, log rank test).*

5 (23%) in group A and 21 (77%) in group B. No significant difference in OS was seen between the two groups (p=0.184) although patients who received the neo-adjuvant immunotherapy showed a trend towards a better survival (Fig. 2A). Cumulative OS after 2 years was 77% (95% CI 74-79) in group A and 66% (95% CI 63-68) in group B. After 5 years, OS in group A was almost twice as much when compared to group B (59%, 95% CI 56-62 and 32%, 95% CI 29-35, respectively).

Among the 31 events (relapse and/or death) observed, 9 (29%) occurred in group A and 22 (71%) in group B. Cumulative 2-year EFS was similar for both treatment arms, being 62% (95% CI 59-65) in group A and 63% (95% CI 59-65) in group B (Fig. 2B). After 5 years, cumulative EFS was 35% (95% CI 32-38) in group A and 25% (95% CI 23-28) in group B (p=0.822).

When the comparison between the two groups was adjusted for age, gender, tumor histology, tumor grade, pT and nodal status (Table III), no statistically significant differences were observed with respect to either OS or EFS.

Multivariate and subgroup analyses

In multivariate analyses, gender, tumor histology, and nodal status were independently associated with OS and/or EFS (Table IV). Patient age, tumor grade and pathological T were not, although an association of borderline significance of the latter two with EFS was observed (p=0.071 for tumor grade and 0.066 for pT). After adjustment for all these prognostic factors, a statistically significant 66% reduction in the hazard of death was found and it was associated with the immunotherapy treatment (HR = 0.34, 95% CI 0.12-1.0; p=0.034). The effect on EFS was less

Table I. *Patient characteristics by treatment group: 13 patients in the immunotherapy (study) group and 32 patients in the control group* *.

Parameter	Study n (%)	Control n (%)	*p* §
Age (years)			
Mean ± s.d.	62.0±5.0	63.0± 9.0	
Median	62.0	64.0	
Range (min. – max.)	56.0 – 71.0	45.0 – 81.0	
Age category			0.244
≤ 60 y	5 (38.5)	11 (34.4)	
61 to 65 y	5 (38.5)	6 (18.8)	
> 65 y	3 (23.0)	15 (46.8)	
Gender			0.254
Male	8 (61.5)	25 (78.1)	
Female	5 (38.5)	7 (21.9)	
Pre-surgery characteristics			
Clinical T			0.202
T1	5 (38.5)	10 (31.3)	
T2	3 (23.1)	16 (50.0)	
> T2	5 (38.4)	6 (18.7)	
Number of stations involved			0.563
1 level	10 (76.9)	27 (84.4)	
> 1 level	3 (23.1)	5 (15.6)	
N bulky			0.094
No	8 (61.5)	28 (87.5)	
Yes	5 (38.5)	4 (12.5)	
Type of surgery			0.163
Pneumonectomy	5 (38.5)	6 (18.8)	
(Bi)-Lobectomy	8 (61.5)	26 (81.2)	
Post-surgery characteristics			
Histology			0.288
Squamous carcinoma	6 (46.2)	20 (62.5)	
Adenocarcinoma	7 (53.8)	10 (31.2)	
Other	-	2 (6.3)	
Grading			0.012
G1 / G2	2 (15.4)	18 (56.2)	
G3	11 (84.6)	14 (43.8)	
Pathological T			0.377
pT0	3 (23.1)	5 (15.6)	
pT1	5 (38.5)	13 (40.6)	
pT2	1 (7.7)	9 (28.1)	
> pT2	4 (30.7)	5 (15.7)	
Tumor size			0.185
0 cm	3 (23.1)	5 (15.6)	
1 – 3 cm	8 (61.5)	15 (46.9)	
> 3 cm	2 (15.4)	12 (37.5)	
Tumor residue			0.500
R0	12 (92.3)	31 (96.9)	
R1	1 (7.7)	1 (3.1)	
Number of stations involved			0.608
0 level	7 (53.8)	18 (56.2)	
1 level	4 (30.8)	6 (18.7)	
> 1 level	2 (15.4)	8 (25.0)	
pN			0.883
pN0	7 (53.8)	18 (56.3)	
pN2	6 (46.2)	14 (43.7)	
Skipped metastasis			0.796
No	11 (84.6)	28 (87.5)	
Yes	2 (15.4)	4 (12.5)	
Extra-nodal metastasis			0.796
No	11 (84.6)	28 (87.5)	
Yes	2 (15.4)	4 (12.5)	
N bulky			0.356
No	13 (100)	30 (93.7)	
Yes	-	2 (6.3)	
Post-surgery adjuvant therapy			0.420
No	4 (30.8)	14 (43.7)	
Yes	9 (69.2)	18 (56.3)	

