

MELANOMA

Prognostic and Predictive Factors in Interferon Immunotherapy

Marna G. Bouwhuis

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MELANOMA

Prognostic and Predictive Factors in Interferon Immunotherapy

Melanoom

Prognostische en predictieve factoren bij interferon immunotherapie

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Chapter I

General Introduction and Aim of the Thesis

1 GENERAL INTRODUCTION

2

3 Melanoma is a malignant tumor of melanocytes and is the most severe form of skin
4 cancer. The name melanoma originates from the Greek word μέλας (melas), meaning
5 black or dark, whereas the suffix 'oma' denotes swelling or tumor. René Laennec was a
6 French physician who was the first to describe melanoma as a disease entity. After being
7 presented in a lecture in 1804 for the Faculté de Médecine de Paris, it was published in
8 1806 as a bulletin.¹ However, the first surgeon to operate on metastatic melanoma was
9 John Hunter in 1787. Microscopic examination of the specimen that was preserved in
10 the Hunterian Museum of the Royal College of Surgeons of England, revealed it to be a
11 metastatic melanoma.²

12

13 Currently, melanoma incidence is rising dramatically worldwide. In the United States,
14 melanoma is the fifth leading cancer in men and the seventh in women.³ Over the past
15 20 years the incidence of melanoma has more than tripled in the white population in the
16 United States. An overview of trends of incidence of cancer in Europe, from mid 1990s to
17 early 2000, showed for melanoma increasing incidence rates for most European coun-
18 tries, especially in females.⁴ In the Netherlands, estimated incidences for males, increased
19 from 5.5 cases per 100,000 inhabitants per year to 12.2 cases per 100,000 inhabitants per
20 year between 1980 and 2002, and for females, incidences increased from 7.8 to 15.0
21 cases per 100,000 inhabitants per year.⁵ A more recent report concerning incidence and
22 mortality of cancer in Europe in the year 2008, showed 84,000 new melanoma cases and
23 20,100 deaths from melanoma.⁶ Estimated numbers of new cases in The Netherlands
24 included 1660 males and 2130 females. The risk factors for developing melanoma are
25 both environmental and genetic.

26

27 The definitive surgical treatment for primary cutaneous melanoma is a wide local exci-
28 sion. Although resection usually controls the primary lesion, melanoma often metasta-
29 sizes to the regional lymph nodes. An elective (immediate) lymph node dissection was
30 not proven to improve survival for the entire patient population.^{7,8} The sentinel lymph
31 node biopsy, introduced by Morton and colleagues in the early 1990s was a promising
32 new staging technique in melanoma.⁹ The assessment of the first draining lymph node
33 from a tumor was suggested to identify subgroups of patients benefiting early comple-
34 tion lymph node dissection.¹⁰ Nevertheless, it is still not demonstrated that an early
35 completion lymph node dissection following positive sentinel node biopsy improves
36 overall survival compared to patients undergoing therapeutic lymph node dissection
37 for palpable lymph nodes.¹¹ Adjuvant radiotherapy can decrease the local recurrence
38 rate but there is no evidence of improved survival. In the palliative setting it is effective
39 in patients with bone, brain and visceral metastases.¹²

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

14
15 Recombinant IFN is a cytokine with a wide range of biological activities, such as direct
16 anti-proliferative/pro-differentiation and protein synthesis-inhibiting antitumor activity,
17 as well as indirect immune-mediated effects, and rendering the tumor more susceptible
18 to host effector cells.¹⁸ In the adjuvant setting, high-dose IFN was approved in the USA
19 and in Europe for stage IIB-III, but is little used in Europe. This treatment was approved
20 in the USA due to the results of the ECOG 1684 trial, showing a recurrence-free survival
21 (RFS) and overall survival (OS) benefit in patients treated with high-dose IFN compared
22 to untreated patients.¹⁹ Numerous trials followed, reporting a consistent and significant
23 effect on RFS but not on OS.^{20,21} An individual patient data meta-analysis confirmed the
24 significant and consistent effect on RFS and a small, but statistically significant, impact
25 of 3% on OS.²²

