MELANOMA

Prognostic and Predictive Factors in Interferon Immunotherapy

Marna G. Bouwhuis

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MELANOMA

Prognostic and Predictive Factors in Interferon Immunotherapy

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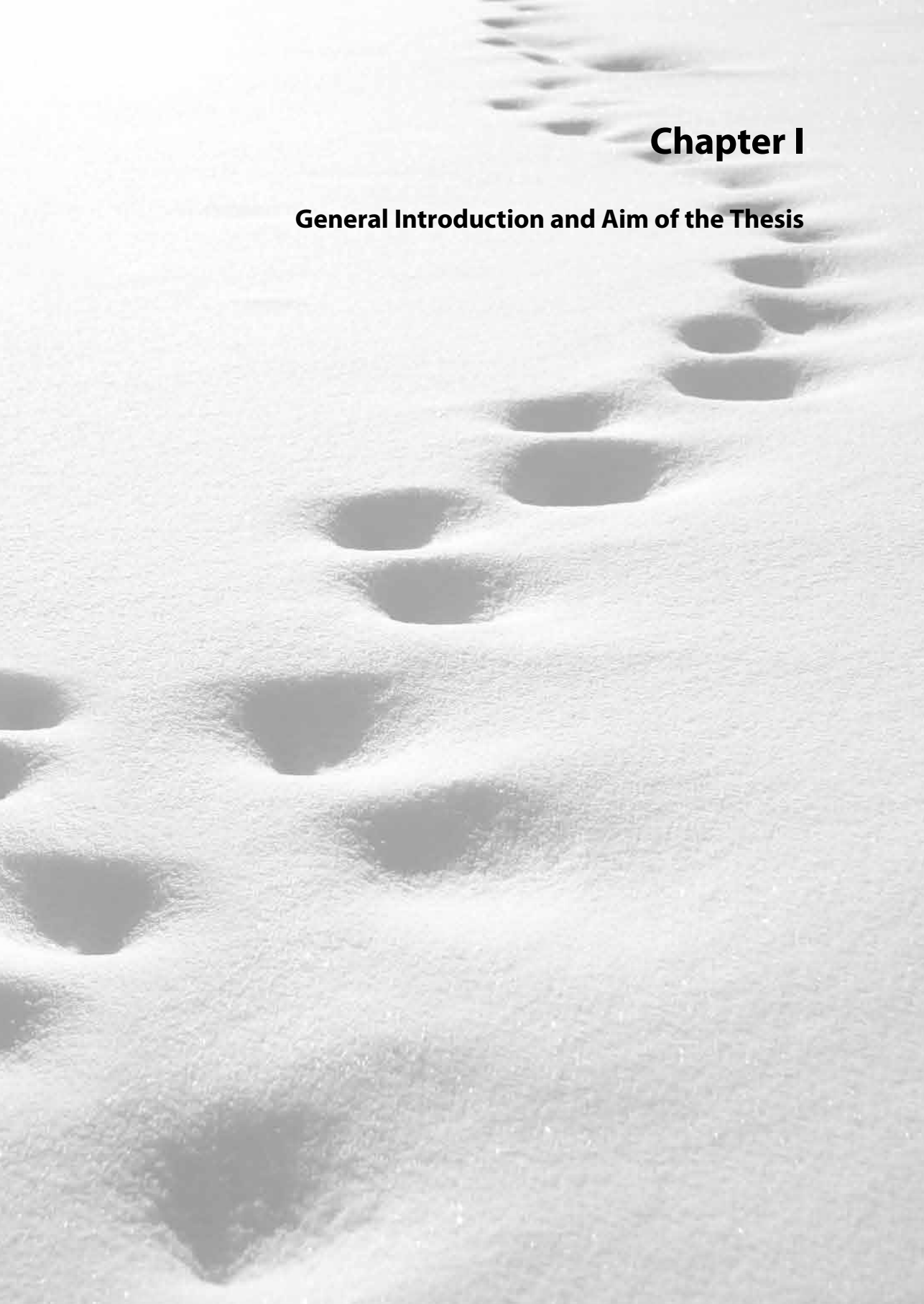

Chapter I

General Introduction and Aim of the Thesis

GENERAL INTRODUCTION

Melanoma is a malignant tumor of melanocytes and is the most severe form of skin cancer. The name melanoma originates from the Greek word μέλας (melas), meaning black or dark, whereas the suffix 'oma' denotes swelling or tumor. René Laennec was a French physician who was the first to describe melanoma as a disease entity. After being presented in a lecture in 1804 for the Faculté de Médecine de Paris, it was published in 1806 as a bulletin.[1] However, the first surgeon to operate on metastatic melanoma was John Hunter in 1787. Microscopic examination of the specimen that was preserved in the Hunterian Museum of the Royal College of Surgeons of England, revealed it to be a metastatic melanoma.[2]

Currently, melanoma incidence is rising dramatically worldwide. In the United States, melanoma is the fifth leading cancer in men and the seventh in women.[3] Over the past 20 years the incidence of melanoma has more than tripled in the white population in the United States. An overview of trends of incidence of cancer in Europe, from mid 1990s to early 2000, showed for melanoma increasing incidence rates for most European countries, especially in females.[4] In the Netherlands, estimated incidences for males, increased from 5.5 cases per 100,000 inhabitants per year to 12.2 cases per 100,000 inhabitants per year between 1980 and 2002, and for females, incidences increased from 7.8 to 15.0 cases per 100,000 inhabitants per year.[5] A more recent report concerning incidence and mortality of cancer in Europe in the year 2008, showed 84,000 new melanoma cases and 20,100 deaths from melanoma.[6] Estimated numbers of new cases in The Netherlands included 1660 males and 2130 females. The risk factors for developing melanoma are both environmental and genetic.

The definitive surgical treatment for primary cutaneous melanoma is a wide local excision. Although resection usually controls the primary lesion, melanoma often metastasizes to the regional lymph nodes. An elective (immediate) lymph node dissection was not proven to improve survival for the entire patient population.[7,8] The sentinel lymph node biopsy, introduced by Morton and colleagues in the early 1990s was a promising new staging technique in melanoma.[9] The assessment of the first draining lymph node from a tumor was suggested to identify subgroups of patients benefiting early completion lymph node dissection.[10] Nevertheless, it is still not demonstrated that an early completion lymph node dissection following positive sentinel node biopsy improves overall survival compared to patients undergoing therapeutic lymph node dissection for palpable lymph nodes.[11] Adjuvant radiotherapy can decrease the local recurrence rate but there is no evidence of improved survival. In the palliative setting it is effective in patients with bone, brain and visceral metastases.[12]

Generally, systemic treatment in melanoma is disappointing. One of the largest meta-analysis of phase II trials for metastatic melanoma demonstrated a median overall survival of only 6 months, a progression free survival of only 1.7 months and a 1-year overall survival rate of only 25%.[13] Various systemic treatment regimens occasionally cured patients with widespread metastatic, but only dacarbazine is a worldwide approved therapy.[14,15] Also, fotemustine is approved in some countries, due to the observation that it prolonged time to occurrence of brain metastases.[16] Cytokine-based therapy with IL-2, based on its ability to produce durable responses, is approved for patients with metastatic melanoma in the USA but not in Europe.[17] Strikingly, combining chemotherapy with immunotherapy was not successful. Twenty-one phase III trials evaluating the addition of interferon-α (IFN) or of IL-2 alone or of the combination of IL-2 and IFN to mono or combination chemotherapy, showed improved response rates, at the cost of significant toxicity, but failed to provide proof for survival benefit.[15]

Recombinant IFN is a cytokine with a wide range of biological activities, such as direct anti-proliferative/pro-differentiation and protein synthesis-inhibiting antitumor activity, as well as indirect immune-mediated effects, and rendering the tumor more susceptible to host effector cells.[18] In the adjuvant setting, high-dose IFN was approved in the USA and in Europe for stage IIB-III, but is little used in Europe. This treatment was approved in the USA due to the results of the ECOG 1684 trial, showing a recurrence-free survival (RFS) and overall survival (OS) benefit in patients treated with high-dose IFN compared to untreated patients.[19] Numerous trials followed, reporting a consistent and significant effect on RFS but not on OS.[20,21] An individual patient data meta-analysis confirmed the significant and consistent effect on RFS and a small, but statistically significant, impact of 3% on OS.[22]

Recently, the desperate situation of waiting for new drugs to break the deadlock of decades, has been changed. For the first time in the history of melanoma, a clearly significant and clinically relevant prolongation of survival was demonstrated with ipilimumab in patients with metastatic melanoma.[23] Results from upcoming phase III trials such as the EORTC 18071 trial of adjuvant ipilimumab, should be awaited. Moreover, advances in drug development based on the inhibition of mutation-driven pathway-signaling, are promising. BRAF (serine-threonine kinase) mutations are found in approximately 60% of melanoma patients.[24] Response rates of around 80% with a selective BRAF inhibitor (PLX4032) were demonstrated in patients with metastatic melanoma.[25,26] Unfortunately, the tumors ultimately recur even with continuous therapy.

Current prognostic markers based on the conventional American Joint Committee on Cancer (AJCC) staging system (TNM) include Breslow tumor thickness, presence of ulcer-

ation and extent of nodal involvement for primary cutaneous melanoma, and furthermore, site of distant metastases and serum lactate dehydrogenase (LDH) in metastatic melanoma.[27] Increasing tumor thickness (originally described by Breslow),[28] correlates with a significantly poorer prognosis. Patients with thin lesions (<0.5 mm) have an excellent 10-year survival of 96%, but survival decreases to 54% for patients with lesions with a thickness of 4.01 to 6.0 mm. Furthermore, outcome in patients with ulcerated primary tumors are worse than in patients with primary melanomas of the same thickness but without ulceration. Another important prognostic factor in localized melanoma is the mitotic index (mitosis/mm^2) with 10-year survival rates of 93% for those with < 1 mitosis/mm^2 and 48% for those with >20 mitosis/mm^2. In stage III, melanoma prognosis depends upon the extent of the nodal tumor burden.[29] Five-year survival rates for patients with micrometastases (detected in the specimen of the completion lymph node dissection following positive sentinel node biopsy) in one, two, or three lymph nodes were 71, 65 and 61% respectively. And for patients undergoing therapeutic lymph node dissection for palpable lymph nodes (macrometastases), 5-year survival rates were 50, 43 and 40% when one, two, or three lymph nodes, respectively, were involved. Prognosis in metastatic patients is poor with 1-year survival rates of 62% for patients with distant skin, subcutaneous, or nodal metastases, 53% for patients with lung metastases and 33% for patients with other visceral metastases or elevated serum LDH. Other prognostic factors that are not included in the AJCC staging system but which are of prognostic importance are age, gender, and anatomic location of the primary tumor. Moreover, the serological biomarkers S100B, melanoma-inhibiting activity (MIA) and YKL-40 were associated with adverse outcome.[30]

The association between tumor immunity and autoimmunity is complex.[31] The immunogenic nature of malignant melanoma is clinically manifested by spontaneous regression and the appearance of vitiligo, which is considered a favorable prognostic factor. Furthermore, spontaneous regression is believed to be more common in melanoma than in any other cancer type. The phenomenon of autoimmunity observed during various forms of immunotherapy, IL-2, IFN and anti-CTLA4 therapy, has been linked to treatment response.[32-34] However, conflicting data have been reported as well, mentioning the effect of a higher rate of immune-related toxicities during prolonged administration of the drug in responders. To understand the link between tumor immunity and autoimmunity in melanoma and to explore its implication on disease susceptibility, prognosis and treatment outcome, remains a challenge.[35]

AIM OF THE THESIS

In melanoma, prognostic markers are needed to subdivide traditional tumor stages into subsets of patients behaving differently in order to achieve personalized treatment. Systemic treatment is still disappointing and predictive factors identifying responders are currently lacking.

This thesis mainly focused on prognostic and predictive factors in stage IIB-III melanoma patients receiving adjuvant (pegylated) interferon-α, conducted as translational research projects from large randomized controlled trials. The cytokines IL-6 and IL-1β and acute phase proteins, ferritin and CRP were analysed in a subset of patients randomized to the European Organization of Research and Treatment of Cancer (EORTC) 18952 trial, evaluating efficacy of intermediate doses of IFN versus observation. To assess the prognostic and predictive value of autoimmunity in IFN-treated patients, autoimmune antibodies were measured in patients participating in the EORTC 18952 trial, the Nordic IFN trial and in patients receiving pegylated (PEG-) IFN in the EORTC 18991 trial. The serum biomarker S100B, known to be of prognostic value in stage IV melanoma, was determined in a subset of patients from the EORTC 18952 trial. The YKL-40 analysis was performed in patients participating in the EORTC 18952, Nordic IFN or the EORTC 18991 trial. Additionally, the influence of genetic variability on melanoma susceptibility and prognosis was evaluated by screening German melanoma patients and German healthy controls for polymorphisms in the CD28/CTLA4/ICOS genes. Finally, immunologic functions as prognostic indicators in patients receiving immunotherapy, including data from the presented studies in this thesis were reviewed. Moreover, the possible impact of statistical and methodological confounders on study results are illustrated in this thesis.

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SECTION ONE

ACUTE PHASE RESPONSE

Chapter II

Sense and Non-sense of Determination of Cytokines in Serum: Cytokines IL-6 and IL-1β in Melanoma Patients in the EORTC 18952 Trial of Adjuvant Interferon α versus Observation and Healthy Controls

Marna G. Bouwhuis, Henk te Velthuis, Diana Wouters, Els de Groot, Stefan Suciu, Antonius J.G. Swaak, Timo L.M. ten Hagen, Alexander M.M. Eggermont, Lucien A. Aarden, on behalf of the EORTC Melanoma Group.

Minor modifications

Submitted

ABSTRACT

Variation of serum levels of cytokines in healthy individuals is dependent on the method used for determination. Since high baseline serum levels of pro-inflammatory cytokines were recently described to be associated with improved outcome in IFN-treated melanoma patients, we performed a prognostic study in melanoma patients treated with or without adjuvant IFN and determined IL-6 and IL-1β serum levels with two independent methods.

In 185 patients, IL-6 and IL-1β serum levels were determined by enzyme-linked immunosorbent assay (ELISA) at baseline and during follow up to 30 months. A selection of 78 samples was also tested by multiplex assay.

Baseline IL-6 levels were raised in 10/185 (4.3%) of the patients as determined by ELISA. During follow-up, 2/155 (1.3%) of the screened sera from the observed patients and 35/745 (4.3%) sera from the IFN-treated patients tested positive for IL-6. Results for IL-6 by ELISA correlated with those from the multiplex method (k = 0.47, CI 0.28-0.66). Since the frequency of elevated IL-6 was very low, we compared the results with the frequency in 50 healthy controls, which was 3/50 (6.0%). Pre-treatment levels of IL-1β (by ELISA) were elevated in 10.3% of the patients. During follow-up, 12,3% (observation) and 11.5% (IFN) of the sera tested positive for IL-1β. Interestingly, 14 patients had elevated IL-1β levels at all or ≥4 time points. In 16/80 (25%) of sera from healthy controls, IL-1β was detectable. Comparisons with the multiplex assay did not agree.

Altogether this led to the hypothesis that the data for IL-1β included false positive test results, possibly due to the existence of heterophilic antibodies in human sera that crosslink the mouse and/or bovine antibodies used in the ELISA. We report on how we reduced this effect, illustrating the complexity of cytokine determination in human sera. The predictive value of IL-6 in IFN-treated patients could not be confirmed since the incidence was too low.

INTRODUCTION

Inflammation and cancer progression are closely related.[1] Inflammatory cytokines and chemokines, which can be produced by tumor cells and tumor-associated leucocytes, whereas platelets may contribute directly to malignant progression.[2] Several studies showed that melanoma cell lines produce various cytokines, growth factors and their receptors.[3,4] Expression of IL-6 was reported as an inhibitor of growth in cell lines from early stage melanoma and as a stimulator of growth in some advance-stage melanoma cell lines.[5,6] In human melanoma, high serum levels of IL-6 at baseline have been associated with poor prognosis in metastatic patients receiving systemic treatment.[7-9]

Melanoma patients with thick primary tumors or lymph nodal involvement are at high risk of recurrence or death.[10] Adjuvant therapy with interferon-α (IFN) has a significant and consistent effect on recurrence-free survival (RFS), but only a marginal and significant impact of 3% on overall survival (OS).[11] Seemingly, only a relatively small proportion of patients benefits from IFN therapy, however a biomarker selecting these patients is currently unavailable. Recent multiplex analysis of a panel of 29 cytokines in serum of high-risk melanoma patients showed the association of higher pre-treatment levels of the pro-inflammatory cytokines IL-1α, IL-1β, IL-6, TNF and the chemokines MIP-1α and MIP-1β with longer (> 5 years) RFS in IFN-treated patients.[12] However, a mean IL-6 baseline level between 2000 and 3000 pg/ml for the group with a RFS longer than 5 years is exceptionally high and was not reported before. Several studies show high difference in serum levels for cytokines, which probably does not only depend on the study population, but also on the method used for detection of cytokines. Two large studies in stage IV melanoma patients report median IL-6 values between 16 and 17 pg/ml and one of these mentioned a range between 0 and 350 pg/ml.[7,13] Another study of 28 metastatic melanoma patients showed that 12 patients had IL-6 levels below 20 pg/ml, 14 patients between 20 and 100 pg/ml and only 2 patients had IL-6 serum levels above 100 pg/ml.[14] Therefore the high IL-6 levels found by Yurkovetsky et al. might indicate false positive test results. The measurement of cytokines in human sera is challenging because of confounding variables, such as heterophilic antibodies in sera that could crosslink the non-human antibodies, i.e. mouse monoclonal antibodies, used in the sandwich ELISA and produce false positive ELISA results. This phenomenon was described for instance for the IL-1β ELISA.[15] IL-1β levels reported for healthy controls vary and are listed in Table 1.

We determined IL-6 and IL-1β levels in serial serum samples of 185 melanoma patients that participated in the EORTC 18952 trial.[16] The ELISA we used for IL-6 determinations correlated with a functional assay for IL-6 in B9 cells.[17] The original aim of the study was to evaluate the prognostic and predictive value of IL-6 and IL-1β measured by ELISA. A subset of 78 samples was also tested by multiplex technique, in order to compare both techniques. We compared the results of this study to the serum detection of IL-6 and IL-1β in healthy controls.

Table 1.

Study	Number of controls	IL-1β Assay	Mean (+/-SD) pg/ml	Median pg/ml	Range pg/ml
Kemik et al.[21]	36	ELISA	276.9 (+/- 132.2)	-	-
Antonelli et al.[22]	43	ELISA	0.8	0.7	0.7 – 1.1
Barak et al.[23]	27	ELISA	316.6	-	170 – 540
Mahajan et al.[24]	10	ELISA	2.15	-	0.87 – 2.98
Yurkovetsky et al.[12]	378	Multiplex	40.2	0.0	0.0 – 2253.5
Watkin et al.[25]	71	ELISA	-	0.41	0 – 1.21
Macri et al.[26]	15	ELISA	33.4 (+/- 7.98)	-	-
Schmitt et al.[27]	23	ELISA	<0.5	<0.5	-
Lyke et al.[28]	251	Multiplex	14.7	-	2.5 – 3355
Yoshida et al.[29]	21	ELISA	0.16 (+/- 0.17)	0.08	0 – 0.61

IL-1β levels in healthy controls.

PATIENTS AND METHODS

Patients and controls

Patients participated in the EORTC 18952 study, evaluating efficacy and toxicity of intermediate doses of adjuvant interferon-α2b in melanoma patients, who had been diagnosed with stage IIB melanoma (Breslow thickness ≥ 4 mm, node negative [N0]), or had undergone curative dissection of regional lymph nodes, either completion lymphadenectomy following positive sentinel node procedure (stage III, N1) or therapeutic lymph node dissection for palpable nodes (stage III, N2).[16] Patients were randomly assigned in a 2:2:1 fashion to receive the following treatment schedules, induction treatment of 4 weeks of 10 million units (MU) IFN s.c. five days a week followed by either 10 MU s.c. three times a week for 1 year (Arm A) or 5 MU s.c. three times a week for 2 years (Arm B). Patients in arm C did not receive treatment.

All available serum samples from a total of nine collaborating centers were collected centrally and stored in –80° C freezers until assayed. The blood samples for this collateral study were drawn at the same time point as for regular follow up tests; before treatment, at the end of the induction phase, at 1, 3, 6, 12, 16, 20 and 24 months during maintenance therapy and furthermore at 30 months follow up (similar for the observation arm).

Informed consent from patients for translational research for identification of prognostic factors in association with these trials was obtained. Control sera were obtained from healthy donors with permission (Sanquin Blood Supply, Amsterdam, The Netherlands).

Cytokine measurements by ELISA

IL-6 and IL-1β serum levels were determined by ELISA kits (Peli-Kine-compact, Sanquin Reagents, Amsterdam, The Netherlands). The IL-6 assay was used as previously described in detail.[18] Sera 1:5 diluted in HPE, were incubated on monoclonal anti-IL-6 coated plates together with polyclonal (sheep) biotinylated anti-IL-6. After washing, incubation with streptavidin-poly HRP (Sanquin, Amsterdam, The Netherlands) and after another washing step incubation with TMB (Merck) followed. The reaction was stopped with H_2SO4 and the colour formation was determined with a spectrophotometer (Labsystems Multiskan Multisoft, Helsinki, Finland). For the IL-1β assay sera were 1:5 diluted in HPE and incubated on plates coated with monoclonal anti-IL-1β in the presence of monoclonal biotinylated anti-IL-1β. The subsequent steps were similar to the IL-6 assay. All determinations were carried out *in duplo* and the detection limits for the IL-6 and the IL-1β assay were 5 and 25 pg/ml, respectively.

IL-1β ELISA alternative inhibition tests

To test the specificity of the positive IL-1β determinations additional measurements were performed. Sera were diluted in a buffer containing 40 µg/mL purified bovine immunoglobulin and 1% normal mouse serum (NMS). Addition of 10 µg/mL anti-IL-1β (similar to the coating), 10 µg/mL anti-IL-6 or 10 µg/mL cetuximab (Merck) was done separately.

Multiplex assay

Invitrogen's Multiplex Bead Immunoassays, 30-Plex assay including: EGF, Eotaxin, FGF-basic, G-CSF, GM-CSF, HGF, IFN-α, IFN-γ, IL-1RA, IL-1β, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40/p70, IL-13, IL-15, IL-17, IP-10, MCP-1, MIG, MIP-1α, MIP-1β, RANTES, TNFα and VEGF, was used conform the manufactures' protocol. Detection limits for IL-6 and IL-1β were 7 and 61 pg/ml, respectively.

RESULTS

Study population

Baseline characteristics of the melanoma patients participating in the EORTC 18952 trial are depicted in Table 2. A total of 1084 sera from 185 melanoma patients were available. Since there was no survival difference between the two IFN-treated groups in this study, these groups were combined.[16]

Table 2.

Variable	EORTC 18952 No of patients (%)
Age (years)	
<35	38 (20.5)
35–49	64 (34.6)
50–65	65 (35.1)
≥65	18 (9.7)
Sex	
Male	104 (56.2)
Female	81 (43.8)
Breslow thickness	
<1.00 mm	13 (7.0)
1.00–1.99 mm	37 (20.0)
2.00–3.99 mm	52 (28.1)
≥4.00 mm	68 (36.8)
Unknown	15 (8.1)
Ulceration status of primary melanoma	
Absent	98 (53.0)
Present	55 (29.7)
Unknown	32 (17.3)
Stage of the disease	
IIB	42 (22.7)
III N1	44 (23.8)
III N2	99 (53.5)
No. of positive lymph nodes	
0	42 (22.7)
1	71 (38.4)
2-4	48 (25.9)
5+	24 (13.0)
Treatment	
Observation	32 (17.3)
13-month IFN	80 (43.2)
25-month IFN	73 (39.5)

Baseline characteristics of the melanoma patients (EORTC 18952 trial).

Interleukin-6

Baseline IL-6 levels were determined by ELISA in 184 patients (baseline sample was not available from 1 patient) of whom 32 were randomly assigned to the observation arm and 152 to one of the IFN groups. In the observation group, 2 out of 32 patients (6.3%) had detectable serum IL-6 levels (≥ 5 pg/ml) with a median of 7 pg/ml (range 6-8 pg/ml), and in the combined IFN group, 8 out of 152 (5.3%) had elevated pretreatment levels with a median of 9 pg/ml, ranging 5-115 pg/ml (Table 3). During follow-up, 2 out of 155 (1.3%) screened sera from the observed patients and 35 out of 745 (4.3%) sera from the IFN-treated patients tested positive for IL-6. Median levels were similar 8 pg/ml, although the range was wider for the treated arm (5-390 pg/ml). Additionally, the occurrence of

Table 3.

		Number of screened sera	Number of IL-6 positive sera ≥5 pg/ml	Median (range) pg/ml
	Controls (n=50)	50	3 (6.0%)	5 (5-6)
IL-6 ELISA	**EORTC 18952**			
	Observation (n=32)			
	Baseline	32	2 (6.3%)	7 (6-8)
	Follow-up	155	2 (1.3%)	8 (7-8)
	IFN (n=153)			
	Baseline	152	8 (5.3%)	9 (5-115)
	Follow-up	745	35 (4.7%)	8 (5-390)
IL-6 MULTIPLEX	**EORTC 18952** (78 sera)	**Number of screened sera**	**Number of IL-6 positive sera ≥7 pg/ml**	**Median (range) pg/ml**
	Sera tested by ELISA			
	Positive	29	28	47 (10-329)
	Negative	49	21	13 (7-84)

IL-6 levels in melanoma patients (n = 185) and healthy controls (n = 50), determined by ELISA. Multiplex assay was performed on a set of 78 sera from the melanoma patients.

elevated IL-6 levels was tested for 50 healthy controls, of these 3 (6.0%) tested positive with a median of 5 pg/ml, ranging 5-6 pg/ml.

A set of 78 serum samples from melanoma patients of the EORTC 18952 trial was also tested by multiplex technique (Table 3). Detection limit of IL-6 in the multiplex assay was 7 pg/ml. In 56 samples a concordance was detected between the multiplex and the ELISA assay; in 22 samples we detected a non-concordance, indicating a moderate agreement, with a kappa of 0.47 (CI 0.28-0.66). When plotting the 28 samples which were detectable in both assays, IL-6 levels appeared higher in the multiplex assay (Figure 1). Maximum IL-6 level by multiplex assay was 329 pg/ml and 115 pg/ml for the same sample tested by ELISA.

One of the aims of this study was to explore the predictive and prognostic value of IL-6 levels on treatment outcome in melanoma patients. However, the occurrence of detectable IL-6 levels at baseline and during follow-up in both treatment groups was too low for statistical analysis.

Interleukin-1β

Baseline IL-1β levels were elevated in 4 out 32 (12.5%) of the patients in the observation arm and in 15 out of 152 (9.9%) patients who received IFN (Table 4). During follow-up, in 12,3% (19/155) and in 11.5% (85/742) of the screened sera, IL-1β was detectable for the observation and the IFN group respectively. Interestingly, 14 patients had elevated IL-1β levels at multiple time points. From these 14 patients, a total of 96 sera were tested of

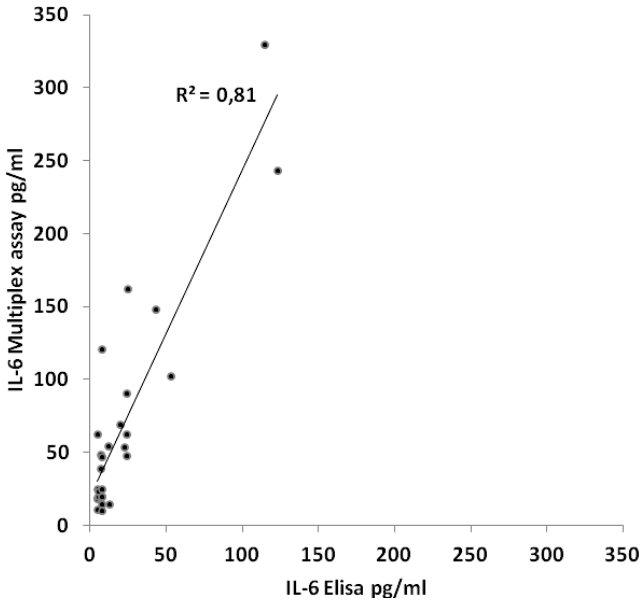

Figure. 1.
Correlation of IL-6 measurements determined by ELISA and multiplex. Only samples detectable in both assays (n=28) are plotted.

Table 4.

		Number of screened sera	Number of IL-1β positive sera ≥25 pg/ml	Median (range) pg/ml
	Controls (n=70)	80	16 (25.0%)	96 (28-5331)
	EORTC 18952			
IL-1β ELISA	**Observation** (n=32)			
	Baseline	32	4 (12.5%)	145 (42-301)
	Follow-up	155	19 (12.3%)	100 (38-364)
	IFN (n=153)			
	Baseline	152	15 (9.9%)	45 (25-181)
	Follow-up	742	85 (11.5%)	52 (25-397)
	EORTC 18952 (78 sera)	**Number of screened sera**	**Number of IL-1β positive sera ≥61 pg/ml**	**Median (range) pg/ml**
IL-1β MULTIPLEX	**Sera tested by ELISA**			
	Positive	24	9	132 (63-1276)
	Negative	54	17	90 (61-390)

IL-1β levels in melanoma patients (n = 185) and healthy controls (n = 70), determined by ELISA. Multiplex assay was performed on a set of 78 sera from the melanoma patients.

which 89 were positive for IL-1β. Moreover, in 16 out of 80 (25.0%) screened sera from 70 healthy controls, IL-1β levels were above the detection limit of the assay. Median IL- 1β levels were 96 pg/ml, ranging from 28 to 5331 pg/ml (Table 4). Two different time points were available for 10 healthy controls, of which 3 controls tested positive at both time points.

Comparing IL-1β levels as determined with ELISA and multiplex method in 78 samples from melanoma patients of the EORTC 18952 trial, IL-1β levels were not in agreement (Table 4). Considering the high number of samples above the detection limit in patients and healthy controls, and the fact that 14 patients (and 3 controls) were positive during the whole study, suggested that several samples showed false positive results.

Heterophilic antibodies in human sera could crosslink the mouse and/or bovine antibodies used in the sandwich ELISA and produce a false positive result in the ELISA. The antibodies used in the ELISAs were cultured in the presence of fetal calf serum and therefore small amounts of bovine immunoglobulins were co-purified with mouse monoclonal antibodies. In an attempt to reduce false positive results we diluted the sera in a buffer containing 40 µg/mL purified bovine immunoglobulin and 1% normal mouse serum (NMS). A total of 104 samples from 35 melanoma patients that tested positive for IL-1β (median 56 pg/ml, range 25-397 pg/ml) in the ELISA with default conditions, were assayed (Table 5). The addition of bovine IgG and NMS resulted in undetectable IL-1β levels in 33 sera from the melanoma patients and a decrease in IL-1β levels in 19 sera (median 28 pg/ml, range 25-70 pg/ml). However, IL-1β levels in half of the sera remained unchanged or were increased (median 241 pg/ml, range 37-2729 pg/ml). IL-1β was detectable in 14 out of 150 (9.3%) tested sera from the control group when bovine IgG and NMS were added (Table 5).

Table 5.

IL-1β	Normal conditions	Median (range) pg/ml	With bovine IgG + NMS	Median (range) pg/ml
EORTC 18952	104 positive IL-1β sera	56 (25 – 397)	33 sera ND 19 sera - 52 sera =/	- 28 (25 – 70) 241 (37 – 2729)
Controls			14/150 (9.3%) positive IL-1β sera	42 (25 – 761)

Samples were tested by IL-1β ELISA as described in Methods. 104 positive sera (≥25 pg/ml) from the patient group were retested in a buffer containing 40 µg/mL purified bovine immunoglobulin and 1% normal mouse serum (NMS).

IL-1β levels in sera from 150 healthy controls were determined by ELISA with bovine IgG and NMS in the buffer.

Whether the IL-1β levels in these samples were truly elevated or still resembled a false positive test result, 20 positive samples were re-tested in the presence of 1) bovine IgG and NMS, 2) in addition of anti-IL-1β which should result in a negative test result,

and 3) in addition of a non-specific monoclonal antibody for this assay (anti-IL-6) to test if this blocking effect is specific for anti-IL-1β (Table 6). Additionally we performed an assay in which the sera were diluted in a buffer containing bovine IgG, NMS and human IgG (Cetuximab), which is a chimeric therapeutic monoclonal antibody, specific for the human epidermal growth factor receptor (EGFR). As previously shown, adding bovine IgG and NMS reduced IL-1β levels in more than half of the sera. Subsequently, the addition of anti-IL-1β did not block IL-1β detection in the majority of the sera. Only for serum sample #1 an undetectable IL-1β level, and for sample #18 a clear decrease in IL-1β level was observed after addition of anti-IL-1β, suggesting the presence of IL-1β in the sample (Table 6). Similar results were found for the remaining samples when anti-IL-6 or Cetuximab was added, suggesting the existence of one or more unknown confounding variables.

Table 6.

20 sera from melanoma patients	1)Normal conditions IL-1β pg/ml	2)bovine IgG + NMS	3)bovine IgG + NMS with anti- IL-1β	4)bovine IgG + NMS with anti-IL6	5)bovine IgG + NMS with Cetuximab
1	29	46	<	34	43
2	336	60	40	57	40
3	81	30	<	<	27
4	37	<	<	<	<
5	40	<	<	<	<
6	81	51	<	<	39
7	69	27	<	<	<
8	181	102	32	26	86
9	59	<	<	<	<
10	133	223	141	118	94
11	29	<	<	<	<
12	27	<	<	<	<
13	30	32	27	30	<
14	38	<	<	<	<
15	43	64	29	32	41
16	45	48	<	<	25
17	95	<	<	<	<
18	230	155	52	118	106
19	397	254	66	53	223
20	316	617	702	785	469

IL-1β blocking assays of 20 positive sera determined by IL-1β ELISA under default conditions. Samples were retested (2-5) under several conditions:

1) Default conditions as described in Methods
2) + 40 µg/mL bovine IgG/1% NMS
3) + 40 µg/mL bovine IgG/1% NMS/10 µg/mL anti-IL-1β
4) + 40 µg/mL bovine IgG/1% NMS/10 µg/mL anti-IL-6
5) + 40 µg/mL bovine IgG/1% NMS/10 µg/mL cetuximab

DISCUSSION

The measurement of cytokines in human sera is widely performed and many cytokines are described as prognostic markers for outcome in patients with several diseases such as sepsis, cancer and acute cardiovascular disorders.[19] IL-6 and IL-1β levels were measured in serial serum samples of 185, stage IIB-III, melanoma patients accrued to the EORTC 18952 trial.[16] The aim of the study was to evaluate the prognostic and predictive value of IL-6 and IL-1β measured by well-established ELISA. However, the number of samples above the detection limit for the IL-6 ELISA in the study group was comparable to the number in a study population of healthy controls, and the incidence was too low to perform statistical analysis. Median IL-6 levels were 6 to 8 pg/ml for the melanoma patients and 5 pg/ml for the control group (Table 3). The multiplex assay yielded slightly higher but comparable results. This indicated that in our study IL-6 cannot be a prognostic marker in stage IIB-III melanoma patients, because the incidence of elevated concentrations is too low.