* *Immunotherapy group = neo-adjuvant immunotherapy subsequent to induction chemotherapy; control group = only induction chemotherapy.*

§ *Pearson Chi-square test for heterogeneity*

Table II. *Analysis of the phenotypic characteristics of lymphoid cells obtained in different phases of the study (Median and Range).*

Surface molecules expressed on cells with physical characteristics of lymphocytes	% of positive in frozen cells	% of positive in thawed cells	% of positive in TIL isolated from the resected tumor
CD45+	98 (95-100)	99 (95-100)	99 (95-100)
CD45+CD2+	78 (65-80)	96 (80-100)	97 (95-100)
CD45+CD3+	77 (60-80)	95 (80-100)	96 (95-100)
CD45+CD3+CD4+	53 (25-65)	52 (40-60)	67 (40-80)
CD45+CD3+CD8+	29 (15-40)	38 (30-50)	33 (30-50)
CD45+CD3- CD16+	7 (5-15)	8 (0-10)	2 (0-5)
CD45+CD3- CD19+	12 (5-15)	6 (0-10)	4 (0-5)
CD45+CD3- CD20+	12 (5-15)	6 (0-10)	5 (0-5)
CD45+CD3+ CD25+	3 (0-10)	5 (0-10)	12 (0-20)
CD45+CD3- CD56+	9 (5-25)	9 (5-15)	6 (0-10)
CD45+CD3+ CD69+	2 (0-5)	2 (0-5)	7 (0-10)
CD45+CD3+ HLA-DR +	3 (0-5)	2 (0-5)	4 (0-10)

pronounced and statistically not significant (HR = 0.77, 95% CI 0.25-2.35; p=0.483).

Subgroup analyses of OS and EFS comparing group A versus group B within strata formed by each prognostic factor, showed no evidence of association between the type of treatment and patient age, gender, tumor grade, pT and pN (p of interaction ranging from 0.216 to 0.915). By contrast, in terms of OS, we found a different treatment effect between patients with squamous cell carcinoma and those with adenocarcinoma (Fig. 3). In patients with squamous cell carcinoma, 5-year OS was 37% (95% CI = 33-40) in group B and 33% (95% CI =30-36) in group A (p=0.494). Conversely, in patients with adenocarcinoma, 5-year OS was 30% (95% CI = 27-33) in group B and 86% (95% CI = 83-88) in group A (p of interaction = 0.041).

DISCUSSION

Stage IIIA-N2 NSCLC represents the most therapeutically challenging and controversial subset of lung cancer (13). This heterogeneous group of patients has been the subject of many clinical trials, indicating that a combined modality approach is the most beneficial treatment (14). In spite of recent improvements in treatment modalities, long term survival in pre-operatively diagnosed N2-NSCLC patients remains poor. Consequently, there is an urgent need for novel therapeutic strategies. Immunotherapy may reasonably be included among these approaches (15).

To our knowledge, this is the first study to investigate the feasibility and potential usefulness of immunotherapy with PBMC and rIL-2 in the neo-adjuvant treatment of patients with resectable, pre-operatively diagnosed, N2-NSCLC. While interpreting the results of our study, we are aware that many fundamental questions remain unanswered: the effects of chemotherapy regimens on the immune system are largely unknown or unexpected (16) and a clear understanding of the mechanisms of our immunological approach is lacking. Nevertheless, when the phenotypic analysis of T cells infiltrating the tumours in group A was compared with that of group B, a clear difference was observed. Indeed, while T cells were virtually absent in patients who did not undergo immunotherapy, these cells were detectable in the samples of patients receiving thawed lymphocytes before surgery. Along this line,