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

37
38 Current prognostic markers based on the conventional American Joint Committee on
39 Cancer (AJCC) staging system (TNM) include Breslow tumor thickness, presence of ulcer-

1 ation and extent of nodal involvement for primary cutaneous melanoma, and further-
2 more, site of distant metastases and serum lactate dehydrogenase (LDH) in metastatic
3 melanoma.²⁷ Increasing tumor thickness (originally described by Breslow),²⁸ correlates
4 with a significantly poorer prognosis. Patients with thin lesions (<0.5 mm) have an excel-
5 lent 10-year survival of 96%, but survival decreases to 54% for patients with lesions with
6 a thickness of 4.01 to 6.0 mm. Furthermore, outcome in patients with ulcerated primary
7 tumors are worse than in patients with primary melanomas of the same thickness but
8 without ulceration. Another important prognostic factor in localized melanoma is the
9 mitotic index (mitosis/mm²) with 10-year survival rates of 93% for those with < 1 mitosis/
10 mm² and 48% for those with >20 mitosis/mm². In stage III, melanoma prognosis depends
11 upon the extent of the nodal tumor burden.²⁹ Five-year survival rates for patients with
12 micrometastases (detected in the specimen of the completion lymph node dissection
13 following positive sentinel node biopsy) in one, two, or three lymph nodes were 71, 65
14 and 61% respectively. And for patients undergoing therapeutic lymph node dissection
15 for palpable lymph nodes (macrometastases), 5-year survival rates were 50, 43 and 40%
16 when one, two, or three lymph nodes, respectively, were involved. Prognosis in meta-
17 static patients is poor with 1-year survival rates of 62% for patients with distant skin,
18 subcutaneous, or nodal metastases, 53% for patients with lung metastases and 33% for
19 patients with other visceral metastases or elevated serum LDH. Other prognostic factors
20 that are not included in the AJCC staging system but which are of prognostic importance
21 are age, gender, and anatomic location of the primary tumor. Moreover, the serological
22 biomarkers S100B, melanoma-inhibiting activity (MIA) and YKL-40 were associated with
23 adverse outcome.³⁰

24

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

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39

1 AIM OF THE THESIS

2

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

7

8 This thesis mainly focused on prognostic and predictive factors in stage IIB-III mela-
9 noma patients receiving adjuvant (pegylated) interferon- α , conducted as translational
10 research projects from large randomized controlled trials. The cytokines IL-6 and IL-1 β
11 and acute phase proteins, ferritin and CRP were analysed in a subset of patients random-
12 ized to the European Organization of Research and Treatment of Cancer (EORTC) 18952
13 trial, evaluating efficacy of intermediate doses of IFN versus observation. To assess the
14 prognostic and predictive value of autoimmunity in IFN-treated patients, autoimmune
15 antibodies were measured in patients participating in the EORTC 18952 trial, the Nordic
16 IFN trial and in patients receiving pegylated (PEG-) IFN in the EORTC 18991 trial. The
17 serum biomarker S100B, known to be of prognostic value in stage IV melanoma, was
18 determined in a subset of patients from the EORTC 18952 trial. The YKL-40 analysis was
19 performed in patients participating in the EORTC 18952, Nordic IFN or the EORTC 18991
20 trial. Additionally, the influence of genetic variability on melanoma susceptibility and
21 prognosis was evaluated by screening German melanoma patients and German healthy
22 controls for polymorphisms in the CD28/CTLA4/ICOS genes. Finally, immunologic func-
23 tions as prognostic indicators in patients receiving immunotherapy, including data from
24 the presented studies in this thesis were reviewed. Moreover, the possible impact of
25 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-1 β in Melanoma Patients in the EORTC 18952 Trial of Adjuvant Interferon α versus Observation and Healthy Controls