Previous studies evaluated the prognostic effect of IL-6 in stage IV melanoma patients and reported higher IL-6 baseline levels.[8,13,14] Since weight loss and elevated serum LDH in stage IV melanoma patients were associated with elevated IL-6 values, it is possible that a more advanced disease stage is correlated with increasing IL-6 levels.[9] Moreover, Moretti et al. showed that average IL-6 levels, although not statistically significant, were higher in metastatic melanomas compared to localized melanomas.[20] On the contrary, average baseline IL-6 levels between 2000 and 3000 pg/ml, determined by multiplex, in stage IIB-III melanoma patients receiving high-dose IFN were associated with favorable prognosis. IL-6 levels between 2000 and 3000 pg/ml are extraordinary high and have not been documented before for melanoma patients. To put into perspective, in our analysis in a similar patient cohort, the highest measured IL-6 concentration during follow-up was 390 pg/ml (by ELISA). This patient had developed multiple distant metastases and was therefore discarded from the study.

The aim of our study was to evaluate the prognostic value of IL-1β in melanoma patients randomized to the EORTC 18952 study. However the results of the IL-1β ELISA were largely influenced by confounding factors as demonstrated in the group of healthy controls. We found an unexpected high positive rate in patients and controls, whereas 14 patients (and 3 controls) remained positive at all measured time points. Furthermore the discrepancy with the multiplex assay suggested that these data included false positive test results. We performed additional analyses to clarify which factors contributed to the false positive results, despite the use of our High Performance blocking buffer (HPE). The addition of bovine IgG and normal mouse serum reduced the number of positive test results to 50%. This blocking effect of bovine IgG and NMS suggested the existence of antibodies in human sera capable of crosslinking the monoclonal antibody

of coat (raised in mice, cultured in the presence of fetal calf serum) and the monoclonal antibody used for detection of IL-1β in this sandwich ELISA. However, when performing the blocking assays, only 2 out of 20 samples showed specific reduction of IL-1β levels in the presence of anti-IL-1β, indicating that there are still other confounding variables responsible for a false positive test result. The IL-6 and the IL-1β ELISA are different in the fact that the IL-6 ELISA uses a monoclonal antibody as coat antibody (mouse) and a polyclonal antibody (sheep) for detection, whereas the IL-1β ELISA uses two monoclonal antibodies for capture and detections. Human anti-mouse antibodies may crosslink more easily in the IL-1β ELISA than in the IL-6 ELISA and therefore obtain more false positive results.

In many studies, cytokine levels are reported to correspond with severe illness.[19] The wide range of cytokine levels reported is large and levels might be influenced by confounders. The concept of heterophilic antibodies interfering with ELISAs is not new. It was previously described for the IL-1β ELISA in a study in which 419 sera from hospitalized patients were screened.[15] The ELISA was sensitive to 35 pg/ml and a sample was considered positive when ≥100 pg/ml. Of the tested samples, 6.7% were positive, including 6 samples with concentrations from 1 to 80 ng/ml. Only few of the positive sera correlated with infectious and/or inflammatory disease. After addition of mouse sera only 2 of 23 previously positive samples remained positive and these were sera from patients with sepsis and polyserositis, respectively. This study, along with our data, demonstrated that the cytokine assays might give false positive results which is largely dependent on the set-up method.

Although ELISAs have become a standard method for analyzing cytokines, results should be interpreted with caution, especially in the case of unexpected high positive rates. The differences in concentrations in the cytokine assays should raise concerns at the user, but also at the manufacturers.

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Chapter III

Changes of Ferritin and CRP Levels in Melanoma Patients Treated with Adjuvant Interferon α (EORTC 18952) and Prognostic Value on Treatment Outcome.

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ABSTRACT

Background: Adjuvant therapy with interferon-α (IFN) only benefits a small subgroup of melanoma patients and a predictive marker selecting responders does not exist. IFN induces increased ferritin and decreased CRP levels; however, an association with treatment effect was not studied.

Methods: Serum was collected from patients participating in the EORTC 18952 trial comparing adjuvant treatment with IFN to observation. Serial ferritin and CRP levels were determined using ELISA, before treatment and up to 24 months. Ferritin levels are influenced by sex and age; therefore ratios of serial ferritin and CRP values with corresponding pre-treatment values were calculated. Cox regression model and landmark method at end of induction and 6 months were used to evaluate the association between ferritin, CRP and distant metastasis-free survival (DMFS).

Results: Baseline ferritin levels were comparable in the 2 treatment groups (P = .92). However, ferritin ratios were significantly higher in IFN-treated patients (N = 96) compared to untreated patients (N = 21), at end of induction (mean: 2.88 vs 0.75; P = .0003) and at 6 months (mean: 3.18 vs 1.02; P = .009). In the IFN arm, higher ferritin ratios at end of induction and at 6 months were not associated with improved outcome (respectively P = .66 and P = .86). Concerning CRP ratios, no differences between the treatment groups, neither an association with DMFS, were observed.

Conclusions: Administration of IFN in melanoma patients induced increased ferritin levels but not in CRP levels. Ferritin and CRP ratios have no prognostic value regarding DMFS.

INTRODUCTION

Ferritin is an important iron-binding protein, composed of 24 subunits consisting of heavy (H) and light (L) chains.[1,2] The light chain is predominant in basic isoferritins, found in liver and spleen. In contrast, heart, kidney and placental ferritins are acidic because they mainly consist of heavy chains, which is also found in cancer cells.[3] Ferritin is an acute-phase protein and its expression is upregulated in inflammation, infection and malignancies. Increased ferritin levels in tumor tissue were reported in several malignancies such as colon cancer, breast cancer and seminoma.[4-6] In melanoma, high levels of L-ferritin were correlated with the metastatic phenotype *in vitro* and down-regulation of L-ferritin reduced the proliferation rate *in vitro* and *in vivo* as well as their invasive potential.[7] Gray and colleagues showed that H-ferritin, expressed by cultured melanoma cells or detected in the circulation of melanoma patients, induced immunosuppressive responses.[8,9] H-ferritin was shown to inhibit the response of lymphocytes stimulated with anti-CD3.[9] Furthermore, elevated serum ferritin levels, as compared to healthy individuals, were reported in melanoma patients with progressive metastatic disease.[10] These findings suggest a potential relation between ferritin and prognosis in melanoma patients.

High serum levels of C-reactive protein (CRP), an acute-phase reactant as well, was associated with shortened survival in metastatic melanoma and resistance to treatment with interleukin-2.[11,12] It is a serum marker, which could discriminate melanoma patients entering AJCC stage IV from patients remaining in AJCC stages I, II or III.[13] Furthermore, a recent study indicated that serum amyloid A and CRP combined were useful prognostic markers, also in early-stage melanoma patients.[14]

Recombinant interferon-α is a cytokine with a wide range of biological activities. Direct anti-proliferative/pro-differentiation, and protein synthesis-inhibiting antitumor activity, as well as indirect immune-mediated effects, rendering the tumor more susceptible to host effector cells, has been described.[15] Adjuvant therapy with interferon-α (IFN) is disappointing with only a minority of melanoma patients who benefits from treatment. A consistent and significant effect on recurrence-free survival (RFS), but not on overall survival (OS), was demonstrated in numerous randomized trials.[16,17] An individual patient data meta-analysis confirmed this significant effect on RFS and a marginal, but statistically significant, impact of 3% on OS.[18]

Normally a fixed correlation between CRP and ferritin is observed both *in vitro* and *in vivo*, with a CRP/ferritin ratio > 100 in favor of CRP.[19,20] However, it was shown that the administration of IFN increased ferritin and decreased CRP levels in melanoma patients.[21] The effect of this dissociation on prognosis was not studied before. Therefore the aim of the present study was to assess the prognostic and predictive significance of ferritin and CRP changes in melanoma patients receiving intermediate doses of IFN or no treatment (European Organization for Research and Treatment of Cancer (EORTC)

18952 trial).[22] Ferritin levels are influenced by sex and age[23] therefore, ratios of increase/decrease (compared to pre-treatment value) were calculated for both ferritin and CRP.

PATIENTS AND METHODS

Patients

Patients participated in the EORTC 18952 study and gave their informed consent for translational research for identification of prognostic factors. Between 1996 and 2000 a total of 1388 patients were enrolled, these were diagnosed with stage IIB melanoma (Breslow thickness ≥ 4 mm, node negative [N0]), or had undergone curative dissection of regional lymph nodes, either completion lymphadenectomy following positive sentinel node procedure (stage III, N1) or therapeutic lymph node dissection for palpable nodes (stage III, N2). Randomization occurred in a 2:2:1 fashion to the following treatment schedules, induction treatment of 4 weeks of 10 million units (MU) interferon-α2b five days a week followed by either 10 MU three times a week for 1 year (Arm A) or 5 MU three times a week for 2 years (Arm B). All were given by subcutaneous injections. Patients in Arm C did not receive treatment.

All available serum samples from a total of eight collaborating centers were collected centrally and stored in –80º C freezers until assayed. The blood samples for this collateral study were drawn at the same time point as for regular follow up tests; before treatment, at the end of the induction phase, and at 1, 3, 6, 12, 16, 20 and 24 months during maintenance therapy (similar for the observation arm).

Enzyme-linked immusorbent assays

CRP serum levels were determined by enzyme-linked immunosorbent assay (ELISA) as described before with some modifications.[24] Plates were coated overnight with polyclonal rabbit anti-human CRP (KH61, Sanquin Reagents) at 2 µg/ml in bicarbonate buffer pH 9.6. After washing, serum samples are diluted 1:4000 and 1:16000 in HPE buffer (Sanquin Reagents) and incubated for 1h. After washing a biotinylated monoclonal antibody to CRP (5G4, Sanquin Reagents)is used at 0.3µg/ml in HPE, followed by streptavidin-HRP. At the chosen sample dilution, detection limit of the ELISA is 0.2 µg CRP/ml serum.

Serum ferritin levels were measured essentially as described before (see also http://www.nibsc.ac.uk/documents/ifu/87-654.pdf) with minor modifications.[25] All incubations were at room temperature, no blocking step was used and serum samples and conjugate were diluted in HPE buffer (Sanquin Reagents). The coating antibody (87/654) the HRP-conjugate (87/662) as well as the recombinant ferritin standard (94/572) were kindly provided by Stephen Poole, NIBSC, Blanche Lane South Mimms Potters Bar Hertfordshire EN6 3QG United Kingdom. Sera were tested at 1:5 and 1:25 dilution. Detection limit at the 1:5 dilution was 5 ng ferritin/ml.

Statistical analysis

Since ferritin levels are influenced by sex and age we used ratios of serial ferritin and CRP values with corresponding pre-treatment values. To explore the effect of the administration of IFN and the dissociation of both acute-phase reactants (an increase in ferritin and a decrease in CRP levels), on prognosis, the ferritin/CRP ratio was calculated: (ferritin at time point t / ferritin at baseline) / (CRP at time point t / CRP at baseline). Distant metastasis-free survival (DMFS) was the time from date of randomization until the date of first distant metastasis or death without distant metastasis, whichever occurred first. The follow-up of patients without distant metastasis was censored at latest visit or contact date. Landmark method, which circumvents guarantee-time bias, based on serum samples available at end of induction and 6 months were used to evaluate the association between ferritin, CRP, ferritin/CRP and DMFS. Cox regression analyses were also used to adjust each analysis by some factors: Breslow thickness, ulceration and stage of the disease. Statistical analyses using SAS 9.1 software (SAS Institute Inc., Cary, NC) were performed at the EORTC Data Center. All tests were two-sided.

RESULTS

Baseline measurements

Baseline ferritin and CRP levels were determined in 138 patients, 77 males (56%) and 61 females (44%). Mean ferritin levels for 35 females younger than 50 years was 35.7 ng/ml (SE 31.1) and for 26 females equal to or older than 50 years 84.6 ng/ml (SE 71.9). For males, mean ferritin levels were 133.5 ng/ml (SE 132.3). Mean CRP levels were 5.1 µg/ml (SE 10.0) for the total patient group, with no differences between females and males. Among all patients, 7 patients had high ferritin levels at baseline (higher than 300 ng/ml for men, 150 ng/ml for women younger than 50 and 300 ng/ml for women older than 50), 14 patients had high CRP levels at baseline (higher than 10 µg/ml) and were excluded from further analysis. Baseline characteristics of the remaining 117 patients are shown in Table 1. The observation group (N = 21) and the IFN group (N = 96) were similar compared to the overall group of patients by treatment arms. In addition, in our study the two groups of patients were not significantly different regarding distant metastasis-free survival, $P = .70$, HR = 1.13 (95% CI = 0.61 to 2.11). This non-significant benefit for the observation group could probably be explained by a favourable distribution of disease stage (less III-N2) and no patients with 5 or more positive lymph nodes in this arm. Median DMFS was 2.76 years and median follow-up was 4.35 years for all 117 patients. Baseline ferritin or CRP levels for the observation group and the IFN group were similar, $P = .92$ and $P = .60$ respectively.

Table 1. Baseline characteristics

Characteristic	Observation (N=21)	IFN# (N=96)	Total (N=117)
	N (%)*	N (%)*	N (%)*
Age (yr)			
<51	13 (62)	61 (64)	74 (63)
51-65	5 (24)	29 (30)	34 (29)
≥65	3 (14)	6 (6)	9 (8)
Sex			
Male	10 (48)	54 (56)	64 (55)
Female	11 (52)	42 (44)	53 (45)
Breslow thickness (mm)			
<1.0	1 (5)	7 (7)	8 (7)
1-1.99	3 (14)	18 (19)	21 (18)
2-3.99	5 (24)	26 (27)	31 (27)
≥4.00	11 (52)	35 (37)	46 (39)
Unknown	1 (5)	10 (10)	11 (9)
Ulceration			
Absent	11 (52)	51 (53)	62 (53)
Present	7 (33)	26 (27)	33 (28)
Unknown	3 (14)	19 (20)	22 (19)
Stage at randomization			
IIB	8 (38)	23 (24)	31 (27)
III-N1 (microscopic)	7 (33)	21 (22)	28 (24)
III-N2 (palpable)	6 (29)	52 (54)	58 (50)
Number of positive lymph nodes			
0	8 (38)	23 (24)	31 (27)
1	8 (38)	39 (41)	47 (40)
2-4	5 (24)	21 (22)	26 (22)
5+	0 (0)	13 (14)	13 (11)
Treatment			
Observation	21 (100)	–	21 (18)
13-month IFN (10 MU)	–	53 (55)	53 (45)
25-month IFN (5 MU)	–	43 (45)	43 (37)
Ferritin (ng/ml): at baseline: mean (SE†)	73.00 (48.34)	74.59 (64.53)	74.31 (61.76)
CRP (ml/ml) at baseline: mean (SE†)	2.30 (1.94)	2.60 (2.45)	2.55 (2.36)

IFN = Interferon-a; MU = million units

* Because of rounding not all percentages equal 100

† SE = Standard Error

Changes of Ferritin, CRP and Ferritin/CRP ratio during follow-up

Ferritin ratios (ferritin level at time point t compared to the baseline ferritin level) remained constant over time in the observation arm (Figure 1). However, means of ferritin ratios in the IFN-treated patients as compared to the untreated patients were significantly higher, at all time points except from the 24-month time point (at end of induction: 2.88 (SE 1.59) versus 0.75 (SE 0.17), $P = .0003$ and at 6 months; 3.18 (SE 3.41) versus 1.02 (SE 0.59), $P = .009$). CRP ratios were similar between treatment groups over time (Figure 2), at end of induction: 1.13 (SE 2.70) versus 0.95 (SE 0.55), $P = .85$ and at

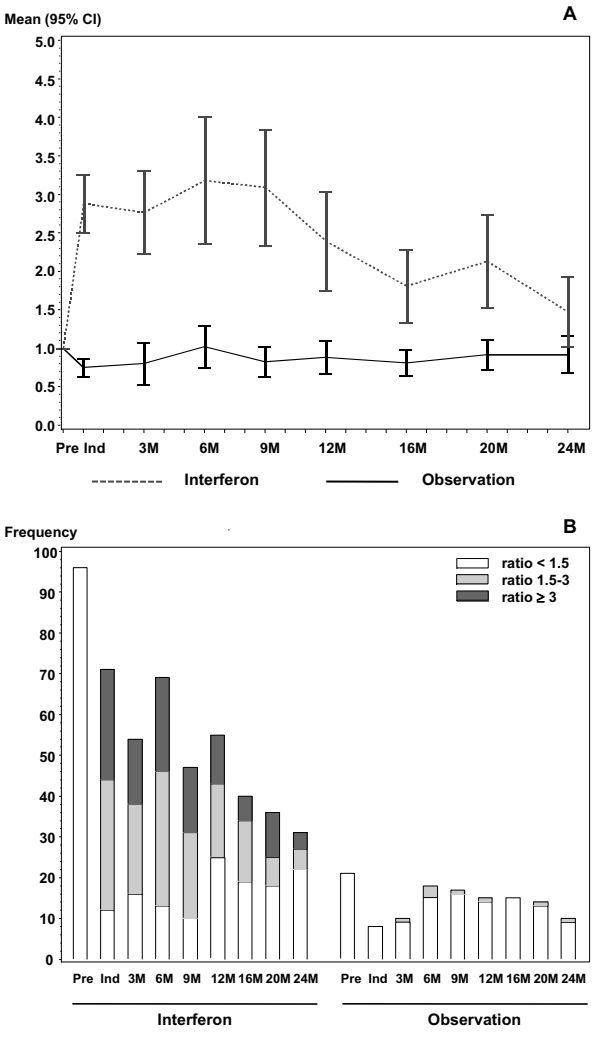

Figure 1. Mean ferritin ratios over time for untreated patients vs patients receiving intermediate doses of IFN are shown in panel **(A)**. Frequency of the serial ferritin ratios; <1.5 / 1.5 – 3 / ≥ 3 are depicted for the two treatment groups in panel **(B)**. Ferritin ratios: ferritin at time point t / ferritin at baseline.

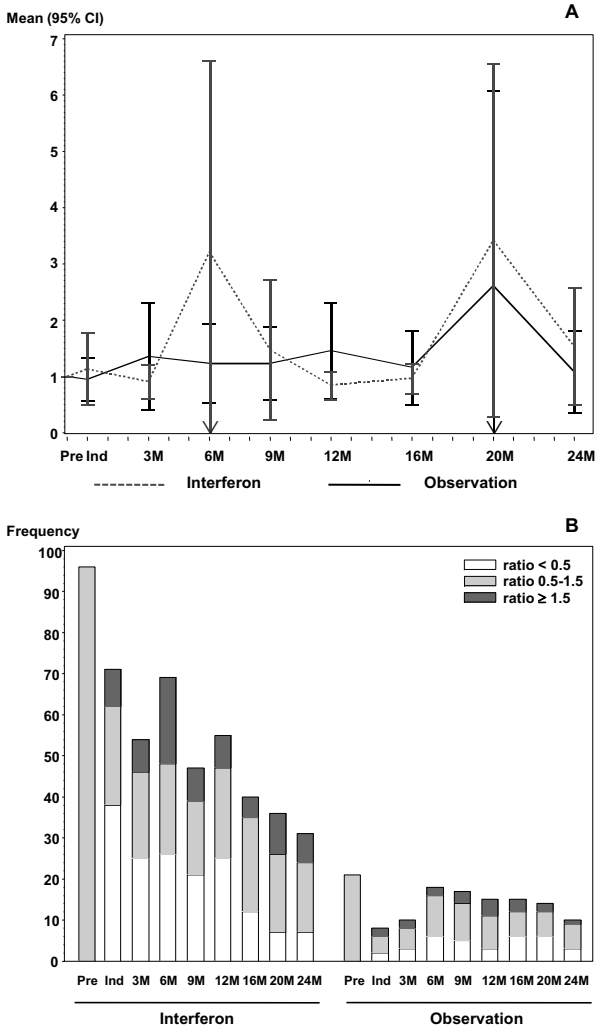

Figure 2. Mean CRP ratios for untreated patients vs IFN-treated patients at base line and up to 24 months are shown in panel **(A)**. Frequency of the CRP ratios; <0.5 / 0.5 – 1.5 / ≥ 1.5 for both treatment arms at the different time points are depicted in panel **(B)**. CRP ratios: CRP at time point t / CRP at baseline.

6 months; 3.21 (SE 14.12) versus 1.23 (SE 1.50), $P = .56$. In the IFN group, a CRP ratio of <0.5 was most frequently observed at end of induction, in 54% of the patients, at 3, 6 and 12 months this was 46%, 38% and 46% respectively. These lower CRP ratios at end of induction could explain the peak observed in the ferritin/CRP ratio analysis at end of induction in the IFN group (Figure 3).

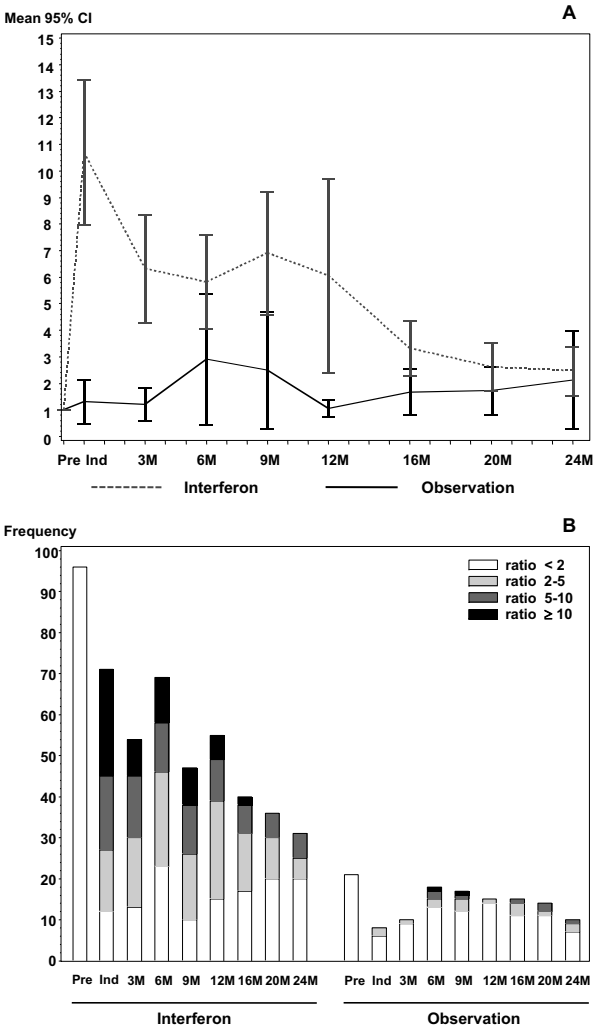

Figure 3. The ferritin/CRP ratios, (ferritin at time point t / ferritin at baseline) / (CRP at time point t / CRP at baseline), for the observation arm and the IFN arm at the different time points are shown in panel **(A)** and the distributions of the ratios, <2 / 2-5 / 5-10 / ≥ 10 for both treatment groups in panel **(B)**.

Landmark analyses for distant metastasis-free survival

Landmark analyses at end of induction and at 6 months indicated that higher ferritin ratios or lower CRP ratios were not associated with improved prognosis in IFN-treated patients (Figure 4). Also, ferritin/CRP ratios at end of induction or at 6 months had no prognostic value (Figure 5). Using each ratio as ordered variable only in the IFN group, the association between ratios of ferritin, CRP or ferritin/CRP and subsequent DMFS was also not significant. In addition, when other cutoffs for these ratios were considered, similar results concerning DMFS were obtained. Also, adjusted analysis by stage of the

disease did not change the results. Since RFS was a secondary endpoint of the original trial we analyzed the effect of ferritin, CRP or ferritin/CRP ratios on RFS as well, yet study results remained unchanged (data not shown).

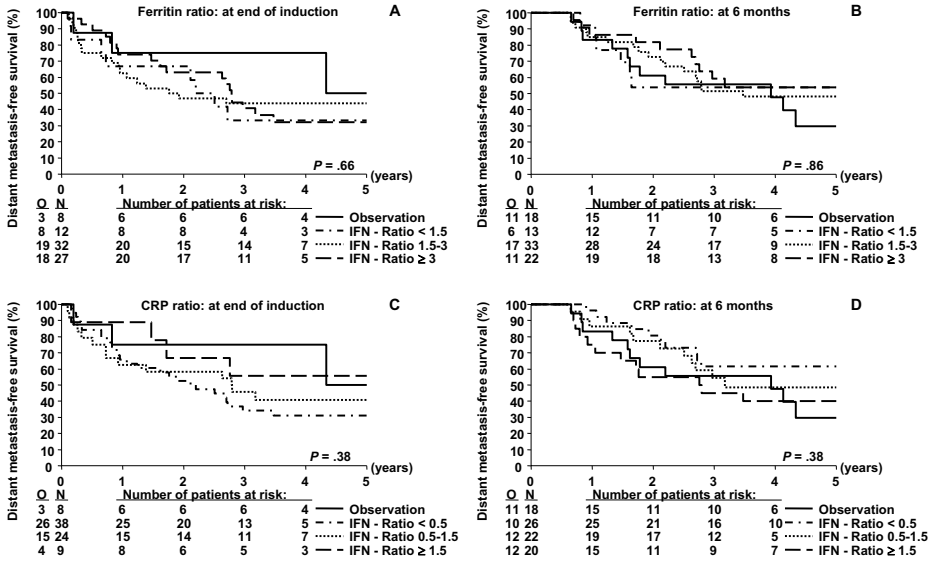

Figure 4. Landmark method at end of induction in panel **(A)** and at six months in panel **(B)** comparing the observation group to the IFN group, according to ferritin ratio, for distant metastasis-free survival. Similarly, CRP ratios at end of induction and at six months are depicted in panels **(C + D)**.

DISCUSSION

Stam and colleagues previously assessed the effects of interferon-α2b administration on the acute-phase response in a subset of 21 melanoma patients of the EORTC 18952 trial.[21] This study showed significant increases in ferritin levels and less pronounced decreases in CRP levels at end of induction and at 6 months. The administration of IFN induced no significant changes in other acute-phase proteins such as secretory phospholipase A_2, alpha1-acid glycoprotein or albumin. Here we extended the analysis in order to explore the association of ferritin and CRP changes with clinical response to IFN therapy. In 117 patients accrued in the EORTC 18952 trial, serial ferritin and CRP levels were determined from baseline, up to 24 months maximum.

Initial ferritin levels were markedly lower in females younger than 50 years old, 35.7 ng/ml, compared to older females, 84.6 ng/ml, or males, 133.5 ng/ml. Reference ranges of haemoglobin and ferritin for women of reproductive age are widely reported showing values that are lower than equivalent aged males. In a recent review by Rushton and Barth this gender difference was addressed.[23] In the present study we calculated

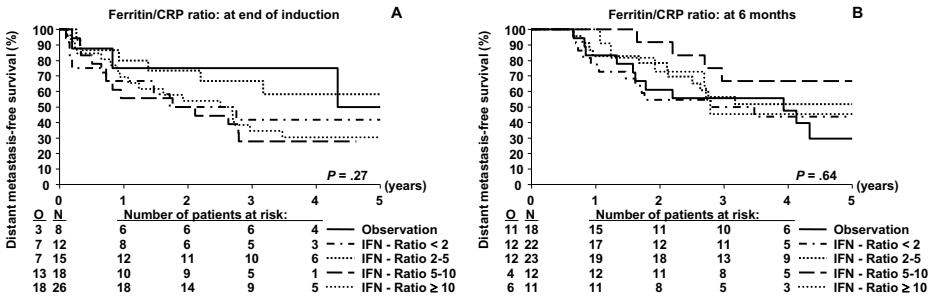

Figure 5. Landmark method for the ferritin/CRP ratio, according to the different categories for patients receiving IFN, compared to untreated patients, are shown in panel **(A)** for end of induction and in panel **(B)** for six months, regarding the DMFS.

ratios, ferritin levels at a time point t during follow-up compared to baseline ferritin levels, circumventing the use of different cutoffs. For consistency we followed the same approach for CRP. Ferritin ratios of patients in the observation group were steady over time, in contrast to the ratios in the IFN-treated patients, which were increased from end of induction and remained high during the treatment period. After 12 months of follow-up a decrease in ferritin ratios was observed, however, only halve of the patients were still on treatment, namely those in the 25-month IFN group. When analyzing the three treatment groups, differences were seen between the 13-month and the 25-month IFN groups (data not shown). During the first year, ferritin ratios were higher in the 13-month IFN (10 MU) group compared to the 25-month IFN (5 MU) group and subsequently during the second year, only ferritin levels in the 25-month IFN group were elevated compared to the observation group. These results suggest a dose-dependent effect of IFN on ferritin levels, the mechanism however is still unclear.

CRP ratios varied slightly during the course of the study but no significant differences between treated and untreated patients were observed. Nevertheless, one could argue that at end of induction relatively more patients in the IFN group had a decrease in CRP ratio accounting for the peak in ferritin/CRP ratio at end of induction. However, higher ferritin or ferritin/CRP ratios could not predict treatment response in patients receiving IFN. Moreover, when considering also 21 patients with high positive CRP or ferritin levels at baseline, the study results were similar and without any effect on prognosis (data not shown).

A limitation of this study is the fact that this is a subgroup analysis comprising of 117 patients from an original series of 1388 patients. Although this subgroup is representative for the total patient group, but as the impact of adjuvant IFN therapy on the outcome is relatively low, in our series of 117 patients there was no difference in DMFS for patients receiving treatment or not as well. Identification of a predictive biomarker is thus only likely if this marker has a strong relationship with treatment benefit and this one is large enough.

It has been over a decade that the E1684 trial, comparing adjuvant high-dose IFN (HDI) to observation, showed an improvement in overall survival in melanoma patients.[26] Numerous randomized trials on IFN treatment followed but there are still many unsolved questions. For instance; how does interferon work and what is the optimal treatment dose or duration? Importantly, which patients are most likely to benefit, rectifying such a toxic treatment? Results from the Hellenic Oncology Group trial of Gogas et al.[27] support the hypothesis of an immunomodulatory mechanism of action rather than a direct cytotoxic effect of IFN. This study showed improved RFS and OS in patients developing clinical and serological manifestations of autoimmunity during treatment with HDI. These findings could not be confirmed by studies from the EORTC and Nordic Melanoma Group, reporting no benefit for patients who became autoantibody positive during treatment with adjuvant IFN or pegylated-(PEG-)IFN.[28,29] Guarantee-time bias is an important confounder when analyzing the prognostic value of any potential biomarker in serial measurements and should be taken into account.[30,31] When correction was made for guarantee-time bias, autoimmunity remained a strong independent prognostic marker in the Gogas's study but it lost significance in the side studies from the EORTC 18952, EORTC 18991 and Nordic IFN trial. Although the same ELISAs (Quanta Lite, Inova Diagnostics) and titers were used in the studies by Gogas et al. and the EORTC/Nordic Melanoma group collaboration, major differences were found for the occurrence of autoantibodies at baseline: 33% and 35% of the patients from the EORTC 18952 study and from the Nordic IFN trial respectively had pre-existing autoantibodies, against 1.5% reported by Gogas et al. This is extremely low considering antinuclear antibodies for instance, with reported prevalences ranging 4 to 35% in healthy individuals, and prevalences up to 40% in cancer patients.

Multiplex analysis of a panel 29 cytokines in serum of high-risk melanoma patients by Yurkovetsky et al.[32] showed the association of higher pre-treatment levels of the proinflammatory cytokines IL-1α, IL-1β, IL-6, TNF and the chemokines MIP-1α and MIP-1β with longer (> 5 years) RFS in IFN-treated patients. Subgroup analysis of the individual patient data meta-analysis by Wheatley et al.[18] suggested that patients with ulcerated primary tumors responded better to adjuvant IFN therapy than patients with non-ulcerated tumors. Also the results of the EORTC 18991 trial,[33] comparing adjuvant pegylated-IFN to observation, suggested a benefit in patients with ulcerated primaries. Furthermore, it seemed that patients with microscopic lymphnodal disease (stage III-N1) benefited from PEG-IFN, in contrast to patients with palpable lymphnode metastases (stage III-N2), in whom no effect of PEG-IFN was observed.

Identification of new biomarkers and confirmatory studies of so far promising biomarkers is needed because these could help us understand the mechanisms of action of interferon-α and could enable tailored treatment to those most likely to benefit. Here we showed that ferritin levels and ferritin/CRP ratios were significantly increased by the administration of IFN, however, these increases did not correlate with clinical response to IFN therapy.

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SECTION TWO

AUTOIMMUNITY

Chapter IV

Autoimmune Antibodies and Recurrence-free Interval in Melanoma Patients Treated with Adjuvant Interferon

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ABSTRACT

Background: Appearance of autoantibodies and clinical manifestations of autoimmunity in melanoma patients treated with adjuvant interferon (IFN)-α2b was reported to be associated with improved prognosis. We assessed the association of the appearance of autoantibodies after initiation of treatment with recurrence-free interval in two randomized trials that compared intermediate doses of IFN to observation for the treatment of melanoma patients.

Methods: Serum levels of anticardiolipin, antithyroglobulin, and antinuclear antibodies were determined using enzyme-linked immunosorbent assays in 187 and 356 patients in the European Organization for Research and Treatment of Cancer (EORTC) 18952 and Nordic IFN trials, respectively, immediately before, and up to 3 years after random assignment. The association of the presence of at least one of the three autoantibodies with risk of recurrence was assessed by three Cox models in patients negative for all three autoantibodies at baseline (125 from the EORTC 18952 trial and 230 from the Nordic IFN trial): 1) a model that considered appearance of autoantibodies as a time-independent variable, 2) one that considered a patient autoantibody positive once a positive test for an autoantibody was obtained, and 3) a model in which the status of the patient was defined by the most recent autoantibody test. All statistical tests were two-sided.

Results: When treated as a time-independent variable (model 1), appearance of autoantibodies was associated with improved recurrence-free interval in both trials (EORTC 18952, hazard ratio [HR] = 0.41, 95% confidence interval [CI] = 0.25 to 0.68, $P < .001$; and Nordic IFN, HR = 0.51, 95% CI = 0.34 to 0.76, $P < .001$). However, on correction for guarantee-time bias, the association was weaker and not statistically significant (model 2: EORTC 18952, HR = 0.81, 95% CI = 0.46 to 1.40, $P = .44$; and Nordic IFN, HR = 0.85, 95% CI = 0.55 to 1.30, $P = .45$); model 3: EORTC 18952, HR = 1.05, 95% CI = 0.59 to 1.87, $P = .88$; and Nordic IFN, HR = 0.78, 95% CI = 0.49 to 1.24, $P = .30$).

Conclusions: In two randomized trials of IFN for the treatment of melanoma patients, appearance of autoantibodies was not strongly associated with improved recurrence-free interval when correction was made for guarantee-time bias.

INTRODUCTION

Several randomized controlled trials have evaluated the effect of adjuvant treatment with interferon-α (IFN) of high-risk melanoma patients on recurrence-free interval and overall survival (OS).[1-3] Pooled analyses of high-dose IFN trials in the United States and meta-analyses of high-, intermediate-, and low-dose IFN trials in both the United States and Europe have demonstrated a consistent and statistically significant effect of IFN treatment on recurrence-free interval but not on OS.[1,2] A recent meta-analysis based on individual patient data also found a statistically significant and consistent effect of IFN treatment on recurrence-free interval and a marginal, but statistically significant, effect of 3% on OS at 5 years.[3] Optimal treatment dose or duration is still not known. These findings suggest the possible existence of a small subgroup of patients responding to IFN therapy. In view of the toxicity and costs of IFN therapy, it would therefore be of great benefit if this subgroup of patients could be identified.

A recent study by Gogas et al. showed that the appearance of autoantibodies and clinical manifestations of autoimmunity in melanoma patients treated with adjuvant high-dose IFN was strongly associated with improved recurrence-free interval and OS.[4] Also, the occurrence of autoimmune thyroid disease in patients receiving low-dose IFN has been reported to be associated with longer recurrence-free interval.[5] Although autoimmunity has been found to be associated with better prognosis in untreated melanoma patients as well, these two trials did not analyze the occurrence, and effects on prognosis, of autoimmunity in an observation group. Therefore, whether the appearance of autoimmunity was associated with the effectiveness of IFN treatment in melanoma patients could not be established.