Table III. *Univariate analysis: comparison of overall survival and event-free survival between the immunotherapy (study) group and the control group, stratified for each prognostic factor.*

Prognostic Factor	Overall survival			Event-free survival		
	Study* N events(%)	Control* N events(%)	$p^{\S}$	Study N events(%)	Control N events(%)	$p^{\S}$
Age group			0.467			0.851
≤ 60 years	2/5 (40.0)	5/11 (45.5)		3/5 (60.0)	7/11 (63.6)	
61 – 65 years	1/5 (20.0)	3/16 (50.0)		3/5 (60.0)	3/6 (50.0)	
> 65 years	2/3 (66.6)	13/15 (86.7)		2/3 (66.6)	13/15 (86.7)	
Gender			0.290			0.967
Male	4/8 (50.0)	17/25 (68.0)		6/8 (75.0)	18/25 (72.0)	
Female	1/5 (20.0)	4/7 (57.1)		2/5 (40.0)	5/7 (71.4)	
Histology¶			0.325			0.695
Squamous carcinoma	4/6 (66.6)	12/20 (60.0)		4/6 (66.6)	13/20 (65.0)	
Adenocarcinoma	1/7 (14.3)	7/10 (70.0)		4/7 (57.1)	8/10 (80.0)	
Tumor grade			0.122			0.969
G1 or G2	0/2 (-)	12/18 (66.6)		2/2 (100)	14/18 (77.8)	
G3	5/11 (45.5)	9/14 (64.3)		6/11 (54.5)	9/14 (64.3)	
Pathological T			0.189			0.857
pT0	0/3 (-)	4/5 (80.0)		0/3 (-)	5/5 (100)	
pT1	2/58 (40.0)	6/13 (47.2)		4/5 (80.0)	7/13 (53.8)	
> pT1	3/5 (60.0)	11/14 (78.6)		4/5 (80.0)	11/14 (79.6)	
Nodal status			0.109			0.527
Negative (pN0)	1/7 (24.3)	9/18 (50.0)		3/7 (42.9)	10/18 (55.6)	
Positive (pN2)	4/6 (66.6)	12/14 (86.7)		5/6 (83.3)	13/14 (92.9)	

* *Immunotherapy group = immunotherapy subsequent to induction chemotherapy; control group = only induction chemotherapy. N = number of events/total number of patients in the subgroup.*

¶ *2 patients bearing a neoplasia other than squamous carcinoma or adenocarcinoma were not included in the analysis as they were both in the control group and had died by the end of the observation period.*

§ *Stratified log-rank test from a Kaplan-Meier survival analysis.*

it should be noted that the significant reduction of cancer mass following neo-adjuvant chemotherapy had completely forbidden any attempt to evaluate the capacity of lymphocytes derived from resected tissue to lyse autologous tumor cells.

We must acknowledge that this is a non-randomized study, underpowered to detect significant differences. Nevertheless, both group A and group B were based on a homogeneous and a highly selected group of patients. It is common knowledge that N2-NSCLC includes subsets of patients with different prognoses and therapeutic options. The present study enrolled only patients with pre-operatively demonstrated N2 disease, who were judged completely resectable and showed a response to induction therapy at the mediastinal node level. Excluded from the study were patients with intra-operatively or post-operatively detected mediastinal node metastases, as well as those with stable or progressive disease after induction chemotherapy. Furthermore, in all patients, the N3 status was excluded by pre-induction mediastinoscopy, while the response to induction therapy was routinely assessed by both CT and PET scan.

Keeping in mind the above-mentioned drawbacks, the present study shows that, for patients with

Table IV. *Multivariate analysis: association of prognostic factors with overall survival and event-free survival.*