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Minor modifications

Submitted

1 ABSTRACT

2

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

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

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

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

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1 INTRODUCTION

2

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

11 Melanoma patients with thick primary tumors or lymph nodal involvement are
12 at high risk of recurrence or death.¹⁰ Adjuvant therapy with interferon- α (IFN) has a
13 significant and consistent effect on recurrence-free survival (RFS), but only a marginal
14 and significant impact of 3% on overall survival (OS).¹¹ Seemingly, only a relatively small
15 proportion of patients benefits from IFN therapy, however a biomarker selecting these
16 patients is currently unavailable. Recent multiplex analysis of a panel of 29 cytokines in
17 serum of high-risk melanoma patients showed the association of higher pre-treatment
18 levels of the pro-inflammatory cytokines IL-1 α , IL-1 β , IL-6, TNF and the chemokines MIP-
19 1 α and MIP-1 β with longer (> 5 years) RFS in IFN-treated patients.¹² However, a mean
20 IL-6 baseline level between 2000 and 3000 pg/ml for the group with a RFS longer than
21 5 years is exceptionally high and was not reported before. Several studies show high
22 difference in serum levels for cytokines, which probably does not only depend on the
23 study population, but also on the method used for detection of cytokines. Two large
24 studies in stage IV melanoma patients report median IL-6 values between 16 and 17 pg/
25 ml and one of these mentioned a range between 0 and 350 pg/ml.^{7,13} Another study of 28
26 metastatic melanoma patients showed that 12 patients had IL-6 levels below 20 pg/ml,
27 14 patients between 20 and 100 pg/ml and only 2 patients had IL-6 serum levels above
28 100 pg/ml.¹⁴ Therefore the high IL-6 levels found by Yurkovetsky et al. might indicate false
29 positive test results. The measurement of cytokines in human sera is challenging because
30 of confounding variables, such as heterophilic antibodies in sera that could crosslink the
31 non-human antibodies, i.e. mouse monoclonal antibodies, used in the sandwich ELISA
32 and produce false positive ELISA results. This phenomenon was described for instance for
33 the IL-1 β ELISA.¹⁵ IL-1 β levels reported for healthy controls vary and are listed in Table 1.

34 We determined IL-6 and IL-1 β levels in serial serum samples of 185 melanoma patients
35 that participated in the EORTC 18952 trial.¹⁶ The ELISA we used for IL-6 determinations
36 correlated with a functional assay for IL-6 in B9 cells.¹⁷ The original aim of the study was to
37 evaluate the prognostic and predictive value of IL-6 and IL-1 β measured by ELISA. A subset of
38 78 samples was also tested by multiplex technique, in order to compare both techniques. We
39 compared the results of this study to the serum detection of IL-6 and IL-1 β in healthy controls.

1 **Table 1.**

2 Study	3 Number of controls	4 IL-1β Assay	5 Mean (+/-SD) pg/ml	6 Median pg/ml	7 Range pg/ml
8 Kemik et al. ²¹	36	ELISA	276.9 (+/- 132.2)	-	-
9 Antonelli et al. ²²	43	ELISA	0.8	0.7	0.7 – 1.1
10 Barak et al. ²³	27	ELISA	316.6	-	170 – 540
11 Mahajan et al. ²⁴	10	ELISA	2.15	-	0.87 – 2.98
12 Yurkovetsky et al. ¹²	378	Multiplex	40.2	0.0	0.0 – 2253.5
13 Watkin et al. ²⁵	71	ELISA	-	0.41	0 – 1.21
14 Macri et al. ²⁶	15	ELISA	33.4 (+/- 7.98)	-	-
15 Schmitt et al. ²⁷	23	ELISA	<0.5	<0.5	-
16 Lyke et al. ²⁸	251	Multiplex	14.7	-	2.5 – 3355
17 Yoshida et al. ²⁹	21	ELISA	0.16 (+/- 0.17)	0.08	0 – 0.61