The measurement of autoantibodies can be used as an index of immune response, which could be an indicator for efficacy of treatment with adjuvant IFN. However, autoantibodies are also commonly detected in healthy persons and (untreated) cancer patients. Antinuclear antibody prevalence, for instance, ranges from 4% to 35% in healthy individuals, and frequencies of up to 40% have been reported in a series of patients with different types of cancer.[6-10] Thus an observation group must be included in trials that seek to determine whether immune response is associated with response to IFN treatment.

We studied whether the occurrence of autoantibodies in melanoma patients receiving intermediate doses of IFN (IFN-α2b) or no treatment was associated with response to treatment. Patients for this translational study were accrued in two large randomized trials: the European Organization for Research and Treatment of Cancer (EORTC) 18952 trial and the Nordic IFN trial. Both of these trials compared treatment with intermediate doses of IFN with observation.[11,12] Because the presence of autoantibodies varies over time, it was crucial to choose a statistical approach that took time to seroconversion

(appearance of autoantibodies) into account. Therefore, we analyzed the relationship between the appearance autoantibodies and recurrence-free interval using three Cox proportional regression models, in two of which the appearance of autoantibodies was treated as a time-dependent variable.

PATIENTS AND METHODS

Patients

The EORTC 18952 study and the Nordic IFN trial were designed to evaluate efficacy and toxicity of intermediate doses of IFN in high-risk melanoma patients. For EORTC 18952, between 1996 and 2000 a total of 1388 patients who had been diagnosed with stage IIB or III melanoma were enrolled.[11] These patients had tumors with Breslow thickness 4 mm and greater and were lymph node-negative (N0), or had undergone curative dissection of regional lymph nodes (either completion lymphadenectomy following positive sentinel node procedure [stage III, N1] or therapeutic lymph node dissection for palpable nodes [stage III, N2]). Patients were randomly assigned in relative proportions of 2:2:1 to 13 months of intermediate high-dose IFN, 25-months of intermediate low-dose IFN, or observation only, respectively. IFN-treated patients received an induction treatment of 4 weeks of 10 million units (MU) IFN, delivered 5 days a week. Induction was followed by either 10 MU IFN three times a week for 1 year or 5 MU IFN three times a week for 2 years, with all treatments delivered subcutaneously.

For the Nordic IFN trial, a total of 855 patients with stage IIB or III melanoma were enrolled between 1996 and 2004. Patients were randomly assigned in equal proportions to three treatment arms: observation only, induction treatment with 10 MU IFN 5 days a week for 4 weeks followed by 10 MU three times a week for 1 year (as in the 13-month IFN arm from the EORTC 18952), or induction treatment followed by 10 MU IFN three times a week for 2 years. All treatments were delivered by subcutaneous injection.[12] All available sera from 27 collaborating centers were collected and autoantibody levels were determined centrally at the Laboratory for Experimental Surgical Oncology, Erasmus University Medical Center, Daniel den Hoed Cancer Center in Rotterdam. The blood samples for this study were drawn at the same time points as for regular follow-up tests, that is, before treatment, at the end of the induction phase (1, 3, 6, 12, 16, 20, and 24 months after the start of treatment), and at 30- and 36- month follow-up. Informed consent from patients for translational research in association with these trials for the purposes of identification of prognostic factors was obtained.

Serum Analysis

Enzyme-linked immunosorbent assays (Quanta Lite; Inova Diagnostics, San Diego, CA) were used to test sera for anticardiolipin (IgG, IgA and IgM classes were assayed simultaneously), antithyroglobulin (IgG), and antinuclear (IgG) antibodies. Following the manufacturer's protocol, moderately positive results were defined as titers of greater than or equal to 1:100, greater than or equal to 1:100, and greater than or equal to 1:40 for anticardiolipin, antithyroglobulin, and antinuclear antibodies, respectively. Corresponding titers for strongly positive results were greater than or equal to 1:200, greater than or equal to 1:170, and greater than or equal to 1:120. All determinations of antibody titer were carried out in duplicate. If tests did not agree, they were repeated. A patient who had a positive test result for any of the three autoantibodies was classified as autoantibody positive. Seroconversion was defined as appearance of autoantibodies during follow-up in patients who were autoantibody negative at baseline.

The assays were validated for reproducibility and concordance with routine, well-standardized enzyme-linked immunosorbent assays used by the Department of Immunopathology, Sanquin, Amsterdam, The Netherlands.[13,14]

Statistical Analysis

Recurrence-free interval was the time from the date of random assignment until the first relapse or death without relapse, whichever occurred first; the follow-up of patients who did not relapse was censored at the latest visit or last contact. Time to appearance of autoantibodies was the time from the date of random assignment until the date a positive test was recorded; the follow-up of patients for whom no positive test was recorded was censored at the latest date of assessment of autoantibody status or date of relapse or last follow-up. The Kaplan-Meier method was used to estimate the survivor function distributions, and the log-rank test was used to test for differences between survival curves.[15] The association of the occurrence of observed autoimmunity with recurrence-free interval was assessed using three Cox models: one in which the appearance of autoantibodies was considered as a time-independent variable and two that considered autoimmunity as a time-dependent variable. In the three models, the prognostic importance of autoantibody status was adjusted for sex and the initial number of positive lymph nodes before random assignment, considered as an ordered categorical variable. Categories for the number of positive lymph nodes were 0, 1, 2–4, and greater than or equal to 5 in the EORTC 18952 study and 0, 1, 2–3, and greater than or equal to 4 in the Nordic IFN study.

In a Cox model in which autoantibody status was considered to be a time-independent variable (Figure 1, model 1), the hazard ratio of the event intensity per time unit in autoantibody-positive patients vs autoantibody-negative patients according to the initial number of lymph nodes and sex was expressed as: $HR = e^{\beta 1 \times \text{autoantibody status}(t) + \beta 2 \times \text{number of positive lymph nodes} + \beta 3 \times \text{sex}}$.

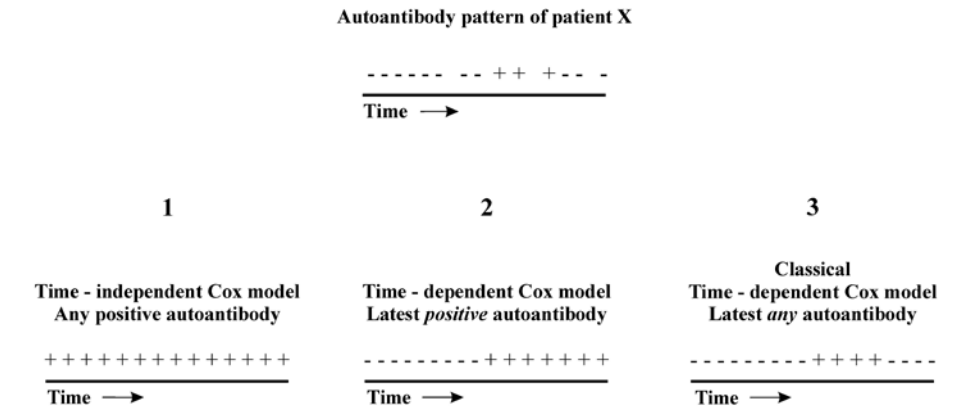

Figure 1. Three statistical approaches to evaluate the effect of autoantibodies on prognosis. The diagram at the top shows the serial results of autoantibody testing in a patient with transient seroconversion. According to model 1, if one or more autoantibody tests are positive, the patient is considered autoantibody positive from baseline, regardless of when seroconversion occurred. This model is biased because patients who are alive and free of relapse for a longer period will have a greater chance to become autoantibody positive than those who had an early relapse. In model 2, the patient is considered autoantibody positive from the moment of seroconversion. This model tests the following hypothesis: the appearance of autoantibodies is indicative of treatment response, irrespective of the duration of seroconversion. Model 3 is the classical time-dependent Cox model. It uses the latest available information regarding autoantibody-status. In contrast to model 2, it tests whether not only appearance but also duration of seroconversion is associated with prognosis. In case of a transient seroconversion in the autoantibody-positive group, the patient returns to the autoantibody-negative group upon testing negative. Model 3 assumes that from that time, the patient has the same prognosis as all other autoantibody-negative patients. Both models 2 and 3 correct for guarantee-time bias.

Autoantibody status (t) was set to 0 if all tests for autoantibodies were always negative, or 1 if an autoantibody test was at least once positive, whatever the moment of seroconversion (ie, autoantibody status (t) is time-independent). Sex was entered as 0 for men and 1 for women. This model provides a biased estimate of the prognostic importance of autoantibody status because in patients who are alive and free of relapse for a longer time period, the possibility of seroconversion will be higher, whereas in those who have an early relapse, seroconversion will be less likely. This model therefore introduces guarantee-time bias.[16]

To determine whether autoantibody status assessed during the course of the study, before time t and before relapse, was associated with the subsequent outcome, two models in which antibody status was considered to be a time-dependent variable were used. In one Cox time-dependent model (Figure 1, model 2), for patients free of relapse just before a time point t, the HR was expressed as above with autoantibody status (t) = 0 if autoantibody tests were always negative before time t and = 1 from the moment the autoantibody status became positive, irrespective of whether it remained positive

or returned to a negative status thereafter; thus the variable is an indicator of the latest positive autoantibody status.

In model 3, for patients free of relapse just before a time point t, the HR was set as above with autoantibody status (t) = 0 if the latest autoantibody status determined before or at time t was negative or t = 1, if the latest autoantibody status determined before or at time t was positive (Figure 1, model 3). Thus, for purposes of risk assessment the most recent value of autoantibody status at time t (determined at that time or earlier if not available at time t) was used.

Both time-dependent models may provide different information, because they discriminate differently for patients who switched from autoantibody-positive back to autoantibody-negative status (transient seroconversion). Model 3 assumes that from the time the patient becomes autoantibody-negative again, she or he has the same prognosis as all other autoantibody-negative patients. Model 2 considers patients once autoantibody-positive as positive, regardless of any subsequent change to autoantibody-negative status.

Based on serial measurements of autoantibody status and the data for each patient on time of recurrence, the hazard ratio and its 95% confidence interval were estimated. The Wald test (standardized coefficient) was used to determine the prognostic value of variables considered in the model. In addition, the landmark method, which also circumvents guarantee-time bias, was used to evaluate the association between autoantibody status based on serum samples available at 6 and 12 months after end of induction and subsequent recurrence-free interval. Because the samples were not all drawn at exactly these time-points, a 60-day time interval window (± 30 days around the theoretical date) was applied. To determine whether the autoantibody status (positive vs negative) has strong impact on the recurrence-free interval, for example, the corresponding hazard ratio is 0.50, a total of 102 events were required to reach a statistical power of approximately 80% (two-sided alpha = .05), considering that, in mean, over time, 30% of patients were in the autoantibody-positive status and 70% in the negative one. Statistical analyses using SAS 9.1 software (SAS Institute Inc., Cary, NC) were performed at the EORTC Data Center. All statistical tests were two-sided.

RESULTS

Patients

All available serum samples from patients accrued in both trials were collected for this study. Baseline autoantibody levels were determined in 187 and 356 patients in the EORTC 18952 and Nordic IFN trials, respectively. There were 62 patients (33%) in the EORTC 18952 study and 126 patients (35%) in Nordic IFN study who were positive at

baseline for at least one of three autoantibodies and thus considered to have developed autoantibodies prior to treatment. No difference in recurrence-free interval between patients with or without pre-existing autoantibodies was observed (Supplementary Figure, Appendix). In the EORTC 18952 study, among the 125 patients who remained after exclusion of those who were autoantibody positive at baseline, 54 patients were randomly assigned to the 13-month IFN group, 48 to the 25-month IFN group, and 23 to the observation group. With a median follow-up of 4.2 years, recurrence-free interval did not differ by a statistically significantly extent between treatment groups (overall *P* = .36) (13-months IFN vs observation, HR = 0.81, 95% CI = 0.46 to 1.40; 25-months IFN vs observation, HR = 0.66, 95% CI = 0.37 to 1.17). Median overall recurrence-free interval among all patients who were free of autoantibodies before treatment was 2.1 years. In the Nordic IFN trial, the 230 patients who did not test positive for one of the three antibodies were randomly assigned as follows: 70 patients to no-adjuvant treatment, 79 patients to IFN treatment for 13 months, and 81 patients to IFN treatment for 25 months. With a median follow-up of 6.8 years, no statistically significant difference in recurrence-free interval across treatment arms was observed (overall *P* = .43; 13-month IFN vs observation, HR = 0.76, 95% CI = 0.50 to 1.17; 25-month IFN vs observation, HR = 0.81, 95% CI = 0.53 to 1.24). Median recurrence-free interval for all 230 patients was 2.46 years.

Seroconversion

Seroconversion, the appearance of autoantibodies in patients who initially tested negative for all three autoantibodies, occurred in 43 (34%) of 125 and 73 (32%) of 230 patients in the EORTC 18952 and Nordic IFN trials, respectively. Frequencies of the three autoantibodies were summarized (Supplementary Table, Appendix). Antinuclear antibodies were the most prevalent in treated as well as untreated patients. Baseline characteristics according to treatment for patients in both trials who initially tested negative for the presence of autoantibodies, were comparable with those of the total patient population from the EORTC 18952 and Nordic IFN trials (Table 1).[11,12] There were no differences in the crude rates of antibody appearance according to age, Breslow thickness, stage at random assignment, or number of positive lymph nodes. However, differences in the crude rates were seen according to treatment, sex, and the presence or absence of ulceration.

In the EORTC 18952 study, 37 (36%) of 102 patients in the IFN arms and six (26%) of 23 patients in the observation arm developed autoantibodies. The rate of seroconversion was higher in the IFN group compared with observation arm, but the difference was not statistically significant (HR for seroconversion = 1.64, 95% CI = 0.69 to 3.88; *P* = .26) (Figure 2, A). In the Nordic IFN study, 63 (39%) of 160 patients in the IFN groups compared with 10 (14%) of 70 patients in the observation group developed autoantibodies (HR for seroconversion = 2.60, 95% CI = 1.33 to 5.07; *P* = .004) (Figure 2, B).

Table 1. Baseline characteristics according to treatment and percentage of autoantibody-positive patients in each category according to the treatment group in the EORTC 18952 and Nordic IFN trials*

	EORTC 18952†		Nordic IFN†	
Characteristic	**Observation**	**IFN-α,‡**	**Observation**	**IFN-α,‡**
	No. (%)	**No. (%)**	**No. (%)**	**No. (%)**
Age, (yr)				
<51	13 (38)	61 (38)	43 (16)	81 (42)
51-65	6 (0)	34 (38)	21 (10)	64 (39)
≥65	4 (25)	7 (14)	6 (17)	15 (27)
Sex				
Male	12 (8)	62 (31)	45 (16)	108 (35)
Female	11 (46)	40 (45)	25 (12)	52 (48)
Breslow thickness (mm)				
<1.00	0 (0)	7 (43)	5 (20)	13 (31)
1.00-1.99	5 (20)	20 (45)	22 (18)	31 (42)
2.00-3.99	6 (33)	34 (29)	21 (10)	47 (36)
≥ 4.00	11 (27)	37 (35)	21 (14)	59 (41)
Unknown	1 (0)	4 (50)	1 (0)	10 (50)
Ulceration				
Absent	12 (8)	61 (34)	42 (17)	83 (36)
Present	8 (38)	31 (45)	19 (11)	45 (47)
Unknown	3 (67)	10 (20)	9 (11)	32 (38)
Stage at random assignment				
IIB	7 (14)	23 (39)	16 (6)	34 (41)
III-N1 (microscopic)	9 (33)	25 (40)		
III-N2 (palpable)	7 (29)	54 (33)		
III-N1/III-N2			54 (17)	126 (39)
No. of positive lymph nodes				
0	7 (14)	23 (39)	16 (6)	34 (41)
1	7 (29)	46 (33)	32 (13)	68 (32)
2-4/2-3	7 (43)	23 (39)	13 (15)	39 (51)
5+/4+	2 (0)	10 (40)	9 (33)	19 (37)
Treatment				
13 months IFN	–	54 (31)	–	79 (34)
25 months IFN (5 MU)	–	48 (42)	–	–
25 months IFN (10 MU)	–	–	–	81 (44)

* EORTC = European Organization for Research and Treatment of Cancer; IFN = interferon; MU = million units.
† The EORTC trial contained 125 patients (23 in the observation arm and 102 in the treatment arms); the Nordic IFN trial contained 230 patients (70 in the observation arm and 160 in the treatment arm).
‡ Treatment was for 13 or 25 months.

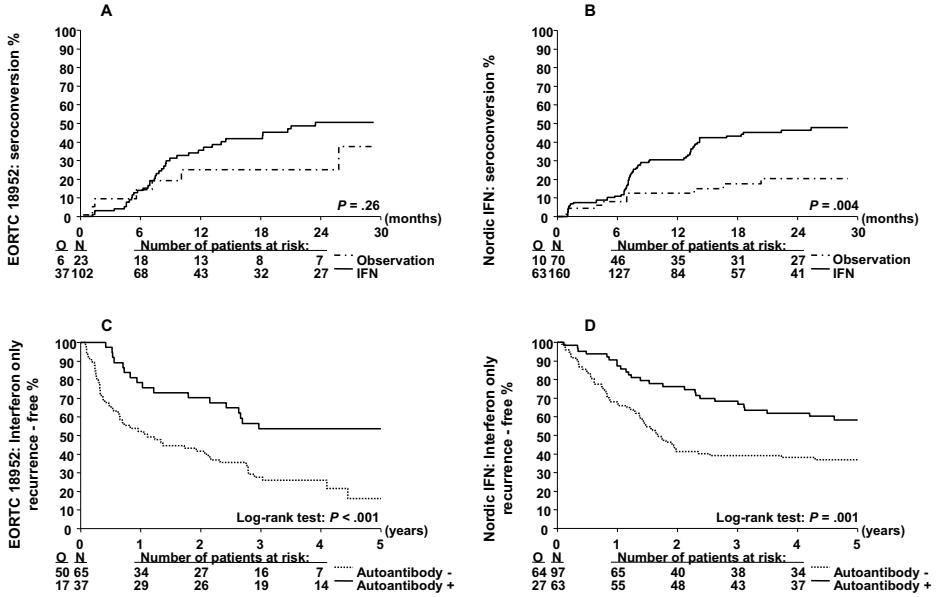

Figure 2.

Time to seroconversion for untreated patients vs patients receiving intermediate doses of IFN-a. **A)** Patients in the EORTC 18952 trial. **B)** Patients in the Nordic IFN trial. **C and D)** For patients randomly assigned to the IFN arm, Kaplan-Meier estimates of recurrence-free interval are shown from initial random assignment according to whether they became autoantibody positive or remained autoantibody negative during the study in the EORTC 18952 trial **(C)** and in the Nordic IFN trial **(D)**. These analyses are subject to "guarantee-time" bias (see the body text); this translates into an initial plateau of the recurrence-free interval curve for the autoantibody-positive group. IFN = interferon; n = number of patients; *o* = observed number of relapses; autoantibody + = positive for the presence of anticardiolipin, antithyroglobulin, or antinuclear antibodies; EORTC = European Organization for Research and Treatment of Cancer.

Seroconversion rates for the IFN-treated patients were similar in both trials and higher in women than in men; percentages of patients in whom autoantibodies were detected were 31% and 35% of the men and 45% and 48% of the women in EORTC 18952 and Nordic IFN, respectively (Table 1). Also, in the observation group of the EORTC 18952 trial, a higher seroconversion rate for women was observed (46% in women vs 8% in men), but this group consisted of only 11 women and 12 men.

In both untreated and treated patients from the EORTC 18952 trial, patients with ulcerated tumors had higher seroconversion rates compared with patients with non-ulcerated tumors. This was also true for the IFN-treated patients in the Nordic IFN trial. However, it is difficult to draw conclusions from these groups because the occurrence of seroconversion in patients with ulcerated tumors was not similar in the observation groups of the two trials, and ulceration status of the primary tumor was frequently unknown.

Autoantibodies were measured for a maximum of 3 years in the EORTC 18952 trial and 2 years in the Nordic IFN trial. In both trials, by 12 months, autoantibodies were detected in half of the patients, and the latest time point at which autoantibodies were initially detected was approximately 2 years.

Association of Seroconversion with Recurrence-Free Interval

In both trials, patients who became autoantibody positive during the study period had a statistically significantly better recurrence-free interval compared with those who remained autoantibody negative. In the EORTC 18952 trial, the 43 autoantibody-positive patients had a statistically significantly ($P < .001$) lower relapse rate than the 82 autoantibody-negative patients (HR = 0.43, 95% CI = 0.27 to 0.71). In the Nordic IFN trial, 73 patients who became autoantibody positive during the course of the trial had a lower relapse rate than the 157 patients who remained autoantibody negative (HR = 0.51, 95% CI = 0.34 to 0.76; $P < .001$). Similar results were observed in IFN-treated patients only. In the EORTC 18952 study (Figure 2, C), the 37 autoantibody-positive patients had statistically significantly better recurrence-free interval compared with the 65 autoantibody-negative patients (HR = 0.39, 95% CI = 0.23 to 0.69; $P < 001$). Kaplan-Meier estimates for the Nordic IFN trial (Figure 2, D) were comparable, showing a statistically significant benefit in recurrence-free interval ($P = .001$) for the 63 autoantibody-positive patients compared with 97 autoantibody-negative patients (HR = 0.48, 95% CI = 0.30 to 0.75).

Using the Cox models, the prognostic importance of the autoantibody status was adjusted for sex and the number of positive lymph nodes because, unlike ulceration, these were independent prognostic factors for recurrence-free interval in this study. The association of sex with recurrence-free interval was more pronounced in the Nordic IFN trial. The results of the Cox model treating antibody status as a time-independent variable (model 1) and adjusting for sex and the number of positive lymph nodes were comparable with those obtained with the log-rank test (autoantibody-positive vs autoantibody-negative patients: HR = 0.41, 95% CI = 0.25 to 0.68 in the EORTC 18952 trial and HR = 0.51, 95% CI = 0.34 to 0.76 in the Nordic IFN trial [Table 2]). These results were similar when only patients in observation or treatment groups were considered.

However, these results were guarantee-time bias driven because patients with a longer recurrence-free interval have a greater chance of developing autoantibodies, and, conversely, early relapses are more likely in autoantibody-negative patients because these patients relapse before autoantibodies develop or can be detected.[15,16] Thus, survival estimates based on Kaplan-Meier curves are biased because they compare all patients who tested positive for autoantibodies to patients who never tested positive, regardless of when seroconversion occurred. To overcome the guarantee-time bias phenomenon, we used two Cox models that treated autoantibody status as a time-dependent variable (Figure 1): a model that used the latest positive autoantibody status and one that used

Table 2. Three Cox proportional hazards regression models showing the association of seroconversion, sex, and number of positive lymph nodes with recurrence-free survival in the EORTC 18952 and Nordic IFN trials*

	Model 1 Any positive autoantibody test (Time-independent Cox model)†		Model 2 Latest positive autoantibody status (Time-dependent Cox model)		Model 3 Latest autoantibody status (Time-dependent Cox model)	
Patient group and variable	**HR (95%CI)**	**P**	**HR (95%CI)**	**P**	**HR (95%CI)**	**P**
EORTC 18952 trial						
All patients						
(N=125, O=86)						
Autoantibody status + vs -	0.41 (0.25-0.68)	< .001	0.81 (0.46-1.40)	.44	1.05 (0.59-1.87)	.88
No. of positive lymph nodes	1.44 (1.13-1.84)	.004	1.37 (1.08-1.74)	.01	1.33 (1.05-1.70)	.02
Sex F vs M	0.90 (0.57-1.41)	.64	0.77 (0.50-1.20)	.25	0.75 (0.48-1.17)	.20
IFN-treated patients						
(N=102, O=67)						
Autoantibody status + vs -	0.37 (0.21-0.66)	< .001	0.81 (0.43-1.51)	.50	0.91 (0.46-1.80)	.78
No. of positive lymph nodes	1.47 (1.11-1.95)	.007	1.40 (1.07-1.84)	.02	1.38 (1.05-1.82)	.02
Sex F vs M	0.82 (0.49-1.38)	.46	0.70 (0.42-1.17)	.18	0.70 (0.42-1.16)	.17
Observed patients						
(N=23, O=19)						
Autoantibody status + vs -	0.89 (0.30-2.65)	.27	1.43 (0.46-4.46)	.54	1.84 (0.60-5.67)	.29
No. of positive lymph nodes	1.49 (0.82-2.73)	.10	1.44 (0.77-2.66)	.25	1.41 (0.76-2.63)	.28
Sex F vs M	0.87 (0.29-2.68)	.81	0.81 (0.27-2.43)	.71	0.84 (0.28-2.52)	.75
Nordic IFN trial						
All patients						
(N=230, O=132)						
Autoantibody status + vs -	0.51 (0.34-0.76)	< .001	0.85 (0.55-1.30)	.45	0.78 (0.49-1.24)	.30
No. of positive lymph nodes	1.25 (1.04-1.50)	.02	1.23 (1.02-1.48)	.03	1.23 (1.02-1.48)	.03
Sex F vs M	0.68 (0.46-1.00)	.05	0.66 (0.45-0.98)	.04	0.66 (0.45-0.97)	.03
IFN-treated patients						
(N=160, O=91)						
Autoantibody status + vs -	0.49 (0.31-0.76)	.002	0.75 (0.46-1.22)	.24	0.66 (0.39-1.11)	.11
No. of positive lymph nodes	1.21 (0.97-1.51)	.09	1.21 (0.97-1.51)	.10	1.20 (0.96-1.50)	.11
Sex F vs M	0.69 (0.43-1.11)	.12	0.66 (0.41-1.06)	.09	0.65 (0.41-1.05)	.07
Observed patients						
(N=70, O=41)						
Autoantibody status + vs -	0.58 (0.23-1.51)	.27	0.99 (0.35-2.83)	.99	1.23 (0.43-3.50)	.70
No. of positive lymph nodes	1.31 (0.95-1.82)	.10	1.28 (0.92-1.77)	.14	1.27 (0.92-1.76)	.15
Sex F vs M	0.65 (0.33-1.27)	.20	0.66 (0.33-1.29)	.22	0.66 (0.34-1.30)	.23

* EORTC = European Organization for Research and Treatment of Cancer; IFN = interferon; HR = hazard ratio; CI = confidence interval; n = number of patients; *o* = observed number of relapses; F = female; M = male; Autoantibody status + = positive for the presence of anticardiolipin, antithyroglobulin, or antinuclear antibodies.

†Results provided by this model are subject to guarantee-time bias (Figure 1).

latest autoantibody status regardless of whether it was positive or negative. When time to seroconversion was taken into account, using model 2, in which the latest positive autoantibody status was entered into the model, all hazard ratios for recurrence-free interval increased compared with model 1, and no statistically significant differences were seen between autoantibody-positive and autoantibody-negative patients. In this model as well, the number of positive lymph nodes was associated with recurrence-free interval. Model 3, which uses the latest autoantibody status, is the most appropriate one to correct for guarantee-time bias. According to this model, autoantibody-positive patients and autoantibody-negative patients did not differ by a statistically significant extent in risk of recurrence. The association of the number of positive lymph nodes and sex with recurrence-free interval remained unchanged between the different Cox models, which is consistent with the idea that the subgroups analyzed are representative and similar.

Landmark analyses for IFN-treated patients at 6 and 12 months confirmed the findings from the models that treated autoantibody status as a time-dependent variable. In the Nordic IFN trial, 39 of 72 autoantibody-negative patients had a relapse at 6 months compared with 11 of the 29 autoantibody-positive patients (HR = 0.59, $P = .12$). At 12 months, results were similar; 26 of 55 autoantibody-negative vs 10 of 28 autoantibody-positive patients relapsed (HR = 0.65, $P = .24$). These analyses lacked statistical power, because in a considerable number of patients autoantibodies were not detected before 12 months at which time some patients had relapsed. In the EORTC 18952 trial, the numbers of patients were even lower: 21 of 36 autoantibody-negative patients had a relapse compared with seven of the 15 autoantibody-positive patients at 6 months (HR = 0.79, $P = .59$). At 12 months, results were similar: nine of 24 autoantibody-negative patients relapsed vs three of seven autoantibody-positive patients (HR = 1.18, $P = .81$).

To explore the effects of using higher cutoff values to dichotomize autoantibody status, an analysis considering patients with a moderate test result for antibodies as negative was performed. Using the higher cutoff values, only 11 of 187 (6%) and 31 of 356 (9%) patients were initially strongly positive in the EORTC 18952 and Nordic IFN trials, respectively. After their exclusion, there remained 176 patients in EORTC 18952 and 325 Nordic IFN patients. Among these patients, 145 and 227 patients received IFN treatment in EORTC 18952 and Nordic IFN, respectively. Although in the EORTC 18952 study, in the 18 (12%) IFN-treated patients with strong seroconversion, there was a trend toward better recurrence-free interval of marginal statistical significance using models 2 and 3 (model 2: HR = 0.46, $P = .07$; model 3: HR = 0.38, $P = .06$), this trend was not observed in the Nordic IFN trial, in which 42 (18%) strongly positive patients did not have statistically significantly improved recurrence-free interval (model 2: HR = 0.79, $P = .39$; model 3: HR = 0.83, $P = .53$; data not shown).

DISCUSSION

Autoimmune conditions including thyroiditis and vitiligo induced by interleukin 2 and/or IFN therapy have been associated with an improved prognosis in melanoma patients.[4,5,17-19] In this study, the appearance of autoantibodies was determined in IFN-treated patients, receiving intermediate doses of interferon-α2b, as well as in untreated patients (observation arms of both trials). Autoantibodies were detected in both treated and untreated patients, but the frequency of autoantibody occurrence increased by 10 and 25 percentage points in the EORTC 18952 and Nordic IFN trials, respectively. The occurrence of autoantibodies in patients who were initially autoantibody negative was associated with a better outcome if a Cox model in which antibody status was time independent was used. However, using the models that treated antibody status as a time-dependent variable and thus corrected for guarantee-time bias, there was no strong association between seroconversion and recurrence-free interval. However, the most important prognostic factor in stage III melanoma patients, the number of positive lymph nodes,[20] was an independent prognostic factor in all models. Furthermore, the results pertaining to the association of seroconversion with recurrence-free interval were similar when only patients in the observation arm or treated patients were considered for analysis, indicating that the appearance of autoantibodies in melanoma patients is neither a prognostic nor a predictive serological marker for treatment outcome.

Our results are not in accordance with the study by Gogas et al.,[4] which demonstrated a statistically significant improvement on both recurrence-free interval (HR = 0.12) and OS for patients with evidence of autoimmunity when correction for guarantee-time bias was applied. Furthermore, we observed that 33% of the patients in the EORTC 18952 study and 35% of those in the Nordic IFN trial had pre-existing autoantibodies, which is in agreement with autoantibody prevalence reported in literature. Antinuclear antibodies are commonly detected in healthy persons, with reported prevalences ranging 4 to 35%,[6-9] but prevalences up to 40% in cancer patients have been reported.[10] Furthermore, the reported prevalence of anticardiolipin and antithyroid antibodies in healthy control subjects ranges between 1 and 15%.[6,21-26] Therefore, an autoantibody prevalence (defined by the presence of one of the three assayed antibodies) of 33 and 35%, reported for our studies at baseline, lies within the normal range. In contrast, the prevalence of 1.5% reported by Gogas et al.[4] is extraordinarily low. The difference between their study and our results cannot be explained by methodological differences because we used the same (validated) assays and titers described by Gogas et al. Moreover, our exploration of higher cutoff values did not change the overall results.

One could argue that patients with pre-existing autoantibodies should not be excluded because this reflects normal variability. We checked the association of pre-existing autoantibodies with recurrence-free interval, and in both trials, no differences

in recurrence-free interval were observed between initially autoantibody-positive and autoantibody-negative patients (Supplementary Figure, Appendix). Thus, autoantibody status before treatment is not a relevant factor in predicting recurrence-free interval.

An additional difference between our study and that of Gogas et al. was that we detected a higher seroconversion rate (36% and 39% in EORTC 18952 and Nordic IFN trials, respectively, compared with 26% in their study). Treatment duration may explain some of the difference because in both trials reported here more seroconversions were observed in patients treated with IFN for 25 months than in those treated for 13 months, and the overall incidence of autoantibodies or autoimmune disorders in the study by Gogas et al. was 28% for the extended treatment group (1-year IFN) vs 24% in the induction group (4-week IFN). The median time to seroconversion was only 3 months in the study by Gogas et al. compared with 6-12 months in the trials reported here. A possible explanation for this difference could be the fact that the induction treatment differed: it consisted of 15 MU IFN delivered intravenously (5 days per week for 4 weeks) in the study by Gogas et al. compared with 10 MU IFN delivered subcutaneously (5 days per week for 4 weeks) in the EORTC 18952 and Nordic IFN trials.

The current study focused on the presence of autoantibodies as an indicator of autoimmune response in contrast to some other studies that also evaluated clinical manifestations of autoimmunity during IFN treatment. Autoimmune diseases are often preceded and/or accompanied by the occurrence of autoantibodies. From the 52 (26%) of the 200 IFN-treated patients with signs of autoimmunity in the study by Gogas et al., only three patients (2%) had clinical manifestations of autoimmunity (vitiligo) without autoantibodies. Therefore, using autoantibodies as an index of immune response seems reasonable.

Our study had some limitations. The EORTC and Nordic trials were originally not designed to determine whether serial serum autoantibody levels are prognostic and predictive markers. It turned out that the number of patients and events reported were sufficient to assess the prognostic importance in the IFN-treated patients. However in the observation groups, especially in the EORTC 18952 trial, the series was quite limited (23 patients), affecting the assessment of the prognostic value of autoantibody levels in this subgroup and, therefore, of its predictive value (differential effect in IFN and observation groups). The group of patients with prolonged recurrence-free interval is relatively small, suggesting that the treatment, if active at all, is only of benefit in a minor patient population. Identification of a potential biomarker is therefore only likely if this marker has a strong relationship with treatment benefit.

The aim of this study was to assess the predictive value of autoantibody determination as a useful tool in selecting patients benefiting from IFN treatment. The results of two similar randomized trials reported here do not suggest that the presence or appearance of autoantibodies is a strong prognostic factor in melanoma patients. Serocon-

version was more frequently observed in patients receiving treatment, yet the time to seroconversion varied greatly. The findings indicate that the assessment of autoimmune antibodies is not a useful tool in selecting patients who would benefit from treatment with intermediate doses of IFN-α2b.

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Chapter V

Phase III Trial Comparing Adjuvant Treatment With Pegylated Interferon Alfa-2b Versus Observation: Prognostic Significance of Autoantibodies–EORTC 18991

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ABSTRACT

Background: Conflicting data have been reported concerning the prognostic value of autoimmune antibodies in patients with melanoma treated with adjuvant interferon alfa-2b (IFN). We evaluated the prognostic significance of autoantibodies in the European Organisation for Research and Treatment of Cancer 18991 trial, comparing long-term administration of pegylated IFN (PEG-IFN) with observation.

Methods: Anticardiolipin, antithyroglobulin, and antinuclear antibodies were determined by enzyme-linked immunosorbent assays in 296 patients before random assignment and every 6 months after random assignment for up to 5 years. Prognostic impact of autoantibodies on recurrence-free survival (RFS) was assessed using the following three Cox models: a model that considered autoantibody appearance as a time-independent variable (model 1); a model that considered a patient to be autoantibody positive from the first positive test (model 2); and a model in which the most recent autoantibody test was used to define the status of the patient (model 3).