	Overall survival		Event-free survival	
Variable	HR (95% CI)*	*P*	HR (95% CI)*	*P*
Age		0.411§‡		0.172§‡
≤ 60 y	1 (ref)		1 (ref)	
61-65 y	0.89 (0.26 – 3.14)		1.21 (0.41 – 3.60)	
> 65 y	1.92 (0.65 – 5.67)		1.58 (0.63 – 3.95)	
Gender		0.034		0.026
Male	1 (ref.)		1 (ref.)	
Female	0.33 (0.11 – 1.0)		0.31 (0.10 – 0.95)	
Histology¶		0.894§‡		0.042
Squamous carcinoma	1 (ref)		1 (ref.)	
Adenocarcinoma	1.08 (0.36 – 3.25)		1.97 (1.05 – 3.71)	
Tumor grade		0.824§‡		0.071
G1/G2	1 (ref)		1 (ref.)	
G3	1.11 (0.42 – 2.94)		0.48 (0.22 – 1.07)	
Pathological T		0.528§‡		0.066
pT0	1 (ref)		1 (ref.)	
pT1	0.47 (0.11 – 1.90)		0.69 (0.21 – 2.31)	
> pT1	0.44 (0.10 – 1.98)		0.32 (0.08 – 1.28)	
Nodal status		< 0.001		0.007
Negative (pN0)	1 (ref.)		1 (ref.)	
Positive (pN2)	5.31 (2.02 – 13.92)		2.17 (1.20 – 3.90)	
Immunotherapy		0.034		0.483§‡
Not administered	1 (ref.)		1 (ref.)	
Administered	0.34 (0.12 – 1.0)		0.77 (0.25 – 2.35)	

* *HR = hazard ratio; 95% CI = 95% confidence interval, ref. = referent subgroup*

§ *From a Cox model in which all variables were initially included as covariates. Covariates not statistically significantly (P>0.05) associated with the outcome were excluded from the model by means of a step-down procedure that was based on likelihood ratio test. All statistical tests were two-sided.*

¶ *2 patients bearing a neoplasia other than squamous carcinoma or adenocarcinoma were not included in the analysis as they were both in the control group and had died by the end of the observation period.*

‡ *Excluded from the final model.*

resectable N2-NSCLC, the neo-adjuvant treatment including chemotherapy and immunotherapy with PBMC and rIL-2 is feasible and well tolerated. These results fulfill the primary aim of the study in that the neo-adjuvant chemo/immunotherapy procedure appears to be safe. In addition, when analyzing the outcome of all patients included in the study merely for exploratory purposes, a statistically significant reduction of the hazard of death was found in patients receiving chemotherapy and immunotherapy, in respect to those who underwent chemotherapy alone. Five-year OS in group A was almost twice as much compared to group B. Although they are rather promising, these results have to be confirmed in further randomized trials based on large patient numbers. The favorable effect of neo-adjuvant immunotherapy was not observed when considering the event-free survival. The discrepancy between

the OS and the EFS curves may result 1) from the small number of patients in the neo-adjuvant chemo/immunotherapy group, 2) from the presence, in this group, of three patients who survived with disease recurrence; the survival of these patients may be due to their rather young age (56, 62 and 64 years) and/or to the fact that their respective post-event observation period was rather short, the relapse occurring at 11, 16 and 38 months from the end of the overall 6-year period of the study.

An additional interesting finding, requiring further investigation but potentially based on experimental evidence (17), is the different efficacy of immunotherapy between patients with squamous cell carcinoma and those with adenocarcinoma. In patients with squamous cell carcinoma, 5-year OS was similar in group A and in group B. Conversely, immunotherapy showed a higher efficacy in patients with adenocarcinoma, the 5-year OS being 86% in group A and 31% in group B.

In conclusion, the favorable toxicity profile of neo-adjuvant immunotherapy with PBMC and rIL-2 maintains the promise of improved tolerance and efficacy of combined induction regimens in patients with resectable N2-NSCLC. The results of the present study are in line with those of previous studies using different immunologic approaches both in the neo-adjuvant and adjuvant treatment of N2-NSCLC patients. Since all these studies support the potential efficacy of immunotherapy, this approach should be considered when planning future combined therapeutic regimens. In addition, the regimen proposed here can be easily carried out even in centers which are not highly specialized, because of the widespread use of leukapheresis and the low cost of this procedure.

However, before starting a phase III study aimed to assess the value of induction immunotherapy with PBMC and rIL-2 in a larger number of N2-NSCLC patients, further knowledge on the interaction between chemotherapy agents and the immune system, as well as a better understanding of the effects of immunotherapy are required.

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