Results: Patients who were autoantibody negative at baseline were analyzed (n = 220). Occurrence of autoantibodies during follow-up was higher in the PEG-IFN–treated patients (18% in the observation arm vs 52% in the PEG-IFN arm). Autoantibody appearance was of prognostic importance by using model 1 (hazard ratio [HR] = 0.56, 95% CI = 0.36 to 0.87; $P = .01$. However, when guarantee-time bias was taken into account using model 2 (HR = 1.19, 95% CI = 0.75 to 1.88; $P = .46$ or model 3 (HR = 1.14, 95% CI = 0.71 to 1.83; $P = .59$), significance was lost. Results were similar when treatment groups were analyzed separately.

Conclusions: Appearance of autoimmune antibodies is neither a prognostic nor a predictive factor for improved outcome in patients with melanoma treated with PEG-IFN.

INTRODUCTION

Patients with melanoma with thick primary tumors or lymph node involvement are at high risk of recurrence or death.[1] The effect of adjuvant treatment after definitive surgery in these patients is still disappointing. Numerous randomized trials have evaluated the effect of adjuvant interferon alfa (IFN-α) on prognosis, demonstrating a consistent and significant effect on recurrence-free survival (RFS) but not on overall survival (OS).[2,3] An individual patient data meta-analysis confirmed the significant and consistent effect on RFS and a marginal, but statistically significant, impact of 3% on OS.[4] Seemingly, only a relatively small proportion of patients benefits from IFN therapy. Because of its significant toxicity and costs it would be of great benefit if one could identify a subgroup of patients who respond to IFN.

The appearance of autoantibodies and clinical manifestations of autoimmunity in patients treated with adjuvant high-dose IFN was shown to strongly correlate with improved RFS and OS.[5] Likewise, in patients receiving low-dose IFN, the occurrence of autoimmune thyroid disease was associated with improved RFS.[6] These studies seemed promising but could not explore the predictive value of autoimmunity on treatment response because no observation arm was analyzed. Previously we assessed the impact of autoantibodies on prognosis in patients with melanoma receiving intermediate doses of IFN-α-2b or no treatment in a side study to the European Organisation for Research and Treatment of Cancer (EORTC) 18952 trial[7] and the Nordic IFN trial.[8] The appearance of autoimmune antibodies was neither a strong prognostic nor a strong predictive indicator.[9] Because the occurrence of autoantibodies varied over time, guarantee-time bias affected the results of the regular log-rank test. Two time-dependent Cox models and the landmark method were used to correct for guarantee-time bias.[10]

The aim of this study was to determine the prognostic or predictive significance of autoantibodies in patients randomly assigned to receive pegylated IFN (PEG-IFN) or no treatment (EORTC 18991).[11] This trial demonstrated a significant treatment benefit in stage III/N1 patients for RFS and distant metastasis-free survival, but no benefit in stage III/N2 patients. Pegylation of IFN-α-2b alters the pharmacokinetics of the drug; renal clearance is decreased, thereby prolonging plasma half-life.[12,13] As a result, less frequent subcutaneous injections, compared with unpegylated IFN, can be given. Moreover, administration of PEG-IFN was shown to be safe and improved efficacy in solid tumors.[14] Few cases have been reported demonstrating autoimmune-related toxicities induced by PEG-IFN;[15-19] however, to our knowledge, systematic determination of autoantibodies was not performed.

PATIENTS AND METHODS

Patients

From June 2000 to August 2003, 1,256 patients diagnosed with stage III melanoma, who underwent either completion lymphadenectomy after positive sentinel node procedure (stage III, N1) or therapeutic lymph node dissection for palpable nodes (stage III, N2), were accrued.[11] Patients were randomly assigned in a 1:1 ratio to receive either long-term therapy with PEG-IFN or no adjuvant treatment (observation). Therapy consisted of an induction treatment of weekly PEG-IFN 6.0 μg/kg subcutaneously for 8 weeks followed by weekly PEG-IFN 3.0 μg/kg subcutaneously for up to 5 years.

Patients' serum samples from 18 collaborating centers were collected, and autoantibody levels were determined at the Laboratory for Experimental Surgical Oncology, Erasmus University Medical Center–Daniel den Hoed Cancer Center in Rotterdam, the Netherlands. Blood samples were obtained before treatment and then every 6 months for up to 5 years, occurring at the same time points as for regular follow-up tests. Informed consent from patients for translational research for identification of prognostic and predictive factors in association with the EORTC 18991 trial was obtained.

Serum analysis

As described previously, enzyme-linked immunosorbent assays (ELISAs; Quanta Lite; Inova Diagnostics, San Diego, CA) were used to test sera for anticardiolipin (screen: immunoglobulin [Ig]G, IgA, and IgM), antithyroglobulin (IgG) and antinuclear (IgG) antibodies.[9] According to the manufacturer's protocol, a moderately positive test result was defined as titers of ≥ 1:100 for anticardiolipin and antithyroglobulin antibodies, and as ≥ 1:40 for antinuclear antibodies; corresponding cutoff values defining a strongly positive test result were ≥ 1:200, ≥ 1:170, and ≥ 1:120, respectively. All determinations were carried out in duplicate. A patient was classified as autoantibody positive if (at least) one of three autoantibody tests was positive. Seroconversion is the appearance of autoimmune antibodies during follow-up in patients who were autoantibody negative at baseline.

Assays for anticardiolipin and antinuclear antibodies were validated because these incidences vary greatly in literature as a result of different methods. We selected 40 samples, 20 positive and 20 negative, determined by the Quanta Lite, ELISA kits, for both anticardiolipin and antinuclear antibodies. These two ELISA kits were tested for reproducibility and concordance with the HeP2 cells (gold standard for antinuclear antibodies) and anticardiolipin IgM and IgG assays, routine well-standardized ELISAs from the Department of Immunopathology, Sanquin, Amsterdam, The Netherlands.[20,21]

Statistical analysis

RFS was the time from date of random assignment until the first relapse or death without relapse; the follow-up of patients without relapse was censored at latest visit/last contact. Time to autoantibody positivity was the time from random assignment until the date of positive autoantibody test; the follow-up of patients for whom no autoantibody positivity was recorded was censored at the latest date of assessment of autoantibodies, relapse, or last follow-up. The Kaplan-Meier method was used to estimate the survivor function distributions.[22] Two-tailed log-rank test was used to test differences between survival curves. As described previously,[9] the prognostic value of serial measurements of autoantibody status (positive vs negative) on RFS, adjusted for the initial number of positive lymph nodes, was assessed using three Cox models–a time-independent Cox model and two different time-dependent Cox models. One time-dependent Cox model considers the patient to be autoantibody positive from the moment the autoantibody status became positive, whether it remained positive or returned to a negative status thereafter; thus, the variable is an indicator of the latest positive autoantibody status. The other time-dependent Cox model uses the most recent autoantibody status. A detailed description of the Cox models is provided as a Data Supplement (Appendix).

On the basis of the data (serial measurements of autoantibody status and the outcome of each patient regarding RFS), an estimate of HR, along with its 95% CI, was calculated; the Wald test (standardized coefficient) was used to determine the prognostic value of variables considered in the model. Additionally, the landmark method was used to evaluate the association between autoantibody status based on serum samples available at 6 and 12 months after end of induction and RFS. Since the samples were not all exactly drawn at these time-points, a 60-day time interval window (± 30 days around the theoretical date) was applied.

To determine whether the autoantibody status has a strong impact on the RFS (ie, the corresponding HR = 0.50), 102 events were required to reach a statistical power of approximately 80% (two-sided $\alpha = .05$), considering that over time, approximately 30% of patients would become autoantibody positive and 70% would remain autoantibody negative. Statistical analyses, using SAS 9.1 software (SAS Institute, Cary, NC), were performed at the EORTC Headquarters.

RESULTS

Patients

All available serum samples from the EORTC 18991 trial were collected for this collateral study. Baseline autoantibody levels were determined in 296 patients; of these, 76 patients (26%) in EORTC 18991 were initially positive for at least one of the three autoantibodies.

These patients had similar RFS compared to patients without pre-existing autoantibodies (Figure 1, A). Among the 220 patients who remained after exclusion of patients who were autoantibody positive at baseline, 107 patients were randomly assigned to receive a 5-year period of PEG-IFN treatment, and 113 were assigned to no adjuvant treatment

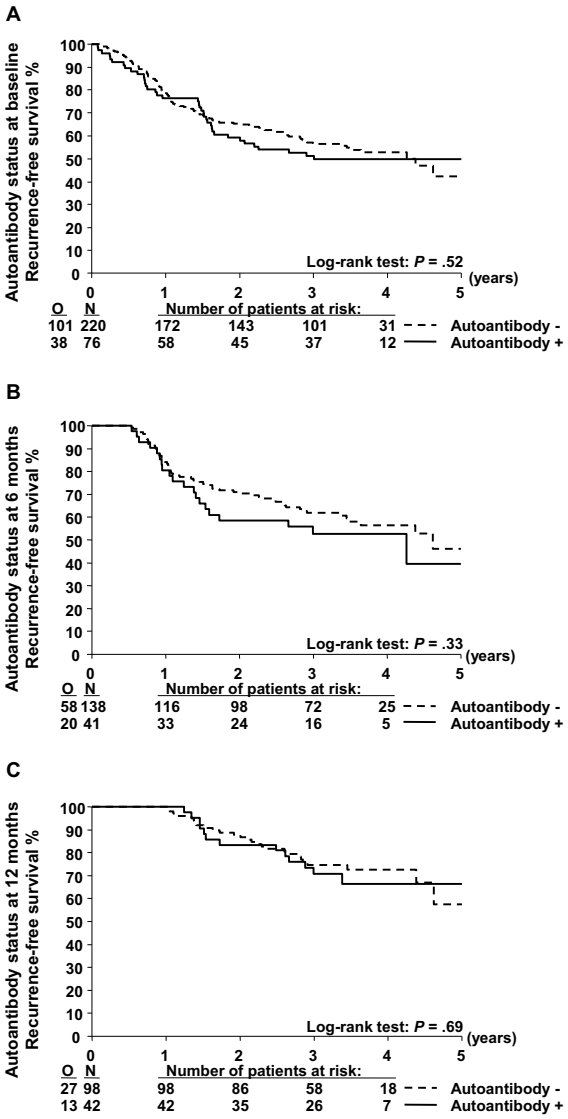

Figure 1.

(A) Kaplan-Meier estimates of recurrence-free survival for patients with or without pre-existing autoantibodies. For all patients who were autoantibody negative at baseline, landmark analyses are shown according to whether they were autoantibody positive or autoantibody negative at **(B)** 6 months or at **(C)** 12 months after random assignment. O, observed events.

(observation). No significant treatment difference on RFS was observed (P = .63); the estimated HR (PEG-IFN vs observation) was 1.10. This could be explained by the fact that there was a greater proportion of stage III/N2 patients and a higher number of positive lymph nodes in the PEG-IFN arm compared with the observation arm. Adjusting the treatment comparison for these variables, the estimated HR was 1.0. Median RFS time for all 220 patients was 4.27 years and the median follow-up time was 3.61 years.

Seroconversion

Seroconversion (the appearance of autoantibodies in initially autoantibody-negative patients) occurred in 76 (35%) of 220 patients. Prevalence of antinuclear antibodies was the highest of the three tested autoantibodies; contributions of the autoantibodies separately are listed in Table 1. Baseline characteristics per treatment arm are listed in Table 2; these were not entirely comparable to the characteristics of the total trial population because, in this side study, relatively more stage III/N1 patients were analyzed (58% in this study vs 43% in the total population).

Overall, there were no differences in the crude rate of appearance of autoantibodies over time according to age, Breslow thickness, ulceration, stage at random assignment, or number of positive lymph nodes. However, differences were seen for treatment and sex. Fifty-six (52%) of 107 patients in the PEG-IFN arm compared with 20 (18%) of 113

Table 1. Autoantibody conversion per treatment arm

Characteristic	Observation N=113	PEG-IFN N=107
	N (%)*	N (%)*
Anticardiolipin antibodies		
Negative	112 (99)	102 (95)
Positive	1 (1)	5 (5)
Antithyroglobulin antibodies		
Negative	109 (97)	88 (82)
Positive	4 (4)	19 (18)
Antinuclear antibodies		
Negative	97 (86)	59 (55)
Positive	16 (14)	48 (45)
Autoantibodies combined		
Negative	93 (82)	51 (48)
Positive	20 (18)	56 (52)

NOTE. Patients with an initial autoantibody-negative status only were included; the autoantibody status in patients before relapse was considered.
Abbreviation: PEG-IFN, pegylated interferon
* Because of rounding, not all percentages total 100%.

Table 2. Baseline characteristics according to autoantibody-status per treatment arm

Characteristic	Observation N=113		PEG-IFN N=107	
	†Autoantibody - N=93	†Autoantibody + N=20	†Autoantibody - N=51	†Autoantibody + N=56
	N (%)*	N (%)*	N (%)*	N (%)*
Age (yr)				
<51	57 (61)	13 (65)	29 (57)	35 (63)
51-65	34 (37)	7 (35)	15 (29)	17 (30)
≥65	2 (2)	0 (0)	7 (14)	4 (7)
Sex				
Male	53 (57)	9 (45)	36 (71)	24 (43)
Female	40 (43)	11 (55)	15 (29)	32 (57)
Breslow thickness (mm)				
<1.5	18 (19)	2 (10)	12 (24)	12 (21)
1.5-3.99	47 (51)	13 (65)	23 (45)	30 (54)
≥4.00	19 (20)	4 (20)	7 (14)	8 (14)
Unknown	9 (10)	1 (5)	9 (18)	6 (11)
Ulceration				
Absent	57 (61)	11 (55)	32 (63)	33 (59)
Present	25 (27)	6 (30)	9 (18)	14 (25)
Unknown	11 (12)	3 (15)	10 (20)	9 (16)
Stage at randomization				
III-N_1 (microscopic)	59 (63)	12 (60)	24 (47)	33 (59)
III-N_2 (palpable)	34 (37)	8 (40)	27 (53)	23 (41)
No. of positive lymph nodes				
1	65 (70)	12 (60)	27 (53)	32 (57)
2-4	20 (22)	6 (30)	15 (29)	19 (34)
5+	7 (8)	2 (10)	9 (18)	5 (9)
Unknown	1 (1)	0 (0)	0 (0)	0 (0)

Abbreviation: PEG-IFN, pegylated interferon

* Patients were autoantibody negative at baseline and either remained negative (autoantibody negative) or tested positive for the presence of anticardiolipin, antithyroglobulin, or antinuclear antibodies during the course of the study (autoantibody positive).

† Because of rounding, not all percentages total 100%.

patients in the observation arm developed autoantibodies. The rate of seroconversion over time was also significantly higher ($P < .001$) in the PEG-IFN group compared with the observation group (HR = 2.05, 95% CI = 1.47 to 2.85; Figure 2, A). Thirty-three (27%) of 122 men versus 43 (44%) of 98 women tested positive for autoantibodies. Women

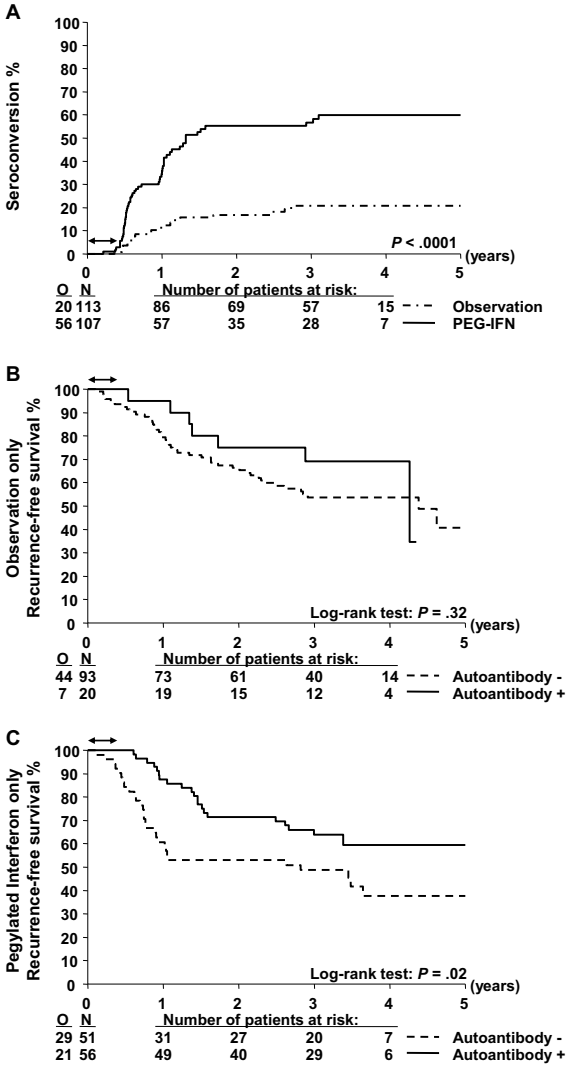

Figure 2.

(A) Time to seroconversion for untreated patients versus patients treated with pegylated interferon (PEG-IFN). Kaplan-Meier estimates of recurrence-free survival comparing patients who became autoantibody positive with patients who remained autoantibody negative, are shown for **(B)** patients in the observation group and **(C)** patients in the PEG-IFN group. Both analyses are subject to guarantee-time bias (see Results); this translates, for instance, into an initial plateau of the autoantibody-positive curve, which is indicated by the arrows. O, observed events.

showed a significantly (P = .005) higher seroconversion rate over time than men (HR = 1.89, 95% CI = 1.20 to 2.98). We observed a higher seroconversion rate in treated patients, separately in males and females, showing that treatment impact was independent of patient sex.

Autoantibodies were measured for a maximum of 5 years, and additionally, a wide spread in time to seroconversion could be observed. The latest time point at which seroconversions initially were detected was approximately 3.5 years. Nevertheless, the majority of seroconversions occurred within 1.5 year (Figure 2, A).

Prognostic impact of autoantibodies on RFS

Biased analysis

Kaplan-Meier estimates of RFS comparing autoantibody-positive with autoantibody-negative patients are depicted for observation patients in Figure 2, B and for patients treated with PEG-IFN in Figure 2, C. In both treatment groups, autoantibody-positive patients had a better RFS compared with autoantibody-negative patients. In the observation group, 20 patients who became autoantibody positive had a lower relapse rate (P = .32) than 93 autoantibody-negative patients (HR = 0.67, 95% CI = 0.30 to 1.48; Figure 2, B). Patients treated with PEG-IFN who became autoantibody positive (n = 56) had significantly better RFS (P = .02) compared with patients who remained autoantibody negative (n = 51; HR = 0.51, 95% CI = 0.29 to 0.90; Figure 2, C).

Using the Cox models, the prognostic significance of the autoantibody status was adjusted for the number of positive lymph nodes, but this information was lacking for one patient, resulting in the analysis of 219 patients. Kaplan-Meier estimates of RFS comparing autoantibody-positive with autoantibody-negative patients were similar to the Cox model, adjusted by the number of positive lymph nodes, which considers seroconversion to be a time-independent covariate (HR = 0.56, 95% CI = 0.36 to 0.87; Table 3).

Unbiased analysis

Guarantee-time bias drove the results mentioned in the previous section.[10] Because patients with longer survival have a higher change for developing autoantibodies, they benefit the autoantibody-positive group. Additionally, early relapses are more likely in autoantibody-negative patients because these patients experience relapse before autoantibodies develop or can be detected, thus disfavoring the autoantibody-negative group. For instance, relapses within the first 6 months occurred in the autoantibody-negative group because the first opportunity to test positive for one of the autoantibodies was at 6 months (Figure 2, indicated by the arrows). Kaplan-Meier estimates are biased since they compare patients who tested positive for autoantibodies with patients who never tested positive, regardless of when seroconversion occurred. To overcome guarantee-time bias, we used two different Cox models that treated the occurrence of autoantibodies as a time-dependent covariate (Data Supplement, Appendix).

Table 3. Prognostic impact of seroconversion on RFS

	Any positive autoantibody test (Time-independent Cox model) †		Latest positive autoantibody status (Time-dependent Cox model)		Latest autoantibody status (Time-dependent Cox model)	
	HR (95%CI)	p-value	HR (95%CI)	p-value	HR (95%CI)	p-value
All patients						
(N=219, O=100)						
Autoantibody status + vs -	0.56 (0.36-0.87)	.01	1.19 (0.75-1.88)	.46	1.14 (0.71-1.83)	.59
No. of positive lymph nodes	1.95 (1.50-2.55)	< .0001	1.86 (1.43-2.42)	< .0001	1.86 (1.43-2.42)	< .0001
PEG-IFN treated patients						
(N=107, 0=50)						
Autoantibody status + vs -	0.52 (0.29-0.91)	.02	1.34 (0.73-2.47)	.34	1.40 (0.76-2.56)	.28
No. of positive lymph nodes	1.66 (1.15-2.39)	.007	1.69 (1.17-2.44)	.005	1.69 (1.17-2.44)	.005
Observed patients						
(N=112, O=50)						
Autoantibody status + vs -	0.54 (0.24-1.22)	.14	1.13 (0.49-2.59)	.77	0.83 (0.29-2.37)	.73
No. of positive lymph nodes	2.13 (1.43-3.16)	.0002	1.98 (1.35-2.91)	.0005	2.02 (1.36-2.98)	.0004

Abbreviations: HR, hazard ratio; PEG-IFN, pegylated interferon. (N = number of patients, O = number of observed events)

* Patients considered autoantibody positive are patients who tested positive for anticardiolipin, antithyroglobulin, or antinuclear antibodies.

† Results provided by this model are subject to guarantee-time bias.

Using two time-dependent Cox models, autoantibody-positive and autoantibody-negative patients did not significantly differ regarding RFS (Table 3). Importantly, the number of positive lymph nodes remained an independent prognostic factor. Landmark analyses for all patients confirmed the results of the time-dependent Cox models, showing no benefit on RFS for patients with an autoantibody-positive status at 6 or 12 months (Figures 1, B and 1, C).

Exploring the impact of higher cutoff values we performed an additional analysis in which moderately positive test was regarded as negative. At baseline, 19 (6%) of 296 patients were strongly positive and were excluded for further analysis. Thus, 277 patients were analyzed; 142 patients received PEG-IFN treatment, and 135 patients received no treatment. Strong seroconversion occurred in 54 of 142 treated patients and in six of 135 untreated patients. Using higher cutoff values, prognostic value on RFS was determined according to the three Cox models, and results are provided in the Data Supplement (Appendix). Strong seroconversion in patients treated with PEG-IFN was an adverse independent prognostic factor (model 2: HR = 1.82, 95% CI = 1.00 to 3.28; $P = .05$; and model 3: HR = 1.94, 95% CI = 1.08 to 3.48; $P = .03$).

Predictive significance of autoantibodies on RFS

We evaluated the predictive value of autoantibodies, and assessed its impact on outcome in both treatment groups separately. Using the time-dependent Cox models, seroconversion in PEG-IFN–treated patients was not associated with improved RFS (model 2: HR = 1.34, 95% CI = 0.73 to 2.47; and model 3: HR = 1.40, 95% CI = 0.76 to 2.56). Similar results were found for untreated patients (model 2: HR = 1.13, 95% CI = 0.49 to 2.59; and model 3: HR = 0.83, 95% CI = 0.29 to 2.37; Table 3).

DISCUSSION

The prognostic value of autoimmune antibodies in melanoma patients treated with adjuvant IFN is still subject for debate. A strong correlation with improved RFS and OS was shown in patients treated with high-dose IFN,[5] which could not be confirmed by our previous study in patients receiving intermediate doses of IFN or no treatment.[9] Treatment with PEG-IFN-α for chronic hepatitis C was associated with autoimmune-related toxicities such as type 1 diabetes mellitus, autoimmune thyroiditis, celiac disease, and systemic lupus erythematosus.[15-19] Nevertheless, systematic measurements of autoimmune antibodies were not carried out in patients receiving PEG-IFN.

We determined anticardiolipin, antithyroid and antinuclear antibodies in patients with melanoma treated with PEG-IFN or no treatment. Autoantibodies were more frequently detected in patients receiving treatment (52% in the PEG-IFN group vs 18% in the observation group). Seroconversion was associated with improved outcome according to the (biased) Cox model that considers the occurrence of autoantibodies as a time-independent covariate. However, once corrected for guarantee-time bias using the time-dependent Cox models, the occurrence of autoantibodies lost significance. However, the number of positive lymph nodes, which is a strong prognostic factor in patients with stage III melanoma,[1] remained an independent prognostic factor throughout all analyses. When analyzing the effect of seroconversion on outcome in observation patients or PEG-IFN–treated patients separately, similar results were found, indicating, although no significance test was used, that seroconversion is also not a predictive serologic marker for treatment outcome.

The same ELISAs and statistical models as in our previous study were used which makes direct comparisons possible. In this study, pre-existing autoantibodies were detected in 26% of the patients, which is slightly lower but similar to the rates of 33% and 35% reported for the EORTC 18952 and Nordic IFN trials, respectively. However, no influence of pre-existing autoantibodies on prognosis was observed. These results are not in accordance with the study by Gogas et al.,[5] which reported a prevalence of 1.5% at baseline. This difference cannot be explained by methodological differences because

we used the same assays and the three tested autoantibodies were also the three most frequently observed autoantibodies by Gogas et al.[5] A prevalence of 1.5% seems extraordinarily low because autoantibodies are also commonly detected in healthy persons. Antinuclear antibody prevalence, for instance, ranges from 4% to 35%,[23-26] whereas in patients with cancer, frequencies up to 40% have been reported.[27] In this study and our previous one, prevalence for antinuclear antibodies was high in both treated and untreated patients. Twenty-six percent of the patients treated with intermediate-dose IFN and 46% of the PEG-IFN treated patients tested positive for antinuclear antibodies. The total rate of seroconversion was also higher in PEG-IFN–treated patients (52%) compared with IFN-treated patients in EORTC 18952 (36%), the Nordic IFN study (39%),[9] and the study by Gogas et al. (24%).[5] This difference could partially be explained by the fact that in the current study, autoantibodies were determined for up to 5 years, compared with 3 years in the EORTC and Nordic studies and 1 year in the study by Gogas et al.,[5] although seroconversions mostly occurred between 6 and 18 months. Possibly a relation exists between pegylation of IFN-α and an immunogenic response resulting in the formation of autoantibodies. Interestingly, the median time to seroconversion was only 3 months in the study by Gogas et al.,[5] which might be explained by the fact that the induction treatment was delivered intravenously, as opposed to subcutaneous administration in this trial.

From the 1,256 patients accrued to the EORTC 18991 trial,[11] serum samples were collected from 296 patients. This represented all available patients' sera from the trial and was not a selected group. However, the median RFS of 4.27 years in this side study is better compared with the RFS of 2.51 years in the total patient population. A relatively greater proportion of N1 versus N2 patients in the collateral study mainly caused this difference (58% vs 42%, respectively); in the total population, the distribution was 43% vs 57%, respectively. The size and difference in outcome for this cohort of patients are limitations of this study. Although the number of events (n=100) was sufficient for robust statistical analysis, it is still possible that we have missed a rather substantial effect as a result of a relatively small sample size.

We assessed the prognostic value of autoimmune antibodies in 839 patients with melanoma from three randomized controlled trials comparing PEG-IFN or IFN treatment with no treatment. None of the studies showed an RFS benefit for patients who developed autoantibodies, whether treated with PEG-IFN/IFN or untreated. Moreover exploring higher cutoff values, the occurrence of autoantibodies was even a detrimental prognostic factor in PEG-IFN–treated patients. However, the mechanism behind these results remains unclear. In addition, the prognostic significance of strong seroconversion in untreated patients is still unclear because this involved only six patients. In conclusion, the results reported here suggest that determination of autoantibodies is not useful in selecting patients who will benefit from treatment with PEG-IFN.

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SECTION THREE

OTHER PROGNOSTIC FACTORS

Chapter VI

Prognostic Value of Serial Blood S100B Determinations in Stage IIB - III Melanoma Patients: a Corollary Study to EORTC Trial 18952

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ABSTRACT

S100B is a prognostic factor for melanoma as elevated levels correlate with disease progression and poor outcome. We determined its prognostic value based on updated information using serial determinations in stage IIB-III melanoma patients.

211 patients who participated in the EORTC 18952 trial, evaluating efficacy of adjuvant intermediate doses of interferon α2b (IFN) versus observation, entered a corollary study. Over a period of 36 months, 918 serum samples were collected. The Cox time-dependent model was used to assess prognostic value of the latest (most recent) S100B determination.

At first measurement, 178 patients had S100B values < 0.2 µg/l and 33 ≥ 0.2 µg/l. Within the first group, 61 patients had, later on, an increased value of S100B (≥ 0.2 µg/l). An initial increased value of S100B, or during follow-up, was associated with worse DMFS; hazard ratio (HR) of S100B ≥ 0.2 versus S100B < 0.2 was 5.57 (95% CI = 3.81 to 8.16), $P < .0001$, after adjustment for stage, number of lymph nodes and sex. In stage IIB patients, the HR adjusted for sex was 2.14 (95% CI = 0.71 to 6.42), whereas in stage III, the HR adjusted for stage, number of lymph nodes and sex was 6.76 (95% CI = 4.50 to 10.16). Similar results were observed regarding overall survival (OS).

Serial determination of S100B in stage IIB-III melanoma is a strong independent prognostic marker, even stronger compared to stage and number of positive lymph nodes. The prognostic impact of S100B ≥ 0.2 µg/l is more pronounced in stage III disease compared with stage IIB.

INTRODUCTION

The S100B protein is an immunohistological marker for malignant melanocytes[1] that was first detected in melanoma cell cultures[2] and is overexpressed in most cells of neuroectodermal origin.[3] S100B is located in the cytoplasm and in the nucleus as a disulfide cross-linked homo- or heterodimers containing primarily Ca^{2+}.[4] It has many cell functions, mainly in cytoskeleton integrity, in cell cycle regulation[5] and apoptosis through its interaction with p53.[6] However, the mechanism by which the protein leaks to the blood is not fully understood but most probably related to cell damage or cell death.[7] This is strongly supported by the fact that immunoreactive S100B is found exclusively in the cytoplasm and nucleus; a limited secretion of the protein has only been reported in the brain and, its substantial release has been found in brain damage and stage IV melanoma.[8,9] Additionally, serum S100B monitoring has been found useful in the latter disease.

While many reports show that S100B blood levels provide a good indication of disease progression as well of response to chemotherapy in stage IV patients,[10-12] it remains of little or limited usefulness in early stages (II & III) where the disease is most often confined to lymph nodes.[13] Guo and colleagues[14] assessed S100B serum levels in 126 melanoma patients and found positive levels in 1.3%, 8.7%, and 73.9% of patients with stage I/II, III, and IV disease, respectively. This illustrates its poor utility in stages I-II-III. However, the progression from the early stages to distant organ involvement is frequently accompanied by a significant increase in S100B levels.[15]

The utility of serial determinations of S100B serum levels in patients with high-risk melanomas (stages IIB-III), using a time-dependent Cox model, has not been reported on until now. As early elevations in S100B levels may precede clinically detectable relapses serial determinations may have a particular prognostic value in the setting of adjuvant systemic therapy. This study was designed to determine the prognostic value of serial serum S100B levels in stage IIB-III melanoma. Patients were enrolled in the randomized phase III EORTC 18952 trial evaluating the efficacy of adjuvant intermediate doses of interferon-α2b.[16] Serum S100B levels were measured during treatment and follow-up, to a maximum of 36 months.

MATERIAL AND METHODS

Patients and sample collection

Patients aged 18 - 75 years with melanoma either stage IIB or stage III (N1, patients with microscopically involved lymph nodes on sentinel node biopsy, or N2, those with palpable tumor-involved nodes) were eligible to be included in the EORTC 18952

study. Patients were randomized between 13-month interferon (IFN), 25-month IFN or observation in a 2:2:1 fashion. IFN treatment comprised a 4-week induction period of 10 million units (MU) s.c. 5 days/week, followed either by 10 MU x 3/week for 1 year or 5 MU x 3/week for 2 years. Clinical/radiological evaluations have been scheduled every 3 months in year 1, every 4 months in year 2, every 6 months in years 3-5, and annually thereafter. In eight European institutions that participated in the EORTC 18952 trial, 211 patients entered this corollary study. Over a 36-month period 918 serum samples were collected (Table 1). According to the study protocol, when patients relapsed they went off study, treatment was discontinued and sampling was also stopped. Occasionally, some samples were collected after relapse. Pretreatment S100B levels were not determined since these are often false positive due to recent surgery.[7] Serum was separated, aliquoted and frozen at -18°C until assayed.

Table 1. Number of patients analyzed at the different time points.

Month	1*	3	6	9	12	16	20	24	30	36
No. of patients	211	113	134	107	104	84	61	49	44	11

* End of induction / observation

S100B determination

S100B was measured in serum samples by an immunoluminometric assay LIA-mat (Sangtec Medical, Sweden), following the manufacturer's instructions. We have already estimated a threshold for pathological S100B levels to be of 0.2 µg/l also based on in vivo non-specific liberation by normal cells mainly endothelial cells.[7]

Statistical analysis

Distant metastasis-free interval (DMFI) was the time interval between the date of randomization until the first appearance of distant metastases; the follow-up of patients who did not develop distant metastases has been censored at the latest visit/last contact. In this S100B study, as no patient died without having developed distant metastases, DMFI was identical to the distant metastasis-free survival (DMFS). Overall survival (OS) was computed from the date of randomization until the date of death or until the last date of follow-up (censored observations). Time to S100B $\geq$ 0.2 µg/l (the considered pathological cutoff level) was the time from randomization until the first date S100B level was $\geq$ 0.2 µg/l; the follow-up of patients for whom S100B did not reach the 0.2 µg/l level has been censored at the latest date of assessment of S100B.

Kaplan-Meier technique was used to estimate survival-type distributions and the standard errors (SE) of the estimates were obtained via the Greenwood formula.[17] Two-tailed log-rank test and generalized Wilcoxon test, which gives more weight to differences occurring at earlier time points, were used to test differences between curves. The

landmark method was used to test the prognostic impact of S100B level at 6 months for the subsequent outcome. Since sampling was scheduled every 3 months during the first year we used a time window of 45 days not to have overlap. The serial measurements of S100B have been performed at several time points (Table 1) with the same schedule for IFN-treated as untreated patients. Since the samples were not exactly drawn at the time points according to study protocol, numbers in Table 1 reflect the closest time points. As mentioned before, for patients who developed distant metastases, generally their S100B levels have not been assessed subsequently after their relapse, therefore the sampling rate is lower towards the end of the study. To determine whether the latest (most recent) value of S100B, which was assessed during the course of the study, before or at the time of distant metastases, has a prognostic impact on the subsequent outcome, the Cox time-dependent model was used.[18] In the Cox time- dependent model, for patients free of event (distant metastasis – for DMFS - or death – for OS) just before a time point t, the HR was set as; $HR = e^{\beta 1 \times S100B(t) + \beta 2 \times Stage + \beta 3 \times Number\ of\ positive\ lymph\ nodes + \beta 4 \times Sex}$

with S100B(t) = 0 if the latest S100B determined before or at time t was < 0.2 µg/l; or = 1, if the latest S100B value determined before or at time t was ≥ 0.2 µg/l. Thus, for purposes of risk assessment the most recent value of S100B at time t (determined at that time or earlier if not available at time t) was used. In the Cox time-dependent model, all available samples/determinations were used, regardless of a time window.

Based on the data (serial measurements of S100B and the outcome of each patient), an estimate of HR along with its 95% confidence interval (CI) has been calculated; the Wald test (standardized coefficient) was used to determine the prognostic value of variables considered in the model, in univariate or multivariate setting (data coding: see table 2). In stage IIB patients, variables stage and number of positive lymph nodes have not been considered. Analyses were censored at 4 years since thereafter only few distant metastases occurred and the time interval between the latest S100B evaluation and a possible event became too long.

SAS 9.1 software (SAS Institute Inc, Cary, NC, USA) was used to perform the statistical evaluation.

RESULTS

Baseline characteristics and S100B values

The distribution of patients according to the treatment protocol, stage of the disease, number of positive lymph nodes, sex and presence of ulcerated primary melanoma, is summarized in Table 2. These characteristics were comparable with those of patients included in the entire 18952 study, and treatment distribution was in accordance with the randomization scheme: 20% randomized in the observation arm and 40% in each

Table 2. Patient distribution and treatment allocated at randomization according to S100B levels at baseline (randomization/end of induction).

Variable	S100B < 0.2µg/l No. (%)	S100B ≥ 0.2µg/l No. (%)	All patients No. (%)
Treatment			
Observation	30 (16.9)	8 (24.2)	38 (18.0)
13-month IFN	74 (41.6)	13 (39.4)	87 (41.2)
25-month IFN	74 (41.6)	12 (36.4)	86 (40.8)
Stage of the disease			
IIB	38 (21.3)	13 (39.4)	51 (24.2)
III N1	40 (22.5)	6 (18.2)	46 (21.8)
III N2	100 (56.2)	14 (42.4)	114 (54.0)
No. of positive lymph nodes			
0	38 (21.3)	13 (39.4)	51 (24.2)
1	75 (42.1)	5 (15.2)	80 (37.9)
2-4	49 (27.5)	9 (27.3)	58 (27.5)
5+	16 (9.0)	6 (18.2)	22 (10.4)
Sex			
Male	95 (53.4)	16 (48.5)	111 (52.6)
Female	83 (46.6)	17 (51.5)	100 (47.4)
Ulceration status of primary melanoma			
Absent	95 (53.4)	23 (69.7)	118 (55.9)
Present	50 (28.1)	8 (24.2)	58 (27.5)
Unknown	33 (18.5)	2 (6.1)	35 (16.6)

IFN group. At the initial measurement time point, corresponding generally to the end of induction/observation period (median of 28 days from randomization), 178 patients had S100B values below 0.2 µg/l and 33 above or equal 0.2 µg/l. A higher incidence of stage IIB patients has been found in those with an initial S100B ≥ 0.2 µg/l than in those with a S100B < 0.2 µg/l: 39% versus 21%. The number of positive lymph nodes did not correlate with S100B increase.

A total of 116 (55.0%) of 211 patients developed distant metastases within 4 years, with a median DMFS of 2.7 years, and 97 (46,0%) of 211 patients died (median OS was not reached). As in this series IFN did not have an impact on the outcome (for DMFS: HR = 1.15 observation arm versus the 1-year IFN arm, HR = 0.92 observation versus 2-year IFN arm, overall *P* value, .55, and for OS: HR = 1.49 observation arm versus the 1-year IFN arm, HR = 1.19 observation versus 2-year IFN arm, overall *P* value, .34), in the subsequent analyses, patients from the three treatment groups were pooled together.

Prognostic significance of S100B ≥ 0.2 µg/l

Among the 178 patients who initially had a S100B < 0.2 µg/l, 61 had, later on, an increased value of S100B (≥ 0.2 µg/l). Therefore, overall, a total of 94 patients (33 + 61) had a S100B level ≥ 0.2 µg/l. Time between random assignment and S100B level ≥ 0.2 µg/l, according to the disease stage, is shown in Figure 1. Within the first 3 months, the

cumulative rate of patients with S100B level ≥ 0.2 µg/l was higher in stage IIB patients than in stage III patients.

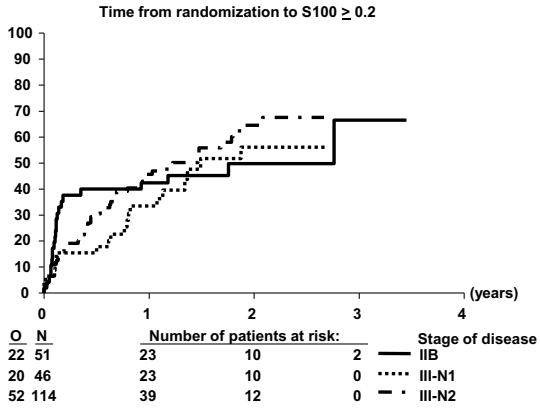

Figure 1.
Time to first S100B level ≥ 0.2 µg/l by initial stage of disease.

A total of 61 patients (65%) from the 94 patients reaching S100B ≥ 0.2 µg/l developed distant metastases, of these, 6 patients (6%) had such a rise after the detection of the metastases. For the remaining 55 patients, median time between S100B ≥ 0.2 µg/l and the development of distant metastases was 94 days (range 0-1580 days). Contrary, from the 117 patients with normal S100B levels, 62 (53%) patients developed distant metastases.

The impact of the initial S100B levels on DMFS and OS was especially seen within one year from randomization, but thereafter the curves converged (Figure 2). According to the Wilcoxon test, a test which gives more weight to differences occurring at earlier time points, there was a significant difference between initial S100B levels and DMFS ($P = .03$). However, when considering the overall curve using the Log-rank test, this difference was not significant ($P = .21$). This temporary effect of elevated S100B levels on prognosis is also illustrated in Figure 3, showing Landmark methods for DMFS and OS at 6 months from randomization; patients with S100B serum level ≥ 0.2 µg/l at 6 months after randomization have, at a short term, a worse prognosis compared to patients with levels < 0.2 µg/l.

In order to evaluate the prognostic value of S100B level ≥ 0.2 µg/l for the different disease stages, we performed an analysis in which we selected the 94 patients with S100B level ≥ 0.2 µg/l, excluding the 6 patients who developed distant metastases before their rise in S100B levels. In these 88 patients, outcome was very different according to the initial stage: stage IIB patients had a high 3-year distant metastasis-free survival (76% at 3 years), whereas stage III-N1 and especially stage III-N2 patients had a

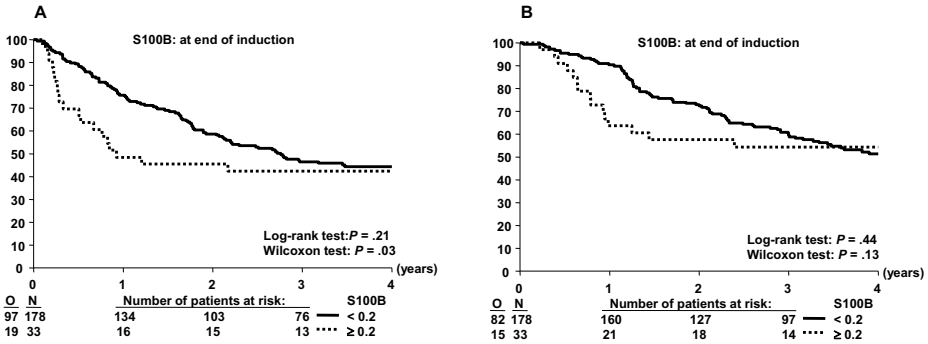

Figure 2.

Kaplan-Meier curves regarding distant metastasis-free survival **(A)** and overall survival **(B)** from randomization according to initial S100B level (< 0.2 vs ≥ 0.2 µg/l). Analyses were censored at 4 years. N=Number of patients at risk. O=Observed number of patients who developed distant metastases **(A)** or who died **(B).**

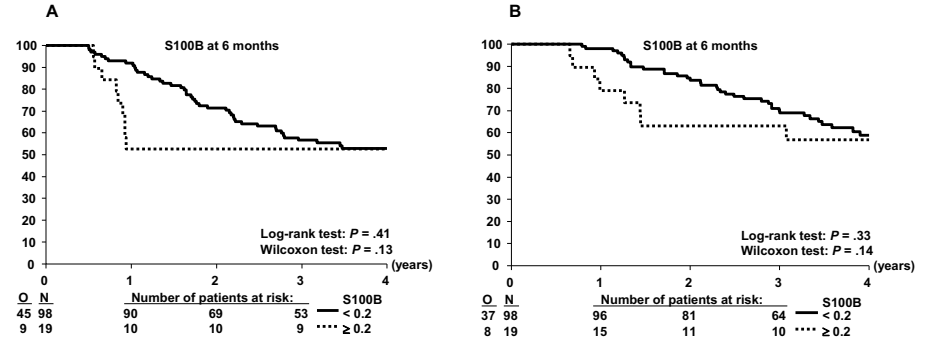

Figure 3.

Landmark method: Kaplan-Meier curves regarding distant metastasis-free survival **(A)** and overall survival **(B)** from randomization according to S100B level (< 0.2 vs ≥ 0.2 µg/l) assessed at 6 months post-randomization. Analyses were censored at 4 years. N=Number of patients at risk. O=Observed number of patients who developed distant metastases **(A)** or who died **(B)**

very poor prognosis: 45% and 23% respectively (Figure 4). This indicates that the effect of increased S100B levels on prognosis is most pronounced in more advanced disease (stage III-N2).

Impact of serial S100B measurements on DMFS and OS

An advantage of serial measurements is the availability of updated information during the course of study. Therefore, a more accurate evaluation of a potential biomarker and its effect on disease outcome is possible. Herein the Cox time-dependent model was used to evaluate the prognostic significance of the latest (most recent) S100B value, in univariate and multivariate setting, as well as according to the initial stage.

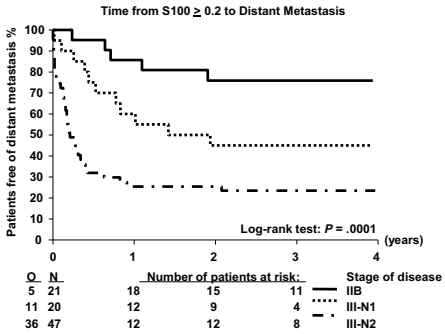

Figure 4.
Kaplan-Meier curves regarding distant metastasis-free survival from the first moment S100B level was ≥ 0.2 µg/l, according to initial stage of disease. Analyses were censored at 4 years. N=Number of patients at risk. O=Observed number of patients who developed distant metastases.

For the entire series of 211 patients, in univariate analysis, the following factors appeared to be of prognostic importance regarding the DMFS: initial stage (stage III-N2 versus stage III-N1 or stage IIB: HR = 3.18, 95% CI = 2.13 to 4.75; $P < .0001$) (Figure 5), number of lymph nodes (0 versus 1 versus 2-4 versus 5+: HR = 1.76, 95% CI = 1.44 to 2.14; $P < .0001$) and sex (male versus female: HR = 1.77, 95% CI = 1.22 to 2.58; $P = .003$).

For DMFS, according to the Cox time-dependent model evaluating all 211 patients, the estimated HR for S100B(t) ≥ 0.2 µg/l versus S100B(t) < 0.2 µg/l comparison was 3.80 (univariate analysis) and, after adjustment for the variables stage, number of lymph nodes and sex, HR was 5.57 (95% CI = 3.81 to 8.16; $P < .0001$, Table 3). Moreover, in the multivariate analysis, HR for disease stage (HR = 2.75) and number of positive lymph nodes (HR = 1.43) were both lower than for S100B (HR = 5.57). In stage IIB patients only,

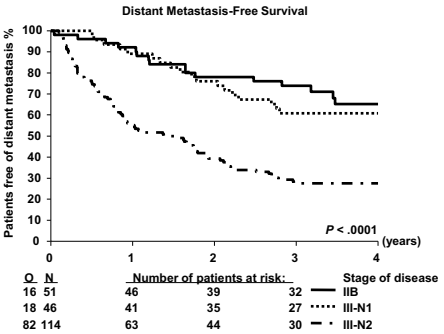

Figure 5.
Kaplan-Meier curves regarding distant metastasis-free survival from randomization according to initial stage of disease. Analyses were censored at 4 years. N=Number of patients at risk. O=Observed number of patients who developed distant metastases.

Table 3. Results of the Cox time-dependent Model

	All patients				Stage IIb				Stage III			
	Hazard Ratio	95% CI		P-value	Hazard Ratio	95% CI		P-value	Hazard Ratio	95% CI		P-value
Distant Metastasis-Free Survival[a]												
Univariate												
S100B[b]	3.80	2.62	5.51	< .0001	1.33	0.46	3.86	.60	6.15	4.11	9.20	< .0001
Multivariate												
S100B[b]	5.57	3.81	8.16	< .0001	2.14	0.71	6.42	.18	6.76	4.50	10.16	< .0001
Stage[c]	2.75	1.68	4.48	< .0001					2.86	1.68	4.88	.0001
Nb of + LN[d]	1.43	1.13	1.83	.004					1.37	1.03	1.81	.03
Sex: Male vs Female	1.94	1.32	2.85	.0007	5.61	1.52	20.68	.01	1.58	1.05	2.37	.03
Overall Survival[a]												
Univariate												
S100B[b]	3.80	2.54	5.67	< .0001	1.36	0.41	4.54	.61	5.48	3.56	8.46	< .0001
Multivariate												
S100B[b]	4.73	3.14	7.12	< .0001	2.73	0.79	9.44	.11	5.46	3.52	8.45	< .0001
Stage[c]	1.92	1.13	3.25	.02					1.87	1.06	3.28	.03
Nb of + LN[d]	1.51	1.17	1.95	.002					1.48	1.10	1.98	.01
Sex: Male vs Female	2.20	1.44	3.35	.0003	15.77	1.93	129.10	.01	1.77	1.14	2.75	.01

a time variable right censored at 4 years
b S100B: 0 = < 0.2, 1 = ≥ 0.2µg/l (Cox time-dependent)
c Stage: 0 = stage IIb (n = 51) or stage III-N1 (n = 46), 1 = stage III-N2 (n = 114)
d Number of positive lymph nodes: 0 = 0, 1 = 1, 2 = 2-4, 3 = 5 or more

the estimated HR for S100B adjusted for sex was 2.14 (95% CI = 0.71 to 6.42; *P* = .18), whereas in stage III patients, the HR for S100B adjusted for stage, number of lymph nodes and sex was 6.76 (95% CI = 4.50 to 10.16; *P* < . 0001, Table 3). Similar results were observed in stage III-N1 and stage III-N2 patients (data not shown).

Regarding OS, the risk of death of those with S100B(t) ≥ 0.2 µg/l was 3.8 higher than the one observed in those with a S100B(t) < 0.2 µg/l. In multivariate analysis, the estimate of the HR was 4.73 (95% CI = 3.14 to 7.12), *P* < .0001); in stage IIB patients the HR was lower than in stage III patients: 2.73 (95% CI = 0.79 to 9.44; *P* = .11) versus 5.46 (95% CI = 3.52 to 8.45; *P* < .0001).

In multivariate analyses, ulceration (presence, absence, unknown) had a weak prognostic impact. Addition of ulceration, however, in the multivariate analyses did not change the prognostic importance of S100B (Supplementary Table, Appendix).

In summary, according to the time-dependent Cox model, which uses the most recent S100B value (whether < 0.2 µg/l or ≥ 0.2 µg/l at that time point), S100B is in both the univariate as well as in the multivariate setting an independent prognostic factor for worse DMFS and OS. This effect is most pronounced in stage III disease, and even stronger than the other important prognostic factors, disease stage and number of positive lymph nodes.

DISCUSSION

We demonstrated in this corollary study to the EORTC 18952 trial in stage IIB-III melanoma patients that serial determinations of S100B serum levels strongly correlate with DMFS and OS. Strikingly, the hazard ratio (HR) for S100B determinations was higher and more significant than the one corresponding to stage, and number of positive lymph nodes, two strong prognostic factors in stage III melanoma.

The use of Cox regression with time-dependent covariates to assess the effect of S100B on the endpoints provides new insights into the prediction value of the marker. Unlike previous studies, several determinations per patient over time could be thus considered and statistically taken into account. However, such an approach focusing on S100B level changes rather than independent single values in time, has been suggested to monitor and predict treatment outcome.[10,11]

Another point to consider was the S100B cutoff level to choose in order to separate "pathological" from "normal" values. Based on previous studies, 0.20 µg/l was chosen also because it allows avoiding possible false positives due to S100B release by damaged vessels after surgery. This is clearly of relevance in this postoperative adjuvant therapy trial, where most patients entered the trial after a full regional lymph node dissection.

The prognostic impact of the baseline S100B on DMFS and OS has been observed, especially within the first year after the assessment (Figure 2). Landmark analysis at 6 months also showed short term (6-12 months) differences between the two groups for both DMFS and OS (Figure 3). These results suggest that a time-window exists within a careful monitoring of S100B can be very helpful to assess the risk of distant metastasis or death. This is further substantiated by the finding that while stage and the number of positive lymph nodes correlated significantly and as expected with DMFS, S100B serial value appeared to be in a Cox time-dependent model not only an independent prognostic factor from these variables but also to have a much higher prognostic importance than these variables. In stage III patients the estimated HR of S100B ≥ 0.20 µg/l versus S100B < 0.20 µg/l, adjusted by these factors and by sex, was very high (6.76, $P < .0001$), but was lower and not significant (2.14, $P = .18$, adjusted by sex only) in stage IIB patients (Table 3). This finding is in accordance with previous studies that were all suggesting

S100B to be a late progression marker but of little value when the disease is still confined to the lymph nodes. This is also consistent with the mechanism of release and the short biological half-life of this marker that is mostly related to a rather substantial cell death.[7] A recent paper by Tarhini et al. reported a weak association of S100B as a time-varying covariate and the risk for mortality.[19] The number of time points in this study was limited: baseline, 4-6 weeks and 12-14 weeks. The low impact of S100B on OS (HR 1.4) can be explained by the long lag-time between the latest evaluation of S100B and the moment a patient is considered in the analysis. Indeed, the S100B has a short-term prognostic value (e.g. Figures 2 and 3), so, when obsolete information on S100B is provided, this leads to a drastic loss in its prognostic value.

Results from the EORTC 18991 trial, evaluating long-term treatment with pegylated IFN-α2b,[20] suggested that treatment with PEG-IFN was more beneficial in stage IIB and stage III-N1 as compared to stage III-N2 patients. Our study of S100B within the framework of the 18952 trial also raises the question whether S100B could identify patients benefiting IFN treatment or not. One could argue that patients receiving IFN should discontinue therapy once S100B levels of $\geq$ 0.2 µg/l have been reached, indicating disease progression and no treatment response. Unfortunately we have no guidance by biomarkers to determine use, dose or duration of adjuvant systemic therapy in melanoma. Gogas and colleagues[21] have reported that patients treated with adjuvant IFN who developed autoantibodies or clinical signs of autoimmunity had a significantly better outcome than patients who did not develop these signs of autoimmunity. The development of markers that might predict who will mount a host immune response could be extremely important. The markers could be used to determine which patients to treat with IFN and for how long. An evaluation of the presence or emergence of autoantibodies in patients who participated in the EORTC 18952 and Nordic IFN trial did not confirm Gogas' observations.[22] Nor did a subsequent similar study in the large EORTC 18991 trial, showing no prognostic or predictive value of autoimmune antibodies in PEG-IFN–treated patients.[23]

In conclusion, time-dependent evaluation of serial blood measurements of S100B showed a very significant prognostic value of S100B, which was even stronger compared to stage and number of positive lymph nodes. Stage III patients with increased S100B levels ($\geq$ 0.2 µg/l) should more frequently be screened for the occurrence of distant metastases.

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Chapter VII

Prognostic and predictive value of serum YKL-40 in stage IIB-III melanoma

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ABSTRACT

Background: To investigate the prognostic and predictive value of elevated serum YKL-40 in high risk melanoma patients stage IIB-III treated with adjuvant interferon-α2b.

Methods: Serum YKL-40 was determined postoperatively in patients with stage IIB-III melanoma included in the Nordic Study (n = 602, baseline serum samples: 452) and EORTC 18952 (n = 246, baseline serum samples: 128), and in EORTC 18991 (n = 386, baseline serum samples: 260).

Results: Univariate analysis showed no association between baseline serum YKL-40 and recurrence-free survival (RFS) in the Nordic Study and EORTC 18952, but in EORTC 18991 baseline YKL-40 was associated with RFS in the patients receiving pegylated interferon (hazard ratio [HR] = 1.6, 95% confidence interval [CI] = 1.1 to 2.5; *P* = .02), but not in the observation group (*P* = .64). Multivariate Cox analysis (including gender, age, stage, ulceration, YKL-40) demonstrated that baseline YKL-40 was associated with overall survival (OS) in the observation group from the Nordic Study and EORTC 18952 (HR = 1.33, 95% CI = 1.01 to 1.74; *P* = .042), but not in the interferon-treated patients (1-year arm: HR = 0.97, 95% CI = 0.76 to 1.25; *P* = .83; 2-year arm: HR = 1.06, 95% CI = 0.83 to 1.34; *P* = .64). In EORTC 18991 multivariate analysis showed no association between baseline YKL-40 and OS. During follow-up increases in YKL-40 were significantly associated with short OS but not with RFS in univariate analysis.

Conclusions: Serum YKL-40 is associated with short OS in untreated high risk melanoma patients stage IIB-III, but not in interferon-treated patients. Increases in YKL-40 during follow-up were related to short OS. This potential role of serum YKL-40 for early detection of disease recurrence has to be validated.

INTRODUCTION

The incidence of melanoma has been rising in the last decade and the estimated number of new cases in 2008 in USA is 62,480 and the number of deaths is 8,420.[1] The prognosis of early-stage melanoma is excellent with 5-year survival of 99%, whereas the prognosis of patients with thick primary melanomas (> 4 mm) or with regional lymph node metastasis remains poor.[2] No clear-cut impact on overall survival has been found using adjuvant interferon after surgery in patients with high-risk melanoma stage II and III, although improved recurrence-free survival is demonstrated.[3-5] No clinical or histological parameters with predictive value of interferon treatment have been identified. It has been suggested that treatment decisions in high-risk melanoma patients should be made on a case-by-case basis.[6] Thus there is a need for new biomarkers for personalized treatment of patients with high-risk melanoma stage IIB or III.

High serum levels of YKL-40 (Chitinase-3-like-1 protein, CHI3L1) has been suggested as a new independent prognostic biomarker of poor survival in patients with melanoma stage I, II and IV[7-9] and in other types of primary and metastatic cancer with highest levels in metastatic disease.[10] High serum YKL-40 predicts low efficacy of anthracycline therapy in patients with first recurrence of breast cancer,[11] and second-line chemoresistance in ovarian cancer patients.[12] Serum YKL-40 may also be useful for monitoring disease progression.[13,14]

YKL-40 is produced by cancer cells, including melanoma cells, and inflammatory cells[15-19] and plays a role in angiogenesis,[20-23] tissue remodelling,[10] fibroblast proliferation,[24] and protects against apoptosis.[25]

Our aim was to test, in 3 different studies, the following hypotheses: 1) postoperative serum YKL-40 is a predictive biomarker of treatment response in patients with high risk melanoma stage IIB and III treated with adjuvant interferon; 2) high serum YKL-40 is a prognostic biomarker of poor survival; and 3) serum YKL-40 has a value in monitoring disease progression during and after treatment.

PATIENTS AND METHODS

Patients

Serum YKL-40 was determined in three prospective, independent studies of patients with high risk melanoma stage IIB–III included in randomised, clinical trials on adjuvant interferon, the Nordic Study,[26] EORTC 18952,[3] and EORTC 18991.[4] YKL-40 was analysed in serum samples collected after definitive surgery, that is, also after surgery for lymph node metastasis or recurrence developed during follow-up, and before treatment with adjuvant interferon or observation, during treatment, and during follow-up. Patients

included in the Nordic Study were similar to those included in EORTC 18952, and were diagnosed with either

- Primary melanoma >4.00 mm thickness without lymph node metastases, or
- Primary melanoma of any thickness with regional lymph node metastases , or
- Recurrence of melanoma in regional lymph nodes during clinical follow-up after surgical intervention.

Patients included in the EORTC 18991 Study had

- Primary melanoma of any thickness with regional lymph node metastases, or
- Recurrence of melanoma in regional lymph nodes during clinical follow-up after surgical intervention.

These patients groups were chosen as they had similar poor prognosis. All patients had ECOG performance status of 0-1, and adequate bone marrow, hepatic, and renal function. Exclusion criteria were melanomas without known primary localisation, non-cutaneous melanoma, evidence of incompletely resected melanoma or distant metastases, other malignancies, heart disease, psychiatric disease or impaired cognitive function, use of corticosteroids, NSAID or other immunomodulatory treatment. As analysis for YKL-40 was not planned in the original protocols comorbidity was not prospectively registered.

The Nordic Study: 855 patients were included between November 1996 and August 2004 from 2 Departments in Denmark, 25 in Sweden, 6 in Norway, and 5 in Finland. Serum was available from 602 of these patients from 2 Departments in Denmark, 14 in Sweden, 2 in Norway, and 1 in Finland. Median follow-up was 92 months (range 46-140 months). Patients were randomised in a 1:1:1 fashion between observation (arm A), treatment with 10 MU interferon-α2b s.c. 5 times per week for 4 weeks, followed by maintenance therapy with 10 MU interferon-α2b s.c. 3 times per week for 1 year (arm B), or maintenance therapy with 10 MU interferon-α2b s.c. 3 times per week for 2 years (arm C). Serum samples were available at baseline up to 70 days after definitive surgery prior to randomization, and after 3, 6, 9, 12, 16, 20, 24, 30, 36, 42, 48, 54 and 60 months, and then once a year for up to 10 years of follow-up. Baseline serum samples were available for YKL-40 determination in 452 patients. A total of 3971 serum samples were collected and the median number of samples from each patient was 6 (range 1-21). The reasons for missing samples were either that the samples were not drawn or lost during storage.

EORTC 18952: 1388 patients were included between May 1996 and June 2000. Serum samples were available from 246 of these patients. Baseline samples were drawn up to 56 days after definitive surgery. There were 85 participating departments from France, Bulgaria, United Kingdom, Switzerland, the Netherlands, and Belgium. Eight of these centres contributed serum samples. Serum samples were drawn at randomisation prior to treatment and after 1, 3, 6, 9, 12, 16, 20, 24 and 30 months. Patients were randomised in a 1:2:2 fashion between observation (arm A), treatment with 10 MU interferon-α2b s.c. 5 times per week for 4 weeks, followed by maintenance therapy with 10 MU

interferon-α2b s.c. 3 times per week for 1 year (arm B), or maintenance therapy with 5 MU interferon-α2b s.c. 3 times per week for 2 years (arm C). Baseline serum samples were available from 128 patients. A total of 783 serum samples were collected, and the median number of samples from each patient was 3 (range 1-11). Median duration of follow-up was 54 months (range 5-81 months).

EORTC 18991: 1256 patients were included between June 2000 and June 2004. Serum samples were available from 386 of these patients. Baseline serum samples were drawn up to 70 days after definitive surgery. There were 99 participating departments from 17 countries (mainly in Europe), of which 16 contributed serum samples. Median follow-up was 43 months (range 25-68 months). Patients were randomly assigned in a 1:1 fashion between observation or treatment with pegylated interferon-α2b 6 µg/kg/week for 8 weeks, followed by maintenance therapy with 3 µg/kg/week for 5 years. Baseline serum samples were available from 260 patients. Serum samples were drawn at baseline prior to treatment and after 6, 12, 18, 24, 30, 36, 42, 48, 54 and 60 months. A total of 1691 serum samples were collected, and the median number of samples from each patient was 5 (range 1-11).

Serum samples in all three studies were collected and processed according to a standard operating procedure (SOP). All patients gave written and oral informed consent according to national guidelines. The studies were approved by the national ethical committees and conducted according to the Declaration of Helsinki. The results of this study are reported in accordance with the REMARK guidelines.[27]

YKL-40 analysis

YKL-40 was determined in duplicates in serum samples by a commercial two-site, sandwich-type enzyme-linked immunosorbent assay (ELISA) (Quidel Corporation, San Diego, California), using streptavidin-coated microplate wells, a biotinylated-Fab monoclonal capture antibody, and an alkaline phosphatase-labeled polyclonal detection antibody. The detection limit is 20 µg/L. The intra-assay coefficients of variations were 5% (at 40 µg/L), 4% (at 104 µg/L), and 4% (at 155 µg/L). The inter-assay coefficients of variations were <5.6% (low control) and <8.7% (high control) in the 194 ELISA kits used for the analysis of the samples. The samples were stored frozen for 2-11 years at -80°C before analysis.

Serum YKL-40 in healthy subjects

The reference interval of serum YKL-40 was determined in 245 (134 women and 111 men, median age 49 years, range 18-79 years) healthy subjects who did not take any medicine, had no known disease or symptoms of joint, metabolic or hormonal diseases. The median serum YKL-40 in these 245 healthy subjects was 43 µg/L (range: 20-184 µg/L; 5th to 95th percentile: 20-124 µg/L) with no difference between men and women.[28] Since

serum YKL-40 increases with age, the normal reference interval for serum YKL-40 was adjusted for age and gender by linear regression with serum YKL-40 as the dependent variable (log transformed) and age and gender as the explanatory variables.

Statistical analysis

Both overall survival (OS), defined as time from randomization to death of any cause, and recurrence free survival (RFS), defined as time from randomization to any event except second primary cancers, were considered important endpoints.

Rank statistics were used to test hypotheses on location. Tests of independence were done using the chi-square test. Serum YKL-40 was log transformed (log2) (HR presented are for a two-fold increase in serum YKL-40), and treated as continuous variables for the uni- and multivariate analyses of survival. The clinical covariates (gender, age, ulceration and stage) were included for multivariable analysis. The Kaplan-Meier method was used to estimate survival probabilities, and the log-rank test was used to test for equality of strata or trend grouping YKL-40 by tertiles. The Cox proportional hazards model was applied for univariate analysis as well as for multivariable analysis of overall survival as well as recurrence free survival. The assumptions for the Cox regression model were assessed using conventional methods. For updated values of serum YKL-40, the Cox proportional hazards model using time dependent covariates was used. The significance level was set to 5%. The SAS® software package (version 9.1; SAS Institute, Cary, NC) was used to manage patient data and to perform all statistical analyses.

RESULTS

Baseline clinical and histological characteristics of the patients are shown in Table 1. The median serum YKL-40 and percentage of patients with elevated YKL-40 (age-corrected) were similar in the three study populations ($P = .15$, Chi-square test). 10%, 18%, and 16% had elevated serum YKL-40 at baseline in the Nordic Study, EORTC 18952, and EORTC 18991, respectively, i.e. in blood taken up to 70 days after operation. Comparison of serum YKL-40 levels in the three studies demonstrated a significant difference ($P = .003$, Kruskal-Wallis test), with lower levels in the Nordic Study compared with the two EORTC studies (these two were not statistically different, $P = .36$). Supplementary Table 1 (Appendix) gives serum YKL-40 in relation to stage and ulceration. 307 (51%) patients in the Nordic Study, 164 (67%) in the EORTC 18952 Study, and 203 (53%) in the EORTC 18991 Study had recurrence during follow-up. 265 (44%) patients in the Nordic Study, 121 (49%) in the EORTC 18952 Study and 139 (36%) in the EORTC 18991 Study died during follow-up.

Table 1. Baseline clinical and histological characteristics of the patients.

		Nordic Study n=602	EORTC 18952 n=246	EORTC 18991 n=386
Gender	Males	390 (65)	138 (56)	230 (60)
	Females	212 (35)	108 (44)	156 (40)
Age	Median (range) years	51 (18-77)	49 (18-75)	48 (18-70)
Stage	N0	124 (21)	67 (27)	0
	N+ sentinel	114 (19)	65 (26)	195 (51)
	N+ palpable	364 (60)	114 (46)	191 (49)
Ulceration	No	323 (54)	123 (50)	201 (52)
	Yes	164 (27)	76 (31)	90 (23)
	Unknown	115 (19)	47 (19)	95 (25)
YKL-40 Baseline*	Normal	406 (90)	105 (82)	219 (84)
	Elevated	46 (10)	23 (18)	41 (16)
YKL-40 Baseline*	Median (range) µg/L	47 (20-1416)	54 (20-281)	53 (20-438)
Breslow thickness	<1.5 mm	145 (24)	46 (18)**	76 (20)
	1.5-3.99 mm	220 (37)	59 (24)***	182 (47)
	≥4.0 mm	200 (33)	105 (43)	78 (20)
	Unknown	37 (6)	36 (15)	50 (13)
Clark's level	I	1 (0.2)		
	II	19 (3)	4(2)	
	III	142 (24)	50 (20)	
	IV	253 (42)	120 (49)	
	V	50 (8)	30 (12)	
	Unknown	137 (23)	42 (17)	

Values are numbers (%).

*Serum samples for determination of YKL-40 at baseline were available from 452 patients in the Nordic Study, from 128 patients in the EORTC 18952 Study and from 260 patients in the EORTC 18991 Study.

**Breslow, <2 mm.

***Breslow, 2-3.99 mm.

Baseline serum YKL-40 and recurrence free survival

Data from The Nordic Study and EORTC 18952 were analysed together, as they were similar regarding inclusion criteria, interferon treatment, and patient characteristics. Results from EORTC 18991 are reported separately, as disease stage and interferon treatment were different.

Univariate analysis showed that baseline serum YKL-40 (log transformed, continuous covariate) in patients in the observation group and interferon groups was not associated with RFS (Nordic Study + EORTC 18952: observation arms, $P = .43$; interferon 1-year arms, $P = .67$; interferon 2-year arms, $P = .76$; EORTC 18991: observation arm, $P = .64$). In patients treated with pegylated interferon, high serum YKL-40 was related with shorter RFS (HR = 1.6, 95% CI = 1.1 to 2.5; $P = .02$).

If all 299 untreated patients included in the 3 studies were combined, baseline serum YKL-40 above the median level was not associated with shorter RFS (HR = 1.09, 95% CI = 0.92 to 1.29; *P* = .35). Supplementary Table 2 (Appendix) gives the results of the multivariate analysis of RFS.

Baseline serum YKL-40 and overall survival

Univariate analysis in the observation group from the Nordic Study and EORTC 18952 demonstrated that baseline serum YKL-40 (log transformed, continuous covariate) was not significantly associated with OS (HR = 1.25, 95% CI = 0.98 to 1.60; *P* = .07). A similar result was found in the observation group in EORTC 18991 (HR = 1.50, 95% CI = 0.91 to 2.45; *P* = .11). When all 299 untreated patients from the 3 studies (Nordic Study [n = 146], EORTC 18952 [n = 22], and EORTC 18991 [n = 131])were combined, high baseline serum YKL-40 was associated with significantly shorter OS (HR = 1.28, 95% CI = 1.05 to 1.57; *P* = .015).

Univariate analysis of interferon-treated patients in the Nordic Study and EORTC 18952 demonstrated that baseline serum YKL-40 was not associated with OS (interferon 1-year: HR = 1.12, 95% CI = 0.88 to 1.42; *P* = .35 and interferon 2-year: HR = 1.08, 95% CI = 0.86 to 1.35; *P* = .54). Whereas in EORTC 18991 baseline serum YKL-40 was associated with OS in patients treated with pegylated interferon (HR = 1.47, 95% CI = 1.06 to 2.04; *P* = .02).

Table 2. Multivariate analyses of overall survival for gender, age, stage, ulceration, and baseline serum YKL-40 in the Nordic Study combined with the EORTC 18952 Study and in the EORTC 18991 Study.

	Nordic Study and EORTC 18952				EORTC 18991			
	Group	**HR**	**95% CI**	**p-value**	**Group**	**HR**	**95% CI**	**p-value**
Gender f vs m	Obs	0.71	0.44-1.13	.14	Obs	0.94	0.49-1.81	.84
Age		0.97	0.80-1.17	.74		1.03	0.77-1.38	.83
N0 vs N+palp.		0.55	0.31-0.97	.004				
N+sent. vs N+palp.		0.37	0.19-0.71			0.65	0.35-1.22	.18
YKL-40		1.33	1.01-1.74	.04		1.31	0.90-1.91	.16
Gender	IFN 1-year	0.61	0.40-0.94	.03	Pegylated IFN	1.91	0.94-3.87	.07
Age		1.24	1.03-1.50	.02		1.08	0.78-1.51	.63
N0 vs N+palp.		0.58	0.34-0.98	.06				
N+sent. vs N+palp.		0.64	0.37-1.10			0.39	0.19-0.77	.007
YKL-40		0.97	0.76-1.25	.83		1.30	0.88-1.90	.19
Gender	IFN 2-year	0.54	0.33-0.87	.01				
Age		1.08	0.88-1.33	.45				
N0 vs N+palp.		0.57	0.33-0.99	.008				
N+sent. vs N+palp.		0.46	0.26-0.81					
YKL-40		1.06	0.83-1.34	.64				

Obs = Observation, IFN = interferon

Table 2 gives the multivariate analysis of OS (including gender, age, stage, ulceration, and serum YKL-40). In the observation group from the Nordic Study and EORTC 18952 baseline serum YKL-40 was associated with OS (HR = 1.33, *P* = .042). This was not found in the interferon-treated patients (1-year: HR = 0.97, *P* = .83; 2- year: HR = 1.06, *P* = .64). In the EORTC 18991 Study baseline serum YKL-40 was not associated with OS (observation: HR = 1.31, *P* = .16; pegylated interferon: HR = 1.30, *P* = .19).

Figure 1 illustrates the Kaplan-Meier curves of OS according to baseline serum YKL-40 in the Nordic Study. Since only few patients had elevated (age-corrected) serum YKL-40 compared to healthy subjects, the median baseline serum YKL-40 level (i.e. after operation) was used as cutoff. Serum YKL-40 above the median in the untreated patients from the Nordic Study and EORTC 18952 was associated with short OS, whereas this was not found in the patients treated with adjuvant interferon. Similar results were found for the patients included in EORTC 18991. Kaplan-Meier curves are showed for the Nordic Study (Figure 1).

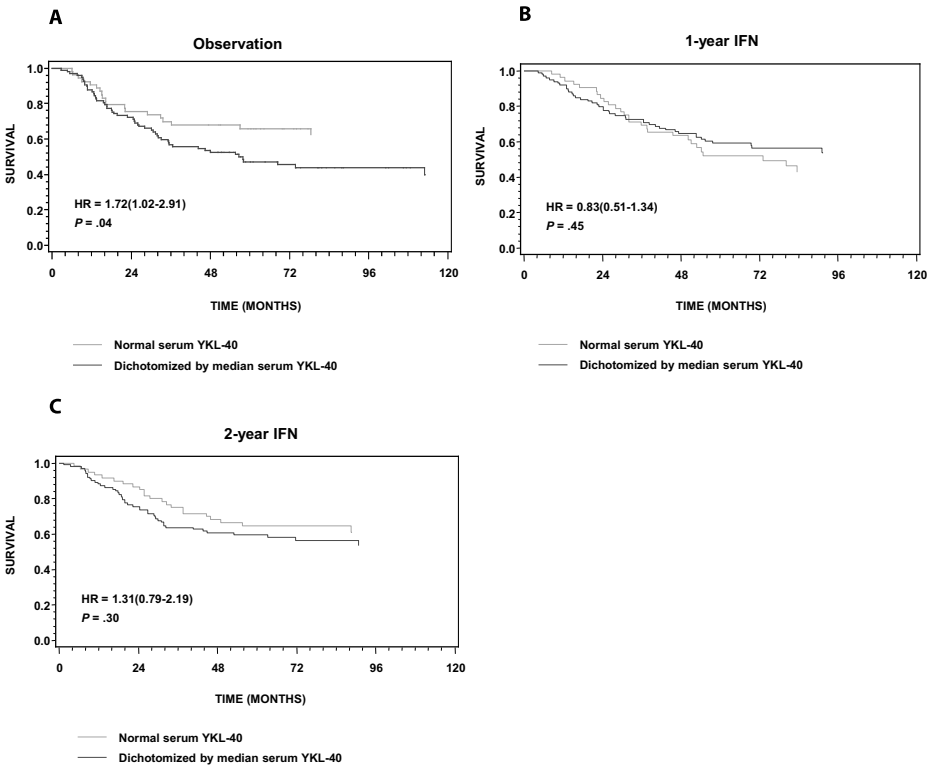

Figure 1. Kaplan-Meier survival curves according to baseline serum YKL-40 in the three treatment arms (**A**: observation group; **B:** patients randomized to one year of adjuvant interferon; **C:** patients randomized to two years of adjuvant interferon) in the Nordic study showing the association between serum YKL-40 above or below the median in baseline samples and survival. Serum YKL-40 was dichotomized according to the median serum YKL-40 level in baseline samples. The P-value refers to the log-rank test for equality of strata.

Serum YKL-40 during follow-up

Patients with baseline serum samples and follow-up serum samples had similar baseline characteristics compared to patients with follow-up serum samples only (data not shown). Analysis of the updated serum YKL-40 levels (latest available measurement, log transformed, continuous covariate, HR for two-fold difference) and OS as well as RFS were done for each study stratified by treatment arm, and similar results were found for each treatment arm. Therefore it was decided to pool the results for each trial adjusted for treatment arm (data not shown).

The Nordic Study: Patients with serum YKL-40 above the median level during follow-up had shorter OS (HR = 1.31, 95% CI = 1.16 to 1.47; $P < .0001$), Table 3. Replacing the serum YKL-40 level with the ratio of serum YKL-40 to the previous measurement of serum YKL-40 demonstrated shorter OS if the ratio increased (HR = 1.43, $P = .002$). For RFS, the results were not significant, Table 3.

EORTC 18952: Patients with serum YKL-40 above the median level during follow-up had no shorter OS or RFS, Table 3. Using the ratio of serum YKL-40 to the previous measurement of serum YKL-40, shorter OS was demonstrated if the ratio increased (HR = 1.84, $P = .043$). For RFS, the results were not significant, Table 3.

EORTC 18991: Patients with serum YKL-40 above the median level during follow-up had shorter OS (HR = 1.33, $P = .004$). Using the ratio of the serum YKL-40 to the previous measurement of serum YKL-40 shorter OS was demonstrated if the ratio increased (HR = 1.39, $P = .044$). For RFS the results were not significant, Table 3.

Table 3. Univariate analysis of serum YKL-40 during treatment and follow-up and overall survival and recurrence-free survival.

		Overall survival			Recurrence-free survival		
		N	**HR (95% CI)**	**P-value**	**N**	**HR (95% CI)**	**P-value**
Nordic Study	Actual value[1]	585	1.31 (1.16-1.47)	< .0001	584	1.12 (1.0-1.26)	.06
	Ratio to previous measurement[2]	546	1.43 (1.13-1.80)	.002	545	1.15 (0.92-1.42)	.22
EORTC 18952	Actual value[1]	138	1.15 (0.89-1.40)	.34	138	1.01 (0.82-1.24)	.94
	Ratio to previous measurement[2]	127	1.84 (1.02-3.31)	.04	127	1.10 (0.62-1.93)	.25
EORTC 18991	Actual value[1]	298	1.33 (1.10-1.62)	.004	298	1.17 (0.99-1.39)	.07
	Ratio to previous measurement[2]	298	1.39 (1.01-1.92)	.04	298	1.07 (0.82-1.39)	.61

1: Serum YKL-40 is entered by its actual value on the log scale (base 2). All patients with at least 1 measurement have been included.

2: The ratio of the updated serum YKL-40 level to the preceding on the log scale. Only patients with at least 2 levels have been included.

DISCUSSION

In the present study we analyzed whether serum YKL-40 had prognostic value in patients with high-risk melanoma stage IIB and III, and if baseline serum YKL-40 had predictive value regarding interferon treatment in this patient population.

Ideally, determining the prognostic value of a biomarker is carried out in a group of patients who have not received any treatment that might influence outcome.[29,30] We had the opportunity to test the prognostic value of serum YKL-40 in a large group of stage IIB-III melanoma patients, who had undergone surgery according to standard clinical guidelines, but not received any adjuvant treatment. We pooled the 299 patients randomized to observation in three studies, and found in univariate analysis that serum YKL-40 postoperatively was significantly associated with OS. In the Nordic Study and EORTC 18952 serum YKL-40 (above the median level of the patients) was an independent parameter of short OS. We had expected stronger association between serum YKL-40 and OS, since Schmidt et al.[9] reported strong independent prognostic value of preoperative serum YKL-40 in stage I-II melanoma. In our study YKL-40 was determined in serum samples drawn after definitive surgery. This is probably the main reason for the lower percentage of patients with elevated serum YKL-40 (compared to healthy subjects) and for the smaller impact on OS in our study on stage IIB-III melanoma than in stage I-II melanoma.

Multivariate analysis in the Nordic Study and EORTC 18952 combined showed that patients with elevated baseline serum YKL-40 above the median level had shorter OS, an impact that disappeared in the interferon-treated patients. This gives rise to speculations on whether baseline serum YKL-40 has predictive value regarding adjuvant treatment with interferon in high-risk melanoma patients. This would rest on the assumption that patients operated for stage IIB-III melanoma with highest postoperative levels of serum YKL-40 defines a high risk sub-group population, who do benefit from interferon therapy, since a causative relationship between YKL-40 and effect of interferon therapy has not been established. If indeed postoperative serum YKL-40 has predictive value regarding adjuvant interferon therapy in stage IIB-III melanoma, an impact on RFS would be expected as adjuvant interferon treatment in multiple trials[3-5,31] with just one exception[32] has yielded positive impact on RFS, but not on OS. However, no impact on RFS was found in our study. The question whether postoperative serum YKL-40 in stage IIB-III melanoma contains predictive value therefore warrants further exploration in prospective clinical studies.

Comparison of serum YKL-40 levels in the three studies showed a significant difference, with lower levels in the Nordic Study compared to the two EORTC studies (these two were not statistically different). In the Nordic Study blood samples were not treated with an enhancer of coagulation, and there was no instruction that serum samples be left

to coagulate before centrifugation. In the two EORTC studies blood samples were drawn into Corvac tubes, which contain silicone for the separation of serum and clot, and was then allowed to coagulate for one hour at room temperature, and then centrifuged to separate clot from serum. YKL-40 is contained in neutrophils and monocytes,[15,16,19] but as no enhancer of coagulation was used in any of the studies it is unlikely that differences in the testing tubes can explain the higher YKL-40 levels seen in the two EORTC studies. Leaving blood samples at room temperature to coagulate for up to three hours does not affect the levels of serum YKL-40.[33]

Baseline serum YKL-40 was elevated in subgroups of the patients – 10% in the Nordic Study, 18% in EORTC 18952, and 16% in EORTC 18991, with median serum YKL-40 levels at 47 µg/L, 54 µg/L, and 53 µg/L, respectively, well within the normal range for healthy subjects. Subjects with elevated serum YKL-40 levels are at risk of dying of multiple diseases. The patients included in this study, however, died almost exclusively of melanoma. Death of other causes than cancer was 3.6% in the Nordic Study (10 of 281 deaths), 2.3% in EORTC 18952 (16 of 681 deaths),[3] and 6.1% in EORTC 18991 (32/525).[4] Different factors might influence the levels of serum YKL-40. The baseline serum YKL-40 might be a reflection of the primary and metastatic tumor cells that are excised, or a reflection of residual tumor lesions. In the first case, levels would be expected to decrease, and in the latter to either stay on the same level or increase in the case of active disease. Diseases characterized by inflammation can influence the levels of serum YKL-40. Detailed information regarding comorbidity is not available, as information on this was not prospectively collected. Analysis of the serum samples for YKL-40 was not planned in the clinical protocols. However, exclusion criteria in the three studies prevented randomization of patients who used corticosteroids, NSAID, or other immunomodulatory treatment, as well as patients with WHO performance status 2-4, and hereby presumably reducing the number of patients with significant comorbidity. As this is a study with a large number of randomized patients, patients with comorbidity are expected to be equally distributed. Storage of serum samples was not under constant surveillance in the beginning, and as a result a number of Norwegian serum samples were thawed unintentionally and discarded. The serum YKL-40 analysis is however very robust and repetitive freezing and thawing of serum samples up to 9 times had no effect on the serum YKL-40.[33-37] Additionally, baseline serum samples were drawn up to 70 days after surgery, carrying the risk that samples drawn early after surgery give rise to falsely elevated serum YKL-40 due to the trauma of surgery or infection related to surgery. However, it must be emphasized that time from definitive surgery to baseline serum sample was equal in the three randomization arms, as expected in this randomized patients population. Furthermore, an increase in serum YKL-40 in connection with infection is rapidly normalized.[38]

Presently, treatment options for patients with metastasizing melanoma are scarce. In the future, as treatment options expand, identifying relapse as early as possible may

become increasingly important. We found that increases in serum YKL-40 during treatment and follow-up was associated with short OS, indicating that serum YKL-40 might be valuable for monitoring patients with melanoma. However, we found no association between increases in serum YKL-40 during follow-up and RFS. A possible explanation for this might be that RFS is an endpoint that consists of death of any cause and locoregional and distant metastasis. Date of death is a reliable parameter, whereas the date of recurrence depends in part upon the timing of follow-up visits and choice of follow-up investigations, making RFS a less reliable endpoint. Serum YKL-40 has little intra-individual variability in healthy subjects in sampling periods for up to 10 years, indicating that it is unlikely that the observed increases in serum YKL-40 are results of pre-analytic conditions, methodological, and normal biological variability.[39] The value of monitoring serum YKL-40 during treatment and follow-up in patients with stage IIB-III melanoma needs confirmation in other studies, and preferably in combination with LDH, S100, and melanoma inhibitory activity which have shown value in detecting especially distant metastases.[40,41]

The exact function of YKL-40 in cancer diseases is unknown. Studies suggest that YKL-40 plays a role in cell proliferation and differentiation, angiogenesis, apoptosis, inflammation and the innate immune response, remodelling of the extracellular matrix and development of fibrosis.[20,22-25,34,42-45] Elevated serum YKL-40, compared to healthy subjects, is found in a subgroup of patients with different types of cancer and elevated serum YKL-40 was an independent prognostic biomarker of short OS.[10,11,13,14,46-53] However, YKL-40 is not cancer specific and co-morbidity should always be considered, since some patients with non-malignant diseases characterized by inflammation and tissue remodelling also have elevated serum YKL-40.[42,54-56]

In conclusion, we found an association between serum YKL-40 and short OS in untreated high-risk stage IIB-III melanoma patients, but not in interferon-treated patients. Increases in serum YKL-40 during treatment and follow-up were associated with short OS but not with RFS. It is unknown whether serum YKL-40 will be proved useful in daily clinical practice for risk assessment of patients with melanoma or in monitoring treatment.

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Chapter VIII

Polymorphisms in the CD28/CTLA4/ICOS Genes; Role in Malignant Melanoma Susceptibility and Prognosis?

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ABSTRACT

The appearance of vitiligo and spontaneous regression of the primary lesion in melanoma patients illustrate a relationship between tumor immunity and autoimmunity. T lymphocytes play a major role both in tumor immunity and autoimmunity. CD28, Cytotoxic T lymphocyte antigen 4 (CTLA4) and inducible costimulator (ICOS) molecules are important secondary signal molecules in the T lymphocyte activation. Single Nucleotide Polymorphisms (SNPs) in the CD28/CTLA4/ICOS gene region were reported to be associated with several autoimmune diseases including, type-1 diabetes, SLE, autoimmune thyroid diseases and celiac disease. In this study we investigated the association of SNPs in the CD28, CTLA4 and ICOS genes with the risk of melanoma. We also assessed the prognostic effect of the different polymorphisms in melanoma patients. Twenty-four tagging SNPs across the three genes and four additional SNPs were genotyped in a cohort of 763 German melanoma patients and 734 healthy German controls. Influence on prognosis was determined in 587 melanoma cases belonging to stage I or II of the disease. In general, no differences in genotype or allele frequencies were detected between melanoma patients and controls. Similarly no association of any polymorphism with prognosis, except for the rs3181098 polymorphism in the CD28 gene, was observed. In addition, individuals with AA genotype for rs11571323 polymorphism in the ICOS gene showed reduced overall survival. However, keeping in view the correction for multiple hypothesis testing our results suggest that the polymorphisms in the CD28, CTLA4 and ICOS genes at least do not modulate risk of melanoma and nor do those influence the disease prognosis in the investigated population.

INTRODUCTION

The association between tumor immunity and autoimmunity is complex.[1] Spontaneous regression is believed to be more common in melanoma than any other cancer type. However, the effect of the phenomenon on prognosis is rather unclear; however, the appearance of vitiligo is considered a favorable prognostic factor. Autoimmune conditions like thyroiditis and vitiligo, induced by interleukin 2 and/or Interferon-α (IFN) therapy have been associated with an improved prognosis in melanoma patients.[2,3] The appearance of autoantibodies or autoimmune manifestations in IFN-treated patients has been reported to be associated with significantly improved recurrence free and overall survival.[4] However, the findings could not be replicated when serum samples were analyzed of patients that were randomized to IFN treatment or observation in the EORTC 18952 and the Nordic Melanoma Group phase III trials.[5]

T lymphocytes play an important role both in tumor immunity as well as in autoimmunity. The CD28, cytotoxic T lymphocyte antigen 4 (CTLA4) and inducible co-stimulator (ICOS) molecules are important secondary signaling molecules involved in the T lymphocyte activation. The genes encoding CD28, CTL4 and ICOS are located within a stretch of 300 kb on chromosome 2q33. Ligation of CD28 molecules with B7-1 (CD80) or B7-2 (CD86) on antigen presenting cells (APCs), stimulate T cell activation and proliferation. CTLA4 counterbalances this effect by competing with CD28 for B7-1/ B7-2 binding and is therefore an important inhibitor of T cell activation.[6,7] CTLA4 is also an established negative regulator of T cell function and proliferation through multiple mechanisms such as reducing interleukin (IL)-2 and IL-2 receptor productions and arresting T cell at the G1-phase of cell cycle.[8] ICOS is another co-stimulatory molecule which is expressed on activated T cells. It binds to a unique ligand, ICOSL, and does not bind to other ligands such as B7-1/ B7-2. Polymorphisms in the CD28/CTLA4/ICOS gene region have been associated with several autoimmune diseases including, type 1 diabetes, SLE, autoimmune thyroid diseases and celiac disease.[9,10] However, a majority of the studies focused on the known CTLA4 polymorphisms. A high prevalence of AA for the CT60 polymorphism in the gene was observed in patients with renal cell cancer and a positive correlation between the polymorphism and tumor grade was also established.[11] The association between the variants in the promoter region of the CTLA4 gene and breast cancer progression has also been reported.[12]

In this study, in order to find an association between polymorphisms in the CD28, CTLA4 and ICOS genes and risk of cutaneous melanoma we screened patients from Germany and ethnically matched healthy controls. The single nucleotide polymorphisms (SNPs) in the three CD28/CTLA4/ICOS genes were selected by tagging approach in order to cover the entire gene regions. Additionally four SNPs reported to be of interest in literature in the CTLA4 gene were also included in the study. The association of variants alleles with prognostic outcome was also determined.

METHODS

Patients and Controls

The study population consisted of 763 melanoma patients from Germany (418 male and 345 female), recruited by the Skin Cancer Unit Mannheim, from 2001 to 2008. Patients with primary cutaneous melanoma with different disease stages that included; 10 cases with in situ melanoma, 615 with stage I/II, 111 stage III, and 12 cases with stage IV of the disease. For 15 patients stage was unknown. Disease staging was performed according to the current AJCC criteria from 2001.[13] Median and mean age of the melanoma cases at diagnosis was 55 and 54 years, respectively. Blood samples from case subjects were taken at their first presentation at the skin cancer unit. DNA was isolated from blood samples using Qiagen mini-preparation kits. Informed consent was obtained from the patients and the study was approved by the institutional ethical review board. Control subjects included 734 healthy German individuals (367 male and 367 female) recruited from blood bank Mannheim, with mean and median age of 60 and 61 years, respectively. They were born in southwest Germany and were matched for ethnicity with cases. The inclusion criteria for controls in the study included cancer free status. The age difference between the cases and controls was statistically significant (T-test; P value < .01), whereas, the gender difference was not statistically significant (χ^2-test; P value > .05).

Genes and SNPs selection

The selection of polymorphisms in the CD28, CTLA4 and ICOS genes was based on inclusion of known non-synonymous SNPs and those located in regulatory regions as reported in the dbSNP database of the National Center for Biotechnology Information, NCBI (http://www.ncbi.nlm.nih.gov/SNP/) or reported in published papers. Additionally, tagging SNPs from each gene region were selected from HapMap data using Haploview software 3.32, with pair-wise r^2 >0.8 for each SNP pair and minor allele frequencies >5% (Figure 1). Ten tagging SNPs in the CD28 gene, five in the CTLA4 gene and 10 in the ICOS gene were selected from HapMap database that covered the three genes completely. Four polymorphisms, rs11571319 (CT61), rs11571302 (JO31), rs7665213 (JO30) and rs11571297 (JO27) in the CTLA4 gene, which have been described to correlate with autoimmune disease(s) were also selected. The investigated polymorphisms span a region of 31.0 kb for the CD28 gene region, 6.1 kb for the CTLA4 gene region and 24.7 kb for the ICOS gene region. In total, 29 polymorphisms in three genes (CD28, CTLA4 and ICOS) were identified.

Validation of the SNPs by DNA sequencing

The validation of the 29 selected polymorphisms was carried out by sequencing a set of 32 DNA samples of control subjects. Sequencing reactions were performed using Big

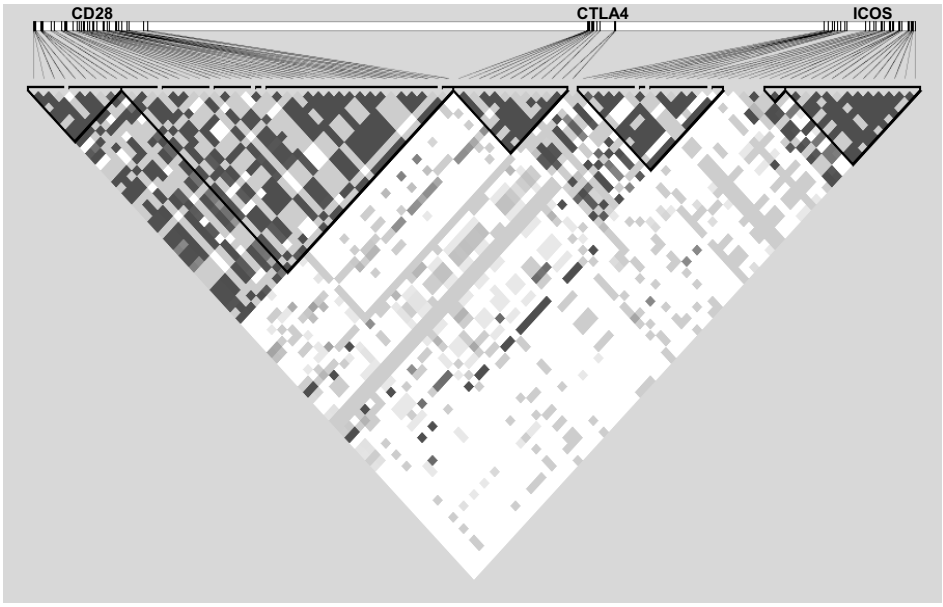

Figure 1.
Haplotype blocks in the genomic region with CTLA-4, CD28 and ICOS genes based on HapMap data.

Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Ca, USA) and the following conditions were used; initial 94°C for 1 min followed by 27 cycles at 96 °C for 16 s, 56°C for 5 s and 60°C for 4 min. Reaction products were run on ABI prism 3100 Genetic analyzer (Applied Biosystems). Primers used in PCR amplification and sequencing reaction are listed in Supplementary Table (appendix).

Genotyping

Genotyping of the validated SNPs was performed by allelic discrimination technique (TaqMan assays, 'by demand or design' Applied Biosystems, Supplementary Table, Appendix). Genotyping for one polymorphism in the ICOS gene (rs4355090) failed and was, thus, excluded from the study. Genotype failure rate was 0.14%, calculated from samples that could not be genotyped after two repeated assays and by direct DNA sequencing. Genotyping data were confirmed by random direct DNA sequencing of 5% of all samples, which showed 100% concordance.

Statistical analysis

The association between malignant melanoma and different genotypes was estimated as odds ratios (OR), 95% confidence intervals (CI) and p-values using SAS version 9.1. Estimates were adjusted for gender and age. Haplotype procedure of SAS/Genetics Software was used to calculate haplotype frequencies in cases and controls. Linkage

disequilibrium (LD) was calculated with Haploview software (www.broad.mit.edu/mpg/haploview/documentation.php). The association between genotypes and different survival parameters, adjusted for age, gender and Breslow thickness, was carried out using proportional hazard regression (Cox) model. Metastases-free survival (MFS) was the time from date of diagnosis until the first metastasis (either lymph node or distant metastasis) and overall survival (OS) was time from diagnosis to death. The follow-up of patients without metastases or who did not decease has been censored at the latest visit/last contact.

RESULTS

Case - control study

The allelic distribution of polymorphisms in the CD28, CTLA4 and ICOS genes was assessed in 763 German melanoma patients and compared with 734 healthy German controls. A total of 28 SNPs were studied and genotype and allele distributions of all the polymorphisms are summarized in Table 1. Genotype frequency in controls for all the polymorphisms was in accordance with the Hardy-Weinberg equilibrium. Minor allele frequency (MAF) for the rs3181098 polymorphism was higher in cases than in controls (OR: 1.18, 95% CI = 1.00 to 1.38; *P* = .05). And for the rs3181100 (C > G) polymorphisms the MAF was lower in cases than controls (OR: 0.83, 95% CI = 0.71 to 0.97; *P* = .02). None of the inferred haplotypes in three genes showed differential distribution between cases and controls (data not shown).

Table 1. Case Control

SNP	Genotype	Cases N=763	(%)	Controls N=734	(%)	OR	95% CI	p-value
CD28								
rs3181098	GG	315	41	331	45			
	AG	331	43	325	44	1.05	0.84 – 1.31	
	AA	117	15	78	11	1.50	1.07 – 2.11	.06
	G-allele	961	63	987	67			
	A-allele	565	37	481	33	1.18	1.00 – 1.38	.05
rs3181100	CC	279	37	229	31			
	CG	368	48	357	49	0.87	0.69 – 1.10	
	GG	116	15	145	20	0.68	0.50 – 0.93	.05
	C-allele	926	61	815	56			
	G-allele	600	39	647	44	0.83	0.71 – 0.97	.02
rs3181101	CC	570	75	559	77			
	CG	175	23	165	23	0.98	0.76 – 1.26	
	GG	17	2	7	10	1.97	0.78 – 4.96	.35
	C-allele	1315	86	1283	88			

Table 1. Continued

SNP	Genotype	Cases N=763	(%)	Controls N=734	(%)	OR	95% CI	p-value
	G-allele	209	14	179	12	1.06	0.85 – 1.33	.61
rs1181390	GG	474	62	467	64			
	GT	257	34	233	32	1.14	0.91 – 1.43	
	TT	32	4	33	5	0.92	0.55 – 1.55	.47
	G-allele	1205	79	1167	80			
	T-allele	321	21	299	20	1.06	0.88 – 1.28	.53
rs1181388	GG	575	76	545	74			
	AG	169	22	170	23	0.98	0.76 – 1.26	
	AA	17	2	18	3	0.90	0.45 – 1.81	.95
	G-allele	1319	87	1260	86			
	A-allele	203	13	206	14	0.97	0.78 – 1.20	.76
rs17533594	AA	483	63	474	65			
	AG	257	34	232	32	1.11	0.89 – 1.40	
	GG	23	3	24	3	0.94	0.52 – 1.73	.63
	A-allele	1223	80	1180	81			
	G-allele	303	20	280	19	1.06	0.88 – 1.28	.55
rs3116494	AA	414	54	393	54			
	AG	307	40	299	41	1.01	0.81 – 1.26	
	GG	42	6	39	5	1.09	0.67 – 1.75	.94
	A-allele	1135	74	1085	74			
	G-allele	391	26	377	26	1.02	0.86 – 1.21	.79
rs3181107	AA	659	86	620	85			
	AG	100	13	106	15	0.92	0.67 – 1.25	
	GG	4	1	6	1	0.74	0.20 – 2.79	.78
	A-allele	1418	93	1346	92			
	G-allele	108	7	118	8	0.91	0.68 – 1.20	.49
rs3116496 (IVS3 +17)	TT	487	64	475	65			
	CT	254	33	231	32	1.10	0.88 – 1.38	
	CC	22	3	24	3	0.89	0.48 – 1.64	.63
	T-allele	1228	81	1181	81			
	C-allele	298	20	279	20	1.04	0.86 – 1.26	.66
CTLA4								
rs16840252	CC	521	68	489	67	1.00		
	CT	218	29	222	30	0.91	0.72 – 1.15	
	TT	23	3	21	3	1.02	0.54 – 1.91	.74
	C-allele	1260	83	1200	82			
	T-allele	264	17	264	18	0.95	0.78 – 1.15	.57
rs5742909 (CT44)	CC	619	81	596	81	1.00		
	CT	136	18	130	18	0.96	0.73 – 1.27	
	TT	8	1	8	1	0.89	0.32 – 2.49	.95
	C-allele	1374	90	1322	90			
	T-allele	152	10	146	10	0.96	0.75 – 1.23	.74
rs231775 (CT42)	AA	289	38	283	39	1.00		

Table 1. Continued

SNP	Genotype	Cases N=763	(%)	Controls N=734	(%)	OR	95% CI	p-value
	AG	369	48	345	47	1.08	0.86 – 1.36	
	GG	104	14	106	14	0.97	0.70 – 1.36	.71
	A-allele	947	62	911	62			
	G-allele	577	38	557	38	1.01	0.87 – 1.18	.90
rs231777	CC	539	71	514	70	1.00		
	CT	208	27	203	28	0.97	0.76 – 1.23	
	TT	15	2	16	2	0.83	0.39 – 1.77	.87
	C-allele	1286	84	1231	84			
	T-allele	238	16	235	16	0.95	0.78 – 1.17	.64
rs3087243 (CT60)	GG	246	32	223	30	1.00		
	AG	355	47	388	53	0.81	0.63 – 1.03	
	AA	162	21	122	17	1.22	0.89 – 1.65	.01
	G-allele	847	56	834	57			
	A-allele	679	45	632	43	1.06	0.91 – 1.23	.45
rs11571319 (CT61)	GG	518	68	488	67	1.00		
	AG	222	29	223	31	0.93	0.73 – 1.17	
	AA	23	3	21	3	1.02	0.54 – 1.92	.81
	G-allele	1258	82	1199	82			
	A-allele	268	18	265	18	0.96	0.79 – 1.16	.64
rs11571302 (JO31)	GG	225	30	210	29	1.00		
	GT	370	49	383	52	0.87	0.68 – 1.12	
	TT	168	22	140	19	1.14	0.84 – 1.55	.15
	G-allele	820	54	803	55			
	T-allele	706	46	663	45	1.05	0.90 – 1.22	.53
rs7665213 (JO30)	GG	228	30	211	29	1.00		
	AG	370	49	383	52	0.87	0.68 – 1.12	
	AA	165	22	137	19	1.13	0.83 – 1.53	.17
	G-allele	826	54	805	55			
	A-allele	700	46	657	45	1.04	0.90 – 1.21	.59
rs11571297 (JO27)	TT	214	28	193	26	1.00		
	CT	376	49	393	54	0.84	0.65 – 1.08	
	CC	173	23	148	20	1.07	0.79 – 1.45	.15
	T-allele	804	53	779	53			
	C-allele	722	47	689	47	1.02	0.88 – 1.18	.81
ICOS								
rs10932029 (+173)	TT	538	71	488	67	1.00		
	CT	204	27	228	31	0.82	0.65 – 1.03	
	CC	21	3	15	2	1.14	0.57 – 2.27	.20
	T-allele	1280	84	1204	82			
	C-allele	246	16	258	18	0.89	0.73 – 1.08	.23
rs4335928	TT	579	76	559	76	1.00		
	CT	170	22	162	22	0.99	0.77 – 1.28	

Table 1. Continued

SNP	Genotype	Cases N=763	(%)	Controls N=734	(%)	OR	95% CI	p-value
	CC	14	2	12	2	1.11	0.50 – 2.47	.96
	T-allele	1328	87	1280	87			
	C-allele	198	13	186	13	1.01	0.80 – 1.26	.96
rs4675374	CC	457	60	436	60	1.00		
	CT	272	36	258	35	0.98	0.78 – 1.23	
	TT	34	5	38	5	0.85	0.52 – 1.40	.82
	C-allele	1186	78	1130	77			
	T-allele	340	22	334	23	0.96	0.80 – 1.14	.62
rs7602383	AA	550	72	533	73	1.00		
	AG	197	26	183	25	1.01	0.79 – 1.29	
	GG	16	2	17	2	0.93	0.46 – 1.90	.98
	A-allele	1297	85	1249	85			
	G-allele	229	15	217	15	0.99	0.81 – 1.23	.95
rs4521021	TT	450	59	451	61	1.00		
	CT	276	36	260	35	1.00	0.80 – 1.25	
	CC	37	5	23	3	1.47	0.83 – 2.60	.41
	T-allele	1176	77	1162	80			
	C-allele	350	23	306	21	1.07	0.89 – 1.28	.45
rs11571323	GG	587	77	534	73	1.00		
	AG	161	21	184	25	0.81	0.63 – 1.04	
	AA	15	2	13	2	1.30	0.60 – 2.84	.18
	G-allele	1335	88	1252	86			
	A-allele	191	13	210	14	0.89	0.71 – 1.10	.29
rs12466129	TT	448	59	451	62	1.00		
	AT	273	36	239	33	1.14	0.91 – 1.43	
	AA	42	6	42	6	0.94	0.59 – 1.51	.47
	T-allele	1169	77	1141	78			
	A-allele	357	23	323	22	1.06	0.89 – 1.27	.54
rs10172036	GG	301	40	283	39	1.00		
	GT	353	46	352	48	0.92	0.73 – 1.15	
	TT	109	14	97	13	1.04	0.75 – 1.45	.63
	G-allele	955	63	918	63			
	T-allele	571	37	546	37	0.99	0.85 – 1.16	.93
rs10183087	AA	461	61	418	57	1.00		
	AC	263	35	277	38	0.85	0.68 – 1.06	
	CC	38	5	37	5	1.04	0.63 – 1.70	.32
	A-allele	1185	78	1113	76			
	C-allele	339	22	351	24	0.92	0.77 – 1.10	.36
rs10932036	AA	611	80	594	81	1.00		
	AT	144	19	129	18	1.09	0.83 – 1.44	
	TT	7	1	7	1	1.12	0.37 – 3.35	.81
	A-allele	1366	90	1317	90			
	T-allele	158	10	143	10	1.08	0.85 – 1.39	.53

Association between polymorphisms and prognosis

The association between polymorphisms and survival parameters was evaluated for melanoma patients in stage I and II. Information regarding metastases-free survival (MFS), overall survival (OS) and Breslow thickness was available for 587 patients (321 male and 266 female). Mean age was 54 years (median 55 years), the mean and median Breslow thickness was 1.84 mm and 1.50 mm, respectively. Ulceration status of the primary tumor was not systematically recorded in the past and is therefore lacking. Age, gender and Breslow thickness were included as covariates in the Cox regression analysis. Overall, on comparing carriers vs non-carriers, no significant differences in OS were observed (Tables 2,3,4). A single SNP in the CD28 gene (rs3181098) showed an association with reduced metastases-free survival (HR 1.34, 95% CI = 1.02 to 1.77). In addition to the carrier vs non-carrier approach, effect of the different genotypes on prognosis was analyzed. According to this analysis one SNP (AA) in the ICOS gene (rs11571323) was associated with reduced overall survival, *P* = .04, HR 3.60, 95% CI = 1.31 to 9.91, however, only 13 (2.2%) melanoma patients carried AA genotype.

Table 2. CD28

			Metastases-Free Survival			Overall Survival		
SNP	**Genotype**	**Cases (%) N=587**	**OR**	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**
rs3181098	GG	247 (42)						
	AG/AA	340 (58)	1.34	1.02 – 1.77	.04	1.18	0.81 – 1.72	.38
rs3181100	CC	212 (36)						
	CG/GG	375 (64)	0.82	0.62 – 1.08	.16	0.82	0.56 – 1.20	.31
rs3181101	CC	440 (75)						
	CG/GG	146 (25)	1.28	0.94 – 1.74	.12	1.21	0.80 – 1.83	.38
rs1181390	GG	360 (61)						
	GT/TT	227 (39)	0.92	0.70 – 1.21	.56	1.16	0.80 – 1.67	.45
rs1181388	GG	440 (75)						
	AG/AA	146 (25)	0.86	0.63 – 1.17	.32	0.82	0.53 – 1.25	.35
rs17533594	AA	372 (63)						
	AG/GG	215 (37)	1.03	0.78 – 1.35	.84	1.23	0.85 – 1.79	.28
rs3116494	AA	319 (54)						
	AG/GG	268 (46)	0.99	0.76 – 1.30	.96	1.13	0.79 – 1.62	.52
rs3181107	AA	506 (86)						
	AG/GG	81 (14)	0.81	0.54 – 1.21	.30	0.73	0.41 – 1.29	.28
rs3116496	TT	378 (64)						
(IVS3 +17)	CT/CC	209 (36)	1.16	0.88 – 1.53	.29	1.39	0.96 – 2.02	.08

Table 3. CTLA4

			Metastases-Free Survival			Overall Survival		
SNP	**Genotype**	**Cases (%) N=587**	**OR**	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**
rs16840252	CC	399 (68)						
	CT/TT	187 (32)	1.11	0.84 – 1.48	.46	1.25	0.85 – 1.82	.26
rs5742909	CC	476 (81)						
(CT44)	CT/TT	111 (19)	1.24	0.88 – 1.72	.22	1.40	0.90 – 2.18	.14
rs231775	AA	219 (37)						
(CT42)	AG/GG	367 (63)	0.99	0.75 – 1.30	.93	0.92	0.63 – 1.33	.64
rs231777	CC	414 (71)						
	CT/TT	172 (29)	1.04	0.78 – 1.39	.79	1.23	0.83 – 1.80	.30
rs3087243	GG	195 (33)						
(CT60)	AG/AA	392 (67)	0.99	0.74 – 1.31	.92	0.93	0.63 – 1.36	.69
rs11571319	GG	397 (68)						
(CT61)	AG/AA	190 (32)	1.10	0.83 – 1.45	.51	1.19	0.81 – 1.73	.38
rs11571302	GG	173 (30)						
(JO31)	GT/TT	414 (71)	0.95	0.71 – 1.27	.72	0.80	0.55 – 1.17	.26
rs7665213	GG	176 (30)						
(JO30)	AG/AA	411 (70)	0.95	0.72 – 1.27	.74	0.83	0.57 – 1.21	.32
rs11571297	TT	163 (28)						
(JO27)	CT/CC	424 (72)	0.87	0.65 – 1.16	.33	0.76	0.52 – 1.11	.16

Table 4. ICOS

			Metastases-Free Survival			Overall Survival		
SNP	**Genotype**	**Cases (%) N=587**	**OR**	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**
rs10932029	TT	412 (70)						
(+173)	CT/CC	175 (30)	0.87	0.65 – 1.17	.36	0.79	0.53 – 1.18	.25
rs4335928	TT	446 (76)						
	CT/CC	141 (24)	1.17	0.86 – 1.60	.32	1.26	0.82 – 1.92	.29
rs4675374	CC	352 (60)						
	CT/TT	235 (40)	1.08	0.82 – 1.42	.58	1.31	0.91 – 1.88	.15
rs7602383	AA	421 (72)						
	AG/GG	166 (28)	1.02	0.76 – 1.37	.88	1.12	0.75 – 1.66	.59
rs4521021	TT	345 (59)						
	CT/CC	242 (41)	0.96	0.73 – 1.26	.77	1.19	0.83 – 1.71	.35
rs11571323	GG	452 (77)						
	AG/AA	135 (23)	1.07	0.78 – 1.46	.70	1.27	0.83 – 1.94	.28
rs12466129	TT	340 (58)						
	AT/AA	247 (42)	0.86	0.66 – 1.13	.28	1.03	0.72 – 1.48	.88
rs10172036	GG	232 (40)						
	GT/TT	355 (61)	1.12	0.85 – 1.50	.42	0.86	0.58 – 1.26	.43
rs10183087	AA	352 (60)						
	AC/CC	234 (40)	0.87	0.66 – 1.15	.33	0.93	0.64 – 1.35	.70
rs10932036	AA	467 (80)						
	AT/TT	119 (20)	0.76	0.54 – 1.06	.11	0.76	0.48 – 1.19	.23

DISCUSSION

The immunogenic nature of malignant melanoma is clinically manifested by spontaneous regression and appearance of vitiligo. The phenomenon of autoimmunity observed during various forms of immunotherapy, IL-2, IFN and anti-CTLA4 therapy, has been linked to treatment response.[2-4] To understand the link between tumor immunity and autoimmunity in melanoma and to explore its implication on disease susceptibility and prognosis remains a challenge.[14] The results from studies evaluating polymorphisms in various autoimmune diseases suggest the existence of a common autoimmune disease locus in the CTLA4 gene.[9]

We genotyped 28 polymorphisms located in the CD28, CTLA4 and ICOS genes in melanoma patients and healthy controls. Use of tagging approach covered the entire loci for all three genes. To the best of our knowledge, the screen for SNPs in the CTLA4 gene was the largest ever performed in melanoma patients (and controls) and the first one for the ICOS and CD28 genes. Our results showed that the variant alleles for two polymorphisms in the CD28 gene (rs3181098 and rs3181100) were differentially distributed in cases and controls. No differences in genotype or allele frequencies were detected between melanoma patients and controls for any other polymorphism. Similarly, carriers of the variant allele for the polymorphism rs3181098 in the CD28 gene showed reduced metastasis free survival and for the polymorphism rs11571323 in the ICOS gene the individuals with variant allele homozygous type were associated with reduced overall survival. However, keeping in view the number of tests carried out in the present study, the observed significant associations would be lost upon multiple hypothesis correction. Moreover, the detected association would also require confirmatory testing in an independent population. One of the limitations of the present study included the lack of pigmentation data, history of sunburns and the existence of statistical significant difference in mean age between cases and controls. Keeping in view the fact that ethnicity and not the age is a major determinant of variant allele frequency, in our study design we ensured complete match between cases and controls for the latter parameter.

Our results are in accordance with a previous study that reported no difference in frequencies of six polymorphisms in the CTLA4 gene in 203 melanoma patients (stage IIB, IIC and III), compared to 288 healthy controls. Also no polymorphism correlated with improved recurrence-free or overall survival.[15] However, several studies have reported association of the CTLA4 polymorphisms with other malignancies.[16] In humans, CTLA4 exists in two isoforms, a full-length isoform and a soluble isoform that lacks exon 3 due to alternative splicing.[17] The CT60 (A/G) polymorphism in the CTLA4 gene is a key susceptibility locus for autoimmune diseases, and the G allele was shown to be correlated with decreased levels of the soluble isoform.[9] The frequency of the AA genotype for CT60 polymorphism was reported to be higher in renal cell carcinoma (RCC) patients than in

controls. In addition, a positive correlation between the AA genotype and tumor grade was also observed, suggesting a role in tumor development.[11] The CT42 polymorphism (49A/G) in exon 1 is the only amino acid (Thr>Ala) altering polymorphism in the CTLA4 gene; and individuals homozygous for the Ala allele were associated with decreased CTLA4 expression on the T cell surfaces.[18] The AA genotype was correlated with increased frequencies in RCC patients and the A allele, in association with the 3'-untranslated region $(AT)_{82}$ alleles, correlated with non-Hodgkin's lymphoma (NHL).[11,19] Interestingly, the GG variant was linked to an increased risk of gastric mucosa-associated lymphoid tissue (MALT) lymphoma.[20] In a study on multiple cancer types, 49A/G polymorphism has been associated with risk of lung, breast and esophageal cancers as well as gastric cardia.[16] CTLA4 with variant Thr allele has been shown to be associated with stronger inhibitory effect on T cell activation than that with common allele. Polymorphisms in the promotor region of the CTLA4 gene were described to modulate expression of the gene.[21] This region contains the CT44 polymorphism (-318 C/T) variant. The CC genotype of the CT44 polymorphism was shown to be correlated with significantly reduced lymph node involvement in breast cancer patients.[12] The T allele was linked to an increased risk of B-CLL but to a decreased risk of MALT lymphoma.[20,22] No correlation was found between the CT44 polymorphism and colon cancer.[23] The chromosomal region 2q33 containing the CTLA4 and CD28 genes has been linked with asthma, however, the association with polymorphisms in the genes was not detected.[24]

Melanoma patients with thick primary tumors and/or nodal involvement are at high risk for relapse or death.[13] However, adjuvant treatment is only beneficial in a small group of these patients. Genetic variability possibly predicts treatment outcome and could be a predictive marker to select the group benefiting from a certain treatment. In this study only stage I and II melanoma patients were evaluated for a possible association between SNPs and prognosis. Since these patients do not frequently receive systemic treatment, we could not assess the predictive value of any of the polymorphisms. Nevertheless, recently it was shown that polymorphisms in the CTLA4 gene were correlated with response in melanoma patients (stage IV) receiving anti-CTLA4 treatment.[25]

In conclusion, from the results of this large study we did not find convincing evidence for association between polymorphisms in the CD28, CTLA4 and ICOS genes and the risk of melanoma, nor with an effect on prognosis. Even two individual polymorphisms showed differential distribution of variant alleles between cases and controls, the effect nevertheless was marginal and a chance factor could not be ruled out. The study was confined to German population, therefore, a strong association of polymorphisms investigated with melanoma susceptibility or disease outcome, in other populations cannot be entirely precluded.

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Chapter IX

General Discussion

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Outcome in melanoma patients with advanced disease is poor and systemic treatment seems to benefit only a subset of patients. Predictive markers identifying these patients are currently not available. Early studies showed an association of immune-related side effects such as vitiligo and autoimmune thyroiditis with response to IL-2 or IFN treatment. However, conflicting data have been reported as well, mentioning the effect of a higher rate of immune-related toxicities during prolonged administration of the drug in responders. Several (immune-related) factors which have been associated with melanoma prognosis will be discussed, thereby focusing on the potential predictive value of these factors in patients receiving immunotherapy. Part one of the discussion reviews the prognostic factors analyzed in this thesis and part two describes other important immune-related prognostic factors such as, white blood cell count, absolute lymphocyte count and human leukocyte antigen.

PART ONE

1. Cytokines

Inflammation and cancer progression are closely related.[1] Inflammatory cytokines and chemokines, which can be produced by tumor cells and/or tumor-associated leucocytes and platelets may contribute directly to malignant progression.[2] Several studies showed that melanoma cell lines produce various cytokines, growth factors and their receptors.[3,4] In metastatic patients receiving systemic treatment, elevated baseline levels of IL-6 were associated with poor prognosis.[5,6] Furthermore, in a limited set of patients with metastatic melanoma, serum IL-6 levels were higher in patients with weight loss, who were anaemic, had elevated LDH levels and in patients who were not responding to chemotherapy.[7] On the contrary, multiplex analysis of a panel 29 cytokines in serum of high-risk melanoma patients showed the association of higher pre-treatment levels of the pro-inflammatory cytokines IL-1α, IL-1β, IL-6, TNF and the chemokines MIP-1α and MIP-1β with longer (> 5 years) RFS in IFN-treated patients.[8] However, a mean IL-6 baseline level between 2000 and 3000 pg/ml for the group with a RFS longer than 5 years is exceptionally high and not reported before in melanoma patients. Suggestive for the existence of a confounding factor, producing a false positive test result.

IL-6 and IL-1β levels were measured by ELISA in serial serum samples of 185 stage IIB-III melanoma patients, accrued to the EORTC 18952 trial (this thesis). Incidence of detectable IL-6 levels in treated and untreated patients was too low (< 5%) to make valid statistical analysis possible, however there was some proof that IL-6 levels > 10 pg/ml were correlated with progressive disease or adverse events (data not shown). The highest measured IL-6 value during follow-up was 390 pg/ml (ELISA), this patient suffered from multiple distant metastases and died one month later. Results from the IL-6

ELISA were comparable with those obtained by multiplex technique. For the IL-1β ELISA we found an unexpected high positive rate in patients and controls and a discrepancy with the multiplex assay, suggesting that these data included false positive test results. The occurrence of heterophilic antibodies in human sera, cross linking the mouse and/or bovine antibodies used in the sandwich ELISA, thereby causing false positive test results, was previously described for the IL-1β ELISA.[9] Therefore, human studies that rely on ELISA detection of cytokine levels alone must be interpreted with caution. Preferably, a confirmatory assay should be performed when determining human cytokine levels.

2. C-reactive protein

High serum levels of C-reactive protein (CRP) were associated with shortened survival in metastatic melanoma and resistance to treatment with interleukin-2.[6,10] It is a serum marker, which could discriminate melanoma patients entering AJCC stage IV from patients remaining in AJCC stages I, II or III.[11] Findeisen et al. showed that serum amyloid A (SAA) and CRP combined were also useful prognostic markers in early-stage melanoma.[12] Serum mass spectrometry revealed a peak at m/z 11.680 differentiating between stage I en IV melanoma, which could later be identified as SAA. In univariate analysis SAA and CRP were prognostic marker in 276 stage I-III melanoma patients ($P = .04$ and $P = .006$ respectively) and in 103 stage IV patients (both $P < .0001$). Multivariate analysis including the well-known prognostic markers and the serum markers; S100b, CRP, LDH and SAA, revealed sex, stage, tumor load as well as S100b, CRP, and SAA as prognostic markers in stage I-IV disease with an interaction between CRP and SAA. A significant prognostic discrimination was found for the combination of these two markers in stage I-III ($P = .01$) and stage IV ($P < .0001$).

Stam et al. previously assessed the effects of interferon α2b on the acute-phase response in a small subset of stage IIB-III melanoma patients participating in the EORTC 18952 trial.[13] They found significant increases in ferritin levels and less pronounced decreases in CRP levels at end of induction and at 6 months in the treated group as compared to baseline levels. We recently extended the analysis in order to explore the association of ferritin and CRP changes over time with clinical response to IFN therapy (this thesis). Ferritin levels in the IFN-treated patients were increased from end of induction and remained high during the treatment period. CRP levels varied slightly during the course of the study but no significant differences between treated and untreated patients were observed. Some patients in the IFN group had decreased CRP levels at end of induction. However, none of these changes were predictive for treatment response in patients receiving IFN (*In press*).

3. Autoimmunity

3.1. IL-2

Hypothyroidism was the first described autoimmune phenomenon associated with a favorable tumor response after treatment with IL-2 and lymphokine-activated killer cells.[14] Tumor regression occurred in five out of seven (71%) patients with laboratory evidence of hypothyroidism in contrast to 5 of the 27 euthyroid patients (19%). Similar observations were reported by others, yet these studies involved a limited set of patients.[15,16] Also, Rosenburg et al. described the strong correlation between the occurrence of vitiligo in patients with metastatic melanoma and response to IL-2 therapy.[17] The occurrence of vitiligo and thyroid dysfunction was evaluated by Phan and colleagues in 372 patients receiving IL-2 treatment.[18] Responders were more likely to develop thyroid dysfunction and vitiligo. The authors limited their evaluation to the presence of thyroid dysfunction and or vitiligo by day 60 in order to correct for the possible confounding factor that long-term immunologic side effects were due to prolonged IL-2 administration. This could occur in responders since they continued on therapy. Thyroid dysfunction was not associated with treatment response anymore, which is in agreement with two previous studies showing the association of a higher incidence of thyroid dysfunction with prolonged IL-2 treatment but no relation with clinical response.[19,20] Since it takes time to develop vitiligo, it is difficult to differentiate between a true association with treatment response and the appearance of vitiligo as a result of prolonged IL-2 treatment (in responders). A similar study by Boasberg et al. demonstrated in a multivariate analysis that vitiligo was predictive for improved survival (HR: 0.50, $P = .04$). Median time to onset of vitiligo was 35 days (ranging 24-202 days). When time to occurrence of vitiligo was taken into account, using it as a time-dependent covariate, vitiligo was not a significant predictor of survival (HR = 0.55, $P = .09$).[21]

3.2. IFN

Gogas et al. were the first to evaluate prognostic significance of autoimmune antibodies and or clinical signs of autoimmunity (vitiligo) in melanoma patients treated with adjuvant high-dose interferon (HDI).[22] Antithyroglobulin, antinuclear or anticardiolipin antibodies were detected in 24% of the patients, and also no more than three patients (2%) developed vitiligo without the occurrence of autoantibodies. Autoimmunity during treatment was associated with a significant RFS and OS benefit according to Kaplan-Meier estimates. Another study in patients treated with low-dose interferon (LDI), also showed a correlation with autoimmunity and a significantly better RFS ($P = .05$) and a trend towards improved OS ($P = .07$).[23] The results of abovementioned trials appeared promising, however, both studies analyzed the effects of autoimmunity on outcome in

patients receiving IFN treatment, no observation group was evaluated. Therefore, the predictive value of autoimmunity still needed to be elucidated.

In contrast, we described the association of autoantibodies in patients receiving intermediate-doses of interferon or no treatment with RFS (this thesis).[24] The analysis was performed in a subset of patients participating in the EORTC 18952 or the Nordic IFN trial.[25,26] At baseline, 33% of the patients in the EORTC 18952 and 35% of the patients in the Nordic IFN study tested positive for antithyroglobulin, antinuclear or anticardiolipin antibodies, however, no difference in RFS between patients with or without pre-existing autoantibodies was observed. During follow-up, autoantibodies were more frequently detected in patients receiving interferon as compared to untreated patients, 36% (EORTC 18952) and 39% (Nordic IFN) versus 26% and 14% respectively in the observation arm of both trials. Seroconversion in IFN-treated patients correlated with improved outcome if a Cox model (model 1) in which antibody status was time independent was used (EORTC 18952, HR = 0.37, $P < .001$ and Nordic IFN, HR = 0.49, $P = .002$). When treating antibody status as a time-dependent variable, no strong association with RFS was found. Furthermore, results from a comparable side study from the ECOG 2696 trial were in agreement with the previous study.[27]

Guarantee-time bias is an important confounder when analyzing the prognostic value of any potential biomarker in serial measurements and should be taken into account.[28,29] Satzger et al. did not perform landmark analyses or time-dependent Cox model analyses to correct for guarantee-time bias and therefore the observation that autoimmunity is beneficial in patients treated with LDI is difficult to interpreter.[23] Moreover, conflicting data were reported when correction was made, autoimmunity remained a strong independent prognostic marker in the Gogas's study but it lost significance in the side studies from the EORTC 18952, Nordic IFN and ECOG 2696 trials.[22,24,27] Although the same ELISAs (Quanta Lite, Inova Diagnostics) and titers were used in the studies by Gogas et al. and the EORTC/Nordic Melanoma group collaboration, major differences were found for the occurrence of autoantibodies at baseline. Thirty-three percent of the patients from the EORTC 18952 study and 35% of those in the Nordic IFN trial respectively had pre-existing autoantibodies, against 1,5% reported by Gogas et al. This seems very low since antinuclear antibodies for instance are commonly detected in healthy persons, with reported prevalences ranging 4 to 35%, and prevalences up to 40% in cancer patients.[30-33] The higher seroconversion rate in the EORTC 18952 and Nordic IFN trials as compared with Gogas's study and the ECOG 2696 trial, could be explained by longer treatment duration in the EORTC 18952 and Nordic IFN trial and a longer sampling period in these trials. Interestingly, the median time to seroconversion was only 3 months in the study by Gogas et al. compared to 6-12 months in the trials reported by the

EORTC/Nordic melanoma group. A possible explanation for this difference could be the fact that the induction treatment comprised a higher dosage and was delivered intravenously (versus subcutaneously) in the study by Gogas et al. One could argue that only clinical manifestations of autoimmunity resemble "true autoimmunity" and should be used to identify possible treatment responders. This seems less likely since autoimmune diseases are often preceded and/or accompanied by the occurrence of autoantibodies. Moreover, from the 52/200 (26%) of the IFN-treated patients with signs of autoimmunity in the study by Gogas et al., only three (2%) of the patients had clinical manifestations of autoimmunity (vitiligo) without autoantibodies. Therefore, using autoantibodies as an index of immune response seems reasonable.

Recently the association of autoimmune antibodies in patients receiving long-term (5 years) treatment with pegylated (PEG)-IFN or no treatment (observation) and prognosis, was described in patients accrued to the EORTC 18991 trial (this thesis).[34] Results were comparable, a correlation with improved outcome (in all patients) according to the (biased) Cox model, yet upon correction for guarantee-time bias using the time-dependent Cox models, the occurrence of autoantibodies lost significance. The effect of seroconversion on outcome in observation patients or PEG-IFN treated patients separately were similar, suggesting that seroconversion is also not a predictive serological marker for treatment outcome.

3.3. Anti-CTLA-4 therapy

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is an important inhibitor of T cell activation.[35,36] Blocking CTLA-4 with neutralizing antibodies, ipilimumab or tremelimumab, is therefore a promising approach to augment antitumor immune responses. Treatment with ipilimumab has been more extensively investigated, showing objective response rates ranging from 5% to 17%.[37-43] Attia et al. were the first to describe the correlation of severe autoimmune side effects such as dermatitis and colitis in 56 stage IV melanoma patients, with clinical response to ipilimumab.[40] Five of the 14 patients with immune-related adverse events (irAEs) exhibited a clinical response, whereas only 2 of the 42 patients without autoimmune side effects responded ($P = .008$). This association has also been reported by others.[41,44] A recent study by Wolchok et al. elicited a dose-dependent effect of ipilimumab on efficacy and safety in patients with advanced melanoma.[39] Best overall response rate (the proportion of patients with a complete or partial response, according to modified WHO criteria), was 11.1% for 10 mg/kg (n=72), 4.2% for 3 mg/kg (n=72), and 0% for 0.3 mg/kg (n=73). IrAEs of grade 3-4 arose in none of the patients in the 0.3 mg/kg group, in 5 patients of the 3 mg/kg group and in 18 patients of the 10 mg/kg group. The most common grade 3-4 adverse events were gastrointestinal immune-related events and generally managed successfully with steroids. This study, however,

did not explore the association between the occurrence of irAEs and clinical response. Another study reported for patients receiving ipilimumab (10 mg/kg) that patients suffering grade 3-4 irAEs at week 24 had a significantly higher clinical response rate as compared to those with grade ≤ 2 irAEs ($P < .01$).[45] Improved overall survival was shown by Hodi et al. in a large phase III study in metastatic melanoma for patients receiving ipilimumab (3 mg/kg) plus glycoprotein 100 (gp100) peptide vaccine compared to patients treated with gp100 alone (HR = 0.68, $P < .001$).[43] The frequency of grade 3 or 4 irAEs was 10 to 15% in the ipilimumab group and 3% in the gp100 alone group, all occurring during the induction and reinduction periods. Among the 94 patients who survived for 2 years many experienced residual effects such as vitiligo (n = 12) and endocrine immune-related adverse events requiring hormone-replacement therapy (n = 8).

Tremelimumab is a newer agent which is generally tolerable and has demonstrated antitumor activity.[46,47] Although ipilimumab and tremelimumab have not been compared directly, it seems that response rates with tremelimumab are lower, which might be explained by suboptimal dosing. Notably is the fact that these patients also developed less immune-related toxicities as compared to ipilimumab-treated patients. The only evidence for a correlation between treatment response and irAEs in 30 patients receiving tremelimumab ($P = .05$) was found by Reuben et al.[48] Five patients suffered from grade 3 diarrhea and 1 patient from grade 3 dermatitis but no grade 4 adverse events were observed. A more recent trial evaluating efficacy and safety in patient treated with tremelimumab, 15 mg/kg, every 90 days, reported an objective response rate of 6.6%, with all responses being durable[47]. Grade 3-4 adverse events included diarrhea (11%), fatigue (2%) and colitis (4%). Furthermore, Camacho et al. showed for the tremelimumab 15 mg/kg arm a treatment-related adverse event rate of 13% including; diarrhea, rash, pruritus, fatigue and nausea, and a serious adverse event rate of 9%.[46] Rates for immune-related adverse events were not mentioned. It is not unthinkable that if tremelimumab dosing would be intensified, the number of immune-related adverse events and the clinical response rates could be increased.

Recently it was shown by Breunis et al. that polymorphisms in the CTLA4 gene were correlated with response in melanoma patients (stage IV) receiving anti-CTLA4 treatment.[49] We genotyped 28 polymorphisms located in the CD28, CTLA4 and ICOS genes in German melanoma patients and German healthy controls (this thesis).[50] None of the polymorphisms were associated with prognosis when corrected for multiple testing. In this study only stage I and II melanoma patients were evaluated for a possible association between SNPs and prognosis. Since these patients do not frequently receive systemic treatment, we could not assess the predictive value of any of the polymorphisms. Our results are in accordance with another study that reported no difference in frequencies

of six polymorphisms in the CTLA4 gene in 203 melanoma patients (stage IIb, IIc and III), compared to 288 healthy controls. Also no polymorphism correlated with RFS, OS or autoimmunity.[51]

3.4. Is it possible to uncouple tumor immunity and autoimmunity?

The immunologic concepts behind concomitant tumor immunity and autoimmunity are complex.[52,53] Although tumor immunity and autoimmunity use similar mechanisms, uncoupling is possible.[54] Various mechanisms have been described.[55-57] Results from a vaccination (AdhDCT) study in mice suggested that tumor immunity and autoimmunity could be separated by modulating the STAT4/STAT6 signaling axis.[58] In Stat4-deficient mice Th1 development and IFNγ production are impaired while Th2 functions are intact. On the contrary, Stat6-deficient T cells cannot differentiate into IL-4 producing Th2 cells but develop into IFNγ producing Th1 cells. Vaccination in Stat6-deficient mice, resulted in antitumor immunity and the occurrence of autoimmunity (vitiligo). However, after depletion of CD8+ T cells, tumor protection was lost but occurrence of vitiligo was not affected, indicating that tumor immunity was dependent upon STAT6 signaling. The opposite was observed in Stat4-deficient mice following depletion of CD8+ T cells. The mice did not develop vitiligo yet antitumor immunity was preserved. Similar studies are awaited for other agents like IL-2, IFN and anti-CTLA-4.

4. S100B

While many reports show that S100B blood levels provide a good indication of disease progression as well of response to chemotherapy in stage IV patients,[59-61] it remains of little or limited usefulness in early stages (II and III) where the disease is most often confined to lymph nodes.[62] Moreover, the progression from the early stages to distant organ involvement is frequently accompanied by a significant increase in S100B levels.[63] Here we evaluated the utility of serial determinations of S100B serum levels in patients with high-risk melanomas, using a time-dependent Cox model. We demonstrated in this corollary study to the EORTC 18952 trial in stage IIB-III melanoma patients that serial determinations of S100B serum levels strongly correlate with DMFS and OS (this thesis).[64] Strikingly, the hazard ratio (HR) for S100B determinations was higher and more significant than the one corresponding to stage, and number of positive lymph nodes, two strong prognostic factors in stage III melanoma. Median time between S100B ≥ 0.2 µg/l and the development of distant metastases was 94 days. Since the prognostic value of S100B was not analyzed separately for the untreated and IFN-treated patients, its predictive significance could not be established. However, one could argue that patients receiving IFN should discontinue therapy once S100B levels of ≥ 0.2 µg/l have been reached, indicating disease progression and no treatment response.

5. YKL-40

High serum levels of YKL-40 has been suggested as a new independent prognostic biomarker of poor survival in patients with melanoma stage I, II and IV, and in other types of primary and metastatic cancer with highest levels in metastatic disease.[65-67] YKL-40 levels were determined in stage IIB-III melanoma patients participating in the EORTC 18952, the Nordic IFN or the EORTC 18991 trial (this thesis). We showed that elevated baseline levels of YKL-40 in observed patients from the Nordic IFN trial and the EORTC 18952 trial was an independent prognostic marker for short OS. Furthermore, a multivariate analysis in the Nordic IFN and EORTC 18952 trial demonstrated that this effect in observed patients was not present in IFN-treated patients, suggesting a possible predictive value of YKL-40. High YKL-40 levels could define a high-risk subgroup population, benefiting IFN therapy. However, these results need to be confirmed, especially since this effect was not described in the patients receiving PEG-IFN. Moreover, in patients receiving treatment, IFN or PEG-IFN, we found that increases in serum YKL-40 during follow-up were associated with decreased OS but not with decreased RFS. The value of monitoring YKL-40 should be further explored, and preferably in combination with other prognostic factors such as LDH and S100B which have shown to be of significance in detecting distant metastases.

PART TWO

6. White blood cell count

Initially in patients with metastatic renal cell carcinoma receiving immunotherapy with IL-2 or IFN, baseline elevated neutrophil counts in peripheral blood were associated with poor survival.[68] A study by Schmidt et al. in 321 stage IV melanoma patients reported similar results for patients treated with IL-2-based immunotherapy.[69] In univariate analyses, elevated neutrophil counts ($P < .001$) and elevated monocyte counts ($P < .001$) were identified as prognostic factors. Entering elevated neutrophils (or monocytes) in a multivariate analysis comparable results were found ($P = .02$). A validation study in stage IV melanoma patients accrued to the EORTC 18951 biochemotherapy trial confirmed these findings.[70] Pretreatment elevated neutrophils count was an independent prognostic factor for reduced overall survival (HR = 1.5, $P = .02$), and a high leukocyte count was an independent prognostic factor of both reduced overall survival (HR = 1.7; $P < .001$) and reduced progression-free survival (HR = 1.5, $P = .008$).

Recently six cases of paraneoplastic granulocytosis were described in a series of 626 patients with metastatic melanoma.[71] These patients were found to have unexplained leukocytosis with neutrophilia. After extensive work-up for infectious disease, serum granulocyte colony-stimulating factor (G-CSF) level was determined and was abnor-

mally elevated in all patients. The degree of leukocytosis directly correlated to the level of serum G-CSF. In three patients the onset of paraneoplastic granulocytosis did not occur with the appearance of metastatic melanoma but at later point in the course of the disease. It is unknown whether G-CSF-secreting melanoma tumors are indicative for a more aggressive tumor phenotype or whether these tumors respond differently to therapy.

7. Absolute lymphocyte count

An early report by Bernengo et al. already provided evidence that melanoma patients with normal lymphocyte counts had a better prognosis compared to those with reduced lymphocyte counts.[72] Furthermore, in patients with metastatic melanoma receiving IL-2, absolute lymphocyte count immediately after therapy was significantly higher in responders compared with non-responders.[18] Moreover a higher change in lymphocyte count, as compared to baseline value, was documented in responders. Generally, rebound lymphocytosis peaked 2 to 5 days after cessation of IL-2. A positive association between lymphocyte count and response was also reported by some others.[73,74]

Recently, in 51 patients with advanced melanoma who were treated with ipilimumab 10 mg/kg, clinical benefit was correlated with absolute lymphocyte count.[45] Patients underwent laboratory testing before each ipilimumab administration. In order to correlate absolute lymphocyte count (ALC) to outcome, patients were stratified based on a cutoff of ≥ 1000/μL (high ALC) versus < 1000 cells/μL (low ALC). At baseline there was a borderline significant trend towards improved overall survival for the high ALC group (P = .06), which remained after correction for LDH. After the first ipilimumab dose, patients with high ALC had a significantly improved overall survival (P < .01), however this association was most pronounced after the second dose of ipilimumab, with a median overall survival of 11.9 months versus 1.4 months and p < .0001. Similar results were found when corrected for LDH. Although this study comprised a relatively small number of patients and no detailed multivariate analysis was performed, the results might have strong implications in future trials and clinical practice. These data suggest that patients with ALC < 1000 cells/μL do not benefit from ipilimumab treatment and could be spared of further toxicity.

8. Human leukocyte antigen

Serological typing for human leukocyte antigen (HLA) class I and class II antigen expression has previously shown to be associated with melanoma prognosis and treatment response.

An early report by Lee et al. suggested that the risk of melanoma incidence or progression could be influenced by HLA-DQB1*0301.[75] Stage I/II melanoma patients with positive HLA-DQB1*0301 status were at an increased risk of developing recurrent

disease compared to stage-matched patients lacking this allele.[76] Also HLA-DRB1*1101 was associated with disease recurrence in patients with localized melanoma.[77] Clinical response in patients with metastatic melanoma receiving IL-2 was described for patients expressing HLA-DQ1.[78] Another report in a larger set of patients could not confirm this beneficial effect of HLA-DQ1, instead, demonstrated an association of homozygosity of HLA-DR and tolerance to IL-2 treatment.[79] In a small study of 32 melanoma patients Scheibenbogen et al. reported an association between HLA-B44 and HLA-Cw7 and response to IL-2, however in a later analysis of 54 patients, the same authors did not find an association between HLA-B44 and response, while HLA-Cw7 remained marginally correlated.[80] Recently, HLA typing in 284 high-risk melanoma patients receiving high-dose adjuvant interferon revealed that the 55 patients expressing HLA-Cw*06 had a better relapse-free and overall survival compared to the 229 patients with a negative HLA-Cw*06 status.[81] When controlling for disease stage in a multivariate analysis, the p-values for the association of HLA-Cw*06 with RFS and OS were .02 and .04 respectively.

Abovementioned studies provide evidence that human leukocyte antigen is associated with melanoma prognosis and treatment response, however many contradicting reports have been published. Especially the older studies included only a limited set of patients and validation in a larger set of patients often failed to confirm the previous findings. Results could be different since patients of various ethnic backgrounds have been analyzed. Also, HLA typing techniques have been improved over the years. Moreover, one should keep in mind that in these kinds of analyses multiple testing should be corrected for. Larger studies are needed to clarify the role of HLA in melanoma prognosis. Ideally, both treated and untreated patients are analyzed to assess its potential predictive value as well.

CONCLUSION

For decades we have been trying to improve survival rates in melanoma patients but have we been really successful? Incidence rates are still increasing, but mortality rates are much more slowly increasing or even flattening out, which is reflective of the fact that increased awareness and a more aggressive diagnostic attitude regarding naevi results in mostly thin, non-metastasizing melanomas being diagnosed at present. So this relative improvement is due to early recognition of thin melanomas that have an excellent prognosis and not because of improvement of systemic treatment since this has been disappointing until only very recently.

Since the overall treatment effect of IFN is marginal it is difficult to identify a predictive marker selecting responders. Immune-related markers were reviewed above; some of these are promising but need further validation. Meta-analysis of the EORTC 18952

and 18991 trials revealed that patients with ulcerated primaries were more sensitive to (PEG-) IFN than patients with non-ulcerated primaries. Moreover, this effect was most pronounced in stage IIB/III-N1 patients.[82] The EORTC 18081 trial, which compares PEG-IFN versus observation in patients with ulcerated primaries > 1mm (stage II), will provide important information concerning the future utility of PEG-IFN.

Only recently, two new drugs changed the whole therapeutic landscape in melanoma, ipilimumab and the highly selective BRAF inhibitor PLX4032 were the first agents showing significant responses in stage IV melanoma.[83] And this seems just the beginning. The first line phase trial in patients with advanced metastatic melanoma (stages IIIC + IV), comparing DTIC vs ipilimumab + DTIC, was recently reported to have been unblinded demonstrating a significant survival benefit for the ipilimumab containing arm, will be presented at ASCO annual meeting in June 2011. Ipilimumab has already been shown to improve survival in 2nd line significantly (doubling of survival at 2 and 3 years) in patients with advanced melanoma, and was approved in March 2011 by the FDA. In first line the results may be even more spectacular. Data from phase II studies with ipilimumab in first line suggest a 2 year survival rate of above 50%. If this is also the case in the pivotal phase III trial to be reported at ASCO, it would probably indicate that survival at 2 and 3 years in the ipilimumab containing arm may be up to three times better than in the DTIC alone arm. Moreover, immune-related adverse events and absolute lymphocyte count were associated with treatment response. Response to the BRAF inhibitor PLX4032 is dependent on the BRAF mutation V600E, present in about 60% of melanoma. When present, response rates up to 80% were described but in absence of the mutation the drug seems not beneficial. The first interim analysis of the worldwide pivotal phase III trial (BRIM3-trial) comparing PLX4032 with DTIC in patients with advanced metastatic melanoma has lead to the unblinding of the study because of a significant impact on both progression-free survival and overall survival in the PLX4032 arm. The detailed results will also be reported at the 2011 ASCO annual meeting.

With the introduction of these new drugs it is essential that we continue the search for prognostic and predictive markers. The advances in understanding of the immune system and the host-tumor interactions should ultimately lead to more effective and tailor-made treatment. Immunotherapy has now proven to provide survival benefit, however it is associated with considerable toxicity. Therefore one of the future's challenges will be to induce more potent tumor immunity balancing autoimmune side effects.

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Chapter X

Summary

The incidence of melanoma is rising dramatically worldwide. This increase is characterized largely by an increase in thin melanomas. Whereas prognosis is excellent for patients with thin, non-ulcerated primary tumors, patients with thick primary tumors or lymph node involvement on the other hand are at high risk of recurrence or death. Adjuvant treatment after definitive surgery in these patients remains disappointing. A consistent effect of adjuvant interferon-α (IFN) on recurrence-free survival (RFS) but only a marginal impact of 3% on overall survival (OS) was reported. Seemingly, only a relative small proportion of patients benefits from IFN therapy. Identification of a subgroup of patients who respond to IFN, to spare others from needless toxicity, would be of great benefit. Several potential prognostic and predictive factors were analyzed in stage IIB-III melanoma patients receiving adjuvant (pegylated) interferon-α and are described in this thesis.

Prognostic factors related to the acute phase response were evaluated in **SECTION ONE** of the thesis. In **chapter II** we report on the cytokine determination of IL-6 and IL-1β in 185 melanoma patients randomized to the EORTC 18952 trial of adjuvant IFN vs observation and healthy controls. IL-6 and IL-1β serum levels were determined by ELISA at baseline and during follow-up up to 30 months. A selection of 78 samples was also tested by multiplex assay. The number of samples above the detection limit for the IL-6 ELISA in melanoma patients was comparable to the number in a study population of healthy controls. Moreover, the incidence was too low to establish the predictive value of IL-6 in IFN-treated patients. An unexpected high rate of elevated IL-1β levels in sera from patients and healthy controls was found and comparisons with the multiplex assay disagreed. This led to the hypothesis that the data for IL-1β included false positive test results, possibly due to the existence of heterophilic antibodies in human sera that crosslink the mouse and/or bovine antibodies used in the sandwich ELISA. We showed that the addition of purified bovine immunoglobulin and 1% normal mouse serum to the buffer decreased the IL-1β levels in half of the tested sera.

Changes of ferritin and CRP levels and prognostic value on treatment outcome were analyzed in **chapter III**. Serial ferritin and CRP levels were determined before treatment and up to 24 months in patients participating in the EORTC 18952 trial. Baseline ferritin levels were comparable in the 2 treatment groups. However, ferritin ratios were significantly higher in IFN-treated patients (N = 96) compared to untreated patients (N = 21), at end of induction (mean: 2.88 vs 0.75; $P = .0003$) and at 6 months (mean: 3.18 vs 1.02; $P = .009$). In the IFN arm, higher ferritin ratios at end of induction and at 6 months were not associated with improved outcome. Concerning CRP ratios, no differences between the treatment groups, neither an association with DMFS, were observed.

In **SECTION TWO**, the prognostic factor autoimmunity was evaluated. The immunogenic nature of melanoma is clinically manifested by spontaneous regression and appearance of vitiligo. Although the effect of the phenomenon on prognosis is

rather unclear, the appearance of vitiligo is considered a favorable prognostic factor. Previously, Gogas et al. reported that the appearance of autoantibodies and clinical manifestations of autoimmunity in melanoma patients treated with adjuvant high-dose IFN was strongly associated with improved RFS and OS. In **chapter IV** the association of autoantibodies and prognosis in patients from two randomized trials, the EORTC 18952 and the Nordic IFN trial is shown. Serum levels of anticardiolipin, antithyroglobulin, and antinuclear antibodies were determined using ELISA. The association of the presence of autoantibodies with risk of recurrence was assessed by three Cox models in patients negative for all three autoantibodies at baseline (125 from the EORTC 18952 trial and 230 from the Nordic IFN trial): 1) a model that considered appearance of autoantibodies as a time-independent variable, 2) one that considered a patient autoantibody positive once a positive test for an autoantibody was obtained, and 3) a model in which the status of the patient was defined by the most recent autoantibody test.

When treated as a time-independent variable (model 1), appearance of autoantibodies was associated with improved RFS in both trials (EORTC 18952, HR = 0.41, $P < .001$; and Nordic IFN, HR = 0.51, $P < .001$). However, on correction for guarantee-time bias, using models 2 and 3, the association was weaker and not statistically significant.

We validated these results in **chapter V** for patients accrued to the EORTC 18991 trial receiving adjuvant pegylated (PEG) IFN or no treatment. Patients who were autoantibody negative at baseline were analyzed (n = 220). Occurrence of autoantibodies during follow-up was higher in the PEG-IFN–treated patients (52%) as compared to the observation arm (18%). Autoantibody appearance was of prognostic importance by using model 1 (HR = 0.56, $P = .01$). However, when guarantee-time bias was taken into account using model 2 (HR = 1.19, $P = .46$) or model 3 (HR = 1.14, $P = .59$), significance was lost. Results were similar when treatment groups were analyzed separately, indicating that the appearance of autoimmune antibodies is neither a prognostic nor a predictive factor for improved outcome in patients treated with PEG-IFN.

In **SECTION THREE** the prognostic significance of S100B, YKL-40 and polymorphisms in the CD28, CTLA4, and ICOS genes are described. Firstly in **chapter VI**, the prognostic value of serial S100B determinations was analyzed in 211 patients who participated in the EORTC 18952 trial. The Cox time-dependent model was used to assess prognostic value of the latest (most recent) S100B determination (model 3). At first measurement, 178 patients had S100B values < 0.2 µg/l and 33 ≥ 0.2 µg/l. Within the first group, 61 patients had, later on, an increased value of S100B (≥ 0.2 µg/l). An initial increased value of S100B, or during follow-up, was associated with worse DMFS; HR of S100B ≥ 0.2 vs S100B < 0.2 was 5.57, $P < .0001$, after adjustment for stage, number of lymph nodes and sex. Similar results were observed regarding OS. We showed that the prognostic impact of S100B ≥ 0.2 µg/l was more pronounced in stage III disease compared with stage IIB.

In **chapter VII**, YKL-40 levels were determined in patients participating in the EORTC 18952, the Nordic IFN or the EORTC 18991 trial. We showed that elevated baseline levels of YKL-40 in observed patients from the Nordic IFN trial and the EORTC 18952 trial was an independent prognostic marker for short OS (HR = 1.33, $P = .04$) . However this effect was not demonstrated in IFN-treated patients. Moreover, in patients receiving treatment, IFN or PEG-IFN, we found that increases in serum YKL-40 during follow-up were associated with decreased OS but not with decreased RFS.

The association of polymorphisms in the CD28, CTLA4 and ICOS genes with the risk of melanoma was investigated in **chapter VIII**. In total, 28 SNPs were genotyped in a cohort of 763 German melanoma patients and 734 healthy German controls. Influence on prognosis was determined in 587 melanoma cases belonging to stage I or II of the disease. In general, no differences in genotype or allele frequencies were detected between melanoma patients and controls. Similarly no association of any polymorphism with prognosis, except for the rs3181098 polymorphism in the CD28 gene, was observed. In addition, individuals with AA genotype for rs11571323 polymorphism in the ICOS gene showed reduced overall survival. However, keeping in view the correction for multiple hypothesis testing our results suggest that the polymorphisms in the CD28, CTLA4 and ICOS genes at least do not modulate risk of melanoma and nor do those influence the disease prognosis.

In **chapter IX** an overview is given of several (immune-related) factors which have been associated with melanoma prognosis, prognostic factors described in this thesis were included. Moreover, the potential predictive value of these factors in patients receiving immunotherapy was addressed.

Chapter XI

SAMENVATTING

De incidentie van het melanoom stijgt wereldwijd dramatisch. Deze incidentie wordt met name gekarakteriseerd door een toename in dunne melanomen. Hoewel de prognose voor patiënten met dunne, niet-geulcereerde primaire tumoren uitstekend is, hebben patiënten met dikke primaire tumoren of lymfkliermetastasen een hoog risico op een recidief of overlijden. Na definitieve chirurgie blijft adjuvante therapie teleurstellend voor deze patiëntengroep. Eerdere studies hebben een consistent effect gerapporteerd van adjuvant interferon-α (IFN) op ziekte-vrije overleving, hoewel maar een marginaal effect van 3% op totale overleving werd gevonden. Blijkbaar heeft slechts een relatief klein deel van de patiënten profijt van therapie met IFN. De identificatie van een subgroep van patiënten die op IFN reageert zou een grote uitkomst zijn om zo anderen onnodige toxiciteit te kunnen besparen. Verscheidene potentiële prognostische en predictieve factoren werden geanalyseerd, bij stadium IIB-III melanoompatiënten die adjuvant behandeld werden met (gepegyleerd) interferon-α. De resultaten van deze onderzoeken staan beschreven in dit proefschrift.

Prognostische factoren die gerelateerd zijn aan de acute fase respons werden geëvalueerd in **DEEL ÉÉN** van dit proefschrift. In **hoofdstuk II** rapporteren we over cytokine bepalingen van IL-6 en IL-1β in 185 melanoompatiënten die hebben deelgenomen aan de EORTC 18952 studie van adjuvant IFN versus observatie, evenals in een gezonde controlegroep. De uitgangswaarden en niveaus van IL-6 en IL-1β serum werden tijdens follow-up tot 30 maanden bepaald middels ELISA. Een selectie van 78 samples werd bovendien ook met behulp van multiplex assay getest. Het aantal samples boven de detectiegrens voor de IL-6 ELISA in melanoompatiënten bleek vergelijkbaar te zijn met het aantal in de groep gezonde controles. Bovendien was de incidentie te laag om de predictieve waarde van IL-6 in IFN behandelde patiënten te bepalen. Er werd een onverwacht hoog aantal verhoogde IL-1β levels in sera van patiënten en gezonde controles gevonden, waarbij de data bovendien niet overeenkwamen met de multiplex assay. Dit heeft tot de hypothese geleid dat de IL-1β data vals-positieve testresultaten bevatten, welke mogelijk het gevolg zijn van heterofiele antilichamen in humaan serum die zich kunnen binden met het muis en/of rund antilichaam dat gebruikt wordt in de sandwich ELISA. Wij toonden aan dat de IL-1β levels verlaagd zijn in de helft van de geteste sera nadat aan de buffer gezuiverd runder immunoglobuline en 1% normaal muizenserum waren toegevoegd.

Veranderingen in ferritine en CRP niveaus en prognostische waarde op het behandelingseffect werden geanalyseerd in **hoofdstuk III**. Opeenvolgende ferritine en CRP levels werden voorafgaand aan de behandeling en tot 24 maanden bepaald bij patiënten die participeerden in de EORTC 18952 studie. Ferritine uitgangswaarden bleken vergelijkbaar te zijn in de 2 behandelingsgroepen. Echter, ferritine ratios waren significant verhoogd in de IFN behandelde patiënten (N = 96) in vergelijking tot de onbehandelde patiënten (N = 21), zowel aan het einde van de inductietherapie (gemiddelde: 2.88 versus 0.75; $P = .0003$) als na 6 maanden (gemiddelde: 3.18 versus 1.02; $P = .009$). In de

IFN groep waren hogere ferritine ratios aan het einde van de inductietherapie en na 6 maanden niet geassocieerd met een verbeterde overleving. Met betrekking tot de CRP ratios werd geen verschil gevonden tussen de behandelingsgroepen, noch werd een associatie met afstandsmetastasen-vrije overleving geobserveerd.

In **DEEL TWEE** werd de prognostische factor auto-immuniteit geëvalueerd. Het melanoom heeft een immunogeen karakter welke zich klinisch manifesteert door spontane regressie en het ontstaan van vitiligo. Auto-immuniteit wordt gezien als een gunstige prognostische factor. Eerder rapporteerden Gogas et al. dat het ontstaan van autoantilichamen en klinische manifestaties van auto-immuniteit in melanoompatiënten, die behandeld werden met hoge dosering adjuvant IFN, sterk geassocieerd was met verbeterde ziekte-vrije en totale overleving. In **hoofdstuk IV** wordt de relatie tussen autoantilichamen en prognose getoond bij patiënten van twee studies, de EORTC 18952 studie en de Nordic IFN studie. Anticardiolipine, antithyroglobuline en antinucleaire antilichamen werden in het serum bepaald middels ELISA. De associatie van de aanwezigheid van autoantilichamen met het risico op recidief werd bepaald bij patiënten met negatieve uitgangswaarden voor alle drie de autoantilichamen (125 van de EORTC 18952 studie en 230 van de Nordic IFN studie), waarvoor gebruik werd gemaakt van drie Cox modellen: 1) een model dat de aanwezigheid van antilichamen beschouwt als een tijds-onafhankelijke variabele, 2) een model dat een patiënt positief beschouwt op het moment dat een positieve testuitslag werd verkregen, en 3) een model waarin de status van de patiënt werd bepaald aan de hand van de meest recente autoantilichaamtest. Indien behandeld als een tijds-onafhankelijke variabele (model 1), was het ontstaan van autoantilichamen geassocieerd met verbeterde ziekte-vrije overleving in beide studies (EORTC 18952, HR = 0.41, $P < .001$; en Nordic IFN, HR = 0.51, $P < .001$). Echter, in modellen 2 en 3 werd gecorrigeerd voor "guarantee-time bias", waaruit bleek dat de associatie zwakker en niet statistisch significant was.

We valideerden deze resultaten in **hoofdstuk V** voor patiënten die deelnamen aan de EORTC 18991 studie en behandeld werden met adjuvant gepegyleerd IFN enerzijds of geen therapie anderzijds. Patiënten die een negatieve uitgangswaarde voor autoantilichamen hadden werden geanalyseerd (n = 220). Het ontstaan van autoantilichamen tijdens follow-up gebeurde vaker bij patiënten die behandeld werden met gepegyleerd IFN (52%) dan bij onbehandelde patiënten (18%). Uit model 1 bleek dat het ontstaan van autoantilichamen een van prognostische waarde heeft (HR = 0.56, $P = .01$). Echter, wanneer gecorrigeerd werd voor "guarantee-time bias" was het niet meer significant (model 2: HR = 1.19, $P = .46$, model 3: HR = 1.14, $P = .59$). De resultaten waren vergelijkbaar wanneer de behandelingsgroepen separaat werden geanalyseerd, waardoor gesuggereerd kan worden dat het ontstaan van autoantilichamen noch een prognostische noch een predictieve factor is voor verbeterde overleving bij patiënten die behandeld zijn met gepegyleerd IFN.

In **DEEL DRIE** wordt de prognostische significantie van S100B, YKL-40 and polymorfismen in de CD28, CTLA4, en ICOS genen beschreven. Allereerst werd in **hoofdstuk VI**

de prognostische waarde van opeenvolgende S100B bepalingen geanalyseerd bij 211 patiënten die participeerden in de EORTC 18952 trial. Het Cox tijds-afhankelijke model (model 3) werd gebruikt om de prognostische waarde van de laatste (meest recente) S100B test te bepalen. Bij de eerste meting hadden 178 patiënten S100B waarden < 0.2 µg/l en 33 ≥ 0.2 µg/l. Van de eerste groep hadden 61 patiënten later een verhoogde S100B (≥ 0.2 µg/l) waarde. Een initieel verhoogde S100B waarde, of tijdens follow-up, was geassocieerd met verslechterde afstandsmetastasen-vrije overleving. Zo was na correctie voor ziektestadium, aantal positieve lymfklieren en geslacht de HR 5.57 (P < .0001) van S100B ≥ 0.2 versus S100B < 0.2. Vergelijkbare resultaten werden gezien voor totale overleving. We toonden tevens aan dat de prognostische invloed van S100B ≥ 0.2 µg/l meer uitgesproken was voor ziektestadium III dan stadium IIB.

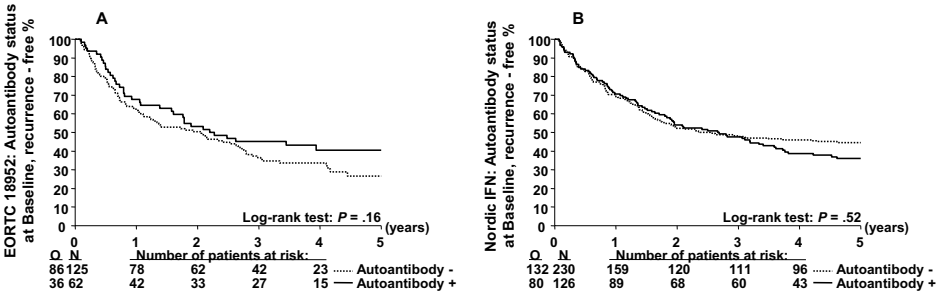
In **hoofdstuk VII** werden YKL-40 niveaus bepaald bij patiënten die deelnamen aan de EORTC 18952, de Nordic IFN of de EORTC 18991 studie. We lieten zien dat een verhoogde uitgangswaarde van YKL-40 in geobserveerde patiënten van de Nordic IFN studie en de EORTC 18952 studie een onafhankelijke prognostische marker voor kortere totale overleving was (HR = 1.33, P = .04). Echter, dit effect werd niet aangetoond voor de IFN behandelde patiënten. Verder zagen we bij patiënten die behandeld werden met IFN of gepegyleerd IFN dat een verhoging van serum YKL-40 tijdens follow-up geassocieerd was met verlaagde totale overleving maar niet met verlaagde ziekte-vrije overleving.

De associatie van polymorfismen in de CD28, CTLA4 en ICOS genen met het risico op melanoom werd onderzocht in **hoofdstuk VIII**. In een cohort van 763 Duitse melanoompatiënten en 734 gezonde Duitse controles werden 28 SNPs gegenotypeerd. Het effect op prognose werd bepaald in 587 melanoompatiënten met ziektestadium I of II. Over het algemeen werden er geen verschillen gedetecteerd in genotype of allel frequenties tussen melanoompatiënten en de controlegroep. Tevens werd geen associatie gevonden tussen de polymorfismen met prognose, behalve voor het rs3181098 polymorfisme in het CD28 gen. Aansluitend zagen we dat patiënten met het AA genotype voor het rs11571323 polymorfisme in het ICOS gen een verlaagde totale overleving hadden. Echter, wanneer rekening gehouden wordt met correctie voor multipel toetsen, suggereren onze resultaten dat de polymorfismen in the CD28, CTLA4 en ICOS genen het risico op melanoom niet moduleren noch de prognose tijdens ziekte beïnvloeden.

In **hoofdstuk IX** wordt een overzicht gegeven van verschillende (immuun-gerelateerde) factoren die geassocieerd zijn met prognose bij het melanoom. Prognostische factoren welke onderzocht zijn in dit proefschrift zijn ook beschreven. De nadruk werd gelegd op de potentiële predictieve waarde van deze factoren bij patiënten die behandeld zijn met immunotherapie.

APPENDIX

DATA COMPLEMENT, CHAPTER IV



Supplemental Figure.
Kaplan-Meier estimates of recurrence-free interval comparing baseline autoantibody-positive to autoantibody-negative patients are shown. N = number of patients; *o* = observed number of relapses; autoantibody + = positive for the presence of anticardiolipin, antithyroglobulin, or antinuclear antibodies. **A)** EORTC 18952 trial. **B)** Nordic IFN trial.

Supplemental Table. Seroconversion according to treatment*

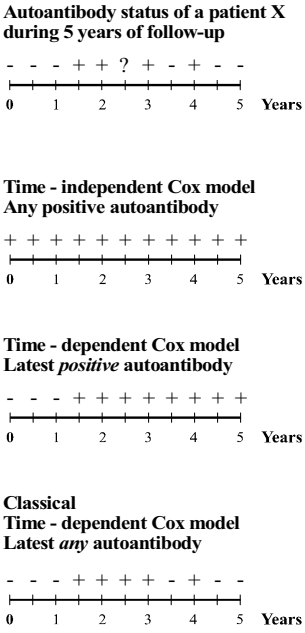
Characteristic	EORTC 18952 N=125		Nordic IFN N=230	
	Observation N=23 N (%)	IFN (13m+25m) N=102 N (%)	Observation N=70 N (%)	IFN (13m+25m) N=160 N (%)
Anticardiolipin antibodies				
Negative	20 (87)	91 (89)	67 (96)	135 (84)
Positive	3 (13)	11 (11)	3 (4)	25 (16)
Antithyroglobulin antibodies				
Negative	21 (91)	92 (90)	69 (99)	138 (86)
Positive	2 (9)	10 (10)	1 (1)	22 (14)
Antinuclear antibodies				
Negative	19 (83)	76 (75)	62 (89)	118 (74)
Positive	4 (17)	26 (26)	8 (11)	42 (26)
Autoantibodies combined				
Negative	17 (74)	65 (64)	60 (86)	97 (61)
Positive	6 (26)	37 (36)	10 (14)	63 (39)

*IFN = interferon-α.

DATA COMPLEMENT, CHAPTER V

Supplementary information

Background information Cox models
The prognostic value of serial measurements of autoantibody status (positive vs negative) observed during the study on the RFS, adjusted for the initial number of positive lymph nodes before randomization (0 vs 1 vs 2-4 vs ≥5), considered as an ordered categorical variable, have been assessed using the Cox models **(See Figure, models 1-3**).



Adapted from Bouwhuis et al. JNCI 2009

Model 1
In this Cox model autoantibody status was considered to be a time-independent variable, the hazard ratio (HR) of the event intensity per time unit in autoantibody-positive patients vs. autoantibody-negative patients according to the initial number of lymph nodes was set as:
$HR=e^{\beta_1 \times \text{autoantibody status(t)} + \beta_2 \times \text{Number of positive lymph nodes}}$

where, autoantibody status (t) = 0, if a patient always tested negative for autoantibodies or (t) = 1 when a patient was at least once positive.

Such model provides a biased estimate of the prognostic importance of autoantibody status: patients who were alive and free of relapse for a longer time period will have a higher chance to become autoantibody-positive, whereas those who had an early relapse did have less chance to have a seroconversion. This guarantee-time bias is well known in clinical trials, for instance the assessment of the relationship between response to treatment and duration of survival[10]. To determine whether autoantibody status assessed during the course of the study, before time t and before relapse, was associated with the subsequent outcome, two models in which antibody status was considered to be a time-dependent variable were used.

Model 2
HR was set as above with autoantibody status (t) = 0 if autoantibody tests were always negative before time t or = 1 from the moment the autoantibody status became positive, whether, thereafter, it remained positive or returned to a negative status; thus the variable is an indicator of the latest *positive* autoantibody status.

Model 3
In model 3, for patients free of relapse just before a time point t, the HR was set as above with autoantibody status (t) = 0 if the latest autoantibody status determined before or at time t was negative or = 1, if the latest autoantibody status determined before or at time t was positive. Thus, for purposes of risk assessment the most recent value of autoantibody status at time t (determined at that time or earlier if not available at time t) was used.

Supplementary Table. Prognostic impact of strong seroconversion on RFS

	Any positive autoantibody test (Time-independent Cox model) †		Latest positive autoantibody status (Time-dependent Cox model)		Latest autoantibody status (Time-dependent Cox model)	
	HR (95%CI)	**p-value**	**HR (95%CI)**	**p-value**	**HR (95%CI)**	**p-value**
All patients						
(N=276, O=128)						
Autoantibody status + vs -	0.76 (0.61-0.96)	.02	1.17 (0.72-1.90)	.54	1.25 (0.76-2.05)	.38
No. of positive lymph nodes	1.91 (1.51-2.43)	< .0001	1.87 (1.47-2.37)	< .0001	1.87 (1.47-2.37)	< .0001
PEG-IFN treated patients						
(N=142, 0=63)						
Autoantibody status + vs -	0.71 (0.53-0.95)	.02	1.82 (1.00-3.28)	.05	1.94 (1.08-3.48)	.03
No. of positive lymph nodes	1.68 (1.21-2.33)	.002	1.78 (1.28-2.47)	.0006	1.77 (1.27-2.46)	.0007
Observed patients						
(N=134, O=65)						
Autoantibody status + vs -	0.87 (0.57-1.32)	.52	0.48 (0.11-2.03)	.31	0.35 (0.05-2.65)	.31
No. of positive lymph nodes	2.06 (1.44-2.95)	< .0001	2.09 (1.46-2.99)	< .0001	2.12 (1.47-3.04)	< .0001

N = number of patients, O = number of observed events, Autoantibody status + are patients who tested strongly positive for anticardiolipin, antithyroglobulin or antinuclear antibodies.

* Results provided by this model are subject to guarantee-time bias (Supplementary information)

Supplementary Table: Results of the Cox Time Dependent Model

	All patients				Stage IIB				Stage III			
	Hazard Ratio	95% Hazard Ratio Confidence Limits		P-value	Hazard Ratio	95% Hazard Ratio Confidence Limits		P-value	Hazard Ratio	95% Hazard Ratio Confidence Limits		P-value
DMFS[a]												
S100B[b]	5.85	3.97	8.63	< .0001	2.19	0.72	6.68	.17	6.98	4.62	10.54	< .0001
Stage[c]	2.78	1.71	4.53	< .0001					2.88	1.69	4.90	< .0001
Nb of + LN[d]	1.45	1.12	1.87	.005					1.37	1.02	1.85	.04
Sex: Male vs Female	1.98	1.34	2.91	.0006	5.08	1.36	19.03	.01	1.62	1.07	2.47	.02
Ulceration: Yes vs No	1.40	0.90	2.15	.13	1.45	0.53	3.98	.48	1.30	0.80	2.12	.29
Ulceration: Unk vs No	1.18	0.70	2.00	.53	0.00	0.00	.	.99	1.16	0.68	1.98	.58
OS[a]												
S100B[b]	4.78	3.15	7.24	< .0001	2.82	0.80	9.93	.11	5.46	3.51	8.51	< .0001
Stage[c]	1.92	1.13	3.25	.02					1.87	1.07	3.29	.03
Nb of + LN[d]	1.53	1.17	1.99	.002					1.49	1.10	2.02	.01
Sex: Male vs Female	2.19	1.43	3.36	.0003	14.71	1.77	122.00	.01	1.78	1.14	2.78	.01
Ulceration: Yes vs No	1.25	0.78	1.99	.36	1.36	0.42	4.37	.61	1.11	0.66	1.87	.70
Ulceration: Unk vs No	1.07	0.60	1.91	.82	0.00	0.00	.	1.00	1.03	0.57	1.85	.92

a time variable right censored at 4 years

b S100B: 0 = < 0.2, 1 = ≥ 0.2 µg/l (Cox time-dependent)

c Stage: 0 = stage IIb (n = 51) or stage III-N1 (n = 46), 1 = stage III-N2 (n = 114)

d Number of positive lymph nodes: 0 = 0, 1 = 1, 2 = 2-4, 3 = 5 or more

Abbreviations: DMFS = Distant Metastasis-Free Survival; OS = Overall Survival; Unk = Unknown

DATA COMPLEMENT, CHAPTER VII

Supplementary Table 1. Serum YKL-40 levels according to stage, ulceration, Breslow thickness and Clark's level.

		Nordic Study			EORTC 18952			EORTC 18991		
		No.	Median (min-max)	P-value	No.	Median (min-max)	P-value	No.	Median (min-max)	P-value
Stage	N0	94	45 (20-259)		26	68 (20-281)		0	0	
	N+ sentinel	105	44 (20-1416)	.51	41	50 (21-206)	.06	149	47 (22-285)	.90
	N+ palpable	267	50 (20-288)		61	54 (20-204)		111	52 (20-438)	
Ulceration	No	248	46 (20-259)		64	52 (22-281)		143	57 (11-285)	
	Yes	132	47 (20-288)	.61	37	58 (14-216)	.88	59	50 (19-438)	.51
	Unknown	81	48 (20-1416)		27	53 (21-197)		58	49 (19-438)	
Breslow thickness	<1.5 mm	106	46 (14-231)		31	51 (14-204)		52	50 (11-285)	
	1.5-3.99 mm	170	49 (12-1416)	.61	37	60 (22-115)		129	53 (11-262)	.71
	≥4.0 mm	150	47 (11-426)		45	58 (20-281)	.29	55	49 (13-438)	
	Unknown	28	45 (13-228)		15	46 (24-206)		24	51 (20-227)	
Clark's level	I	1	21							
	II	16	41 (14-183)		2	38 (36-39)				
	III	112	46 (16-1416)		28	50 (22-206)				
	IV	204	48 (11-888)		61	54 (20-259)	.57			
	V	38	47 (20-426)	.66	13	56 (14-281)				
	Unknown	80	51 (13-228)		24	65 (24-197)				
	Missing	3	69 (48-106)							

Supplementary Table 2. Multivariate analyses of recurrence free survival for gender, age, stage and baseline serum YKL-40 in the Nordic Study combined with the EORTC 18952 Study and in the EORTC 18991 Study.

	Nordic Study and EORTC 18952				EORTC 18991			
	Group	HR	95% CI	p-value	Group	HR	95% CI	p-value
Gender f vs. m	Obs	0.76	0.48-1.21	.25	Obs	1.12	0.66-1.89	.67
Age		0.98	0.82-1.16	.77		1.11	0.88-1.40	.40
N0 vs. N+palp.		0.64	0.39-1.04	.003				
N+sent. vs. N+palp.		0.34	0.19-0.62			0.97	0.58-1.63	.92
YKL-40		1.13	0.89-1.44	.32		0.93	0.69-1.26	.66
Gender	IFN 1-year	0.67	0.41-1.10	.11	Pegylated IFN	0.63	0.35-1.12	.11
Age 10 years		1.14	0.97-1.35	.12		1.22	0.92-1.61	.16
N0 vs. N+palp.		0.57	0.35-0.94	.009				
N+sent. vs. N+palp.		0.51	0.30-0.86			0.54	0.30-0.95	.034
YKL-40		0.96	0.77-1.21	.76		1.17	0.84-1.64	.35
Gender	IFN 2-year	0.95	0.59-1.53	.83				
Age		0.98	0.82-1.17	.82				
N0 vs. N+palp.		0.76	0.46-1.26	.13				
N+sent. vs. N+palp.		0.63	0.40-1.01					
YKL-40		1.01	0.80-1.26	.97				

Obs = Observation, IFN = interferon

DATA COMPLEMENT, CHAPTER VIII

Supplementary table.

SNP ID	Region type	Position on Gene	SNP	Forward primer	Reverse primer	Taq Man assay
rs3181098	CD28 promotor	-1042	G/A	tgagacaccaaggggctttt	ataggatggggacaggttgtg	C_27467172_10
rs3181100	CD28 intron 1	587	C/G	tgtaagtagattggctctgga	cttcgcatggattatttcat	C_2821000_10
rs3181101	CD28 intron 1	615	C/G	idem rs3181100	idem rs3181100	C_27469464_10
rs1181390	CD28 intron 1	1258	G/T	aaattaacttcggaaaatcac	ctctgtactgccaaaataaga	C_8806607_10
rs1181388	CD28 intron 1	4532	G/A	tgggtggttttgtgactg	taagggagcagctcaaagtta	C_2821002_10
rs17533594	CD28 intron 1	9776	A/G	cttatggcccagctaaatg	ttcctacacaggcagacacta	C_32900233_10
rs3116494	CD28 intron 2	20602	A/G	ctgacattgagcgggagagta	ttcctgagtcttaacccattagac	C_27464981_10
rs3181107	CD28 intron 2	22307	A/G	cctagttccagccctgaga	gaatcaaaaggggatgagaag	C_27467173_10
rs3116496 (IVS3 +17)	CD28 intron 3	23093	T/C	gtggagtcctggcttgc	aatgccttctgggaaatctaa	C_25922478_10
rs16840252	CTLA4 promotor	-1147	C/T	gaggcatttggtgagtatt	agacaggaccaatgatctaac	C_32900355_10
rs5742909 (CT44)	CTLA4 promotor	-319	C/T	caagggctcagaaagttag	gaagccgtgggtttag	C_27834180_10
rs231775 (CT42)	CTLA4 exon 1	49	A/G	gaacaccgctcccataaag	gccagccaagccagatt	C_2415786_20
rs231777	CTLA4 intron 1	923	C/T	tacacggcttaaaatgatgag	cactaaatgcggtcacactc	C_2415784_10
rs3087243 (CT60)	CTLA4 3′	6254	G/A	tcagtatctggtggagtct	caactgtaatgcctgtgat	C_3296043_10
rs11571319 (CT61)	CTLA4 3′	6273	G/A	idem rs3087243	idem rs3087243	C_30981401_10
rs11571302 (JO31)	CTLA4 intergenic	10269	G/T	ccatccatctccatcccaagt	tttgcgatcatcccctgaaat	by design
rs7665213 (JO30)	CTLA4 intergenic	10744	G/A	tttttcttcctgcttgtcatt	aaaggtgctcacaggagagta	by design
rs11571297 (JO27)	CTLA4 intergenic	12338	T/C	ctggtcagccgagattgtgat	cccccagcgattcagagtg	C_3296036_10
rs10932029 (+173)	ICOS intron 1	231	T/C	attacgcacccaaaagacagt	ctatggtgccctggacatt	C_430013_10
rs4335928	ICOS intron 1	443	T/C	idem rs10932029	idem rs10932029	C_26263275_10
rs4675374	ICOS intron 1	1041	C/T	ctctttcacactgagccctat	catacaccatctggcacag	C_29934539_10
rs7602383	ICOS intron 1	4915	A/G	tctctgtaaatggcatctca	aagttcatatccccagtttct	C_29052409_10
rs4521021	ICOS intron 1	15038	T/C	aatgggggaaaaatctg	gagaacaggtgcagtctacat	C_377746_10
rs11571323	ICOS intron 1	17307	G/A	ctaataattttcccaaacaat	ctgcctcagcctctta	C_30981464_10
rs12466129	ICOS intron 1	17444	T/A	idem rs11571323	idem rs11571323	C_8166476_30
rs10172036	ICOS intron 4	22746	G/T	aaggcaatggagaggggaaag	tcggcagtaccaaggcagtc	C_26263283_10
rs10183087	ICOS 3′UTR	22787	A/C	idem rs10172036	idem rs10172036	C_30421029_10

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