18 IL-1 β levels in healthy controls.

19 **PATIENTS AND METHODS**

20 **Patients and controls**

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

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

36 Informed consent from patients for translational research for identification of prognos-
 37 tic factors in association with these trials was obtained. Control sera were obtained from
 38 healthy donors with permission (Sanquin Blood Supply, Amsterdam, The Netherlands).
 39

1 Cytokine measurements by ELISA

2 IL-6 and IL-1 β serum levels were determined by ELISA kits (Peli-Kine-compact, Sanquin
3 Reagents, Amsterdam, The Netherlands). The IL-6 assay was used as previously described
4 in detail.¹⁸ Sera 1:5 diluted in HPE, were incubated on monoclonal anti-IL-6 coated plates
5 together with polyclonal (sheep) biotinylated anti-IL-6. After washing, incubation with
6 streptavidin-poly HRP (Sanquin, Amsterdam, The Netherlands) and after another wash-
7 ing step incubation with TMB (Merck) followed. The reaction was stopped with H₂SO₄
8 and the colour formation was determined with a spectrophotometer (Labsystems Mul-
9 tiskan Multisoft, Helsinki, Finland). For the IL-1 β assay sera were 1:5 diluted in HPE and
10 incubated on plates coated with monoclonal anti-IL-1 β in the presence of monoclonal
11 biotinylated anti-IL-1 β . The subsequent steps were similar to the IL-6 assay. All determi-
12 nations were carried out *in duplo* and the detection limits for the IL-6 and the IL-1 β assay
13 were 5 and 25 pg/ml, respectively.

14

15 IL-1 β ELISA alternative inhibition tests

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

21

22 Multiplex assay

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

28

29

30 RESULTS

31

32 Study population

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

37

38

39

1 **Table 2.**

2 Variable	EORTC 18952
	No of patients (%)
4 Age (years)	
5 <35	38 (20.5)
6 35–49	64 (34.6)
7 50–65	65 (35.1)
8 ≥65	18 (9.7)
9 Sex	
10 Male	104 (56.2)
11 Female	81 (43.8)
12 Breslow thickness	
13 <1.00 mm	13 (7.0)
14 1.00–1.99 mm	37 (20.0)
15 2.00–3.99 mm	52 (28.1)
16 ≥4.00 mm	68 (36.8)
17 Unknown	15 (8.1)
18 Ulceration status of primary melanoma	
19 Absent	98 (53.0)
20 Present	55 (29.7)
21 Unknown	32 (17.3)
22 Stage of the disease	
23 IIB	42 (22.7)
24 III N1	44 (23.8)
25 III N2	99 (53.5)
26 No. of positive lymph nodes	
27 0	42 (22.7)
28 1	71 (38.4)
29 2–4	48 (25.9)
30 5+	24 (13.0)
31 Treatment	
32 Observation	32 (17.3)
33 13-month IFN	80 (43.2)
34 25-month IFN	73 (39.5)

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

29 Interleukin-6

30 Baseline IL-6 levels were determined by ELISA in 184 patients (baseline sample was not
 31 available from 1 patient) of whom 32 were randomly assigned to the observation arm
 32 and 152 to one of the IFN groups. In the observation group, 2 out of 32 patients (6.3%)
 33 had detectable serum IL-6 levels (≥ 5 pg/ml) with a median of 7 pg/ml (range 6–8 pg/ml),
 34 and in the combined IFN group, 8 out of 152 (5.3%) had elevated pretreatment levels
 35 with a median of 9 pg/ml, ranging 5–115 pg/ml (Table 3). During follow-up, 2 out of 155
 36 (1.3%) screened sera from the observed patients and 35 out of 745 (4.3%) sera from the
 37 IFN-treated patients tested positive for IL-6. Median levels were similar 8 pg/ml, although
 38 the range was wider for the treated arm (5–390 pg/ml). Additionally, the occurrence of

39

1 **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)	
EORTC 18952				
IL-6 ELISA	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)

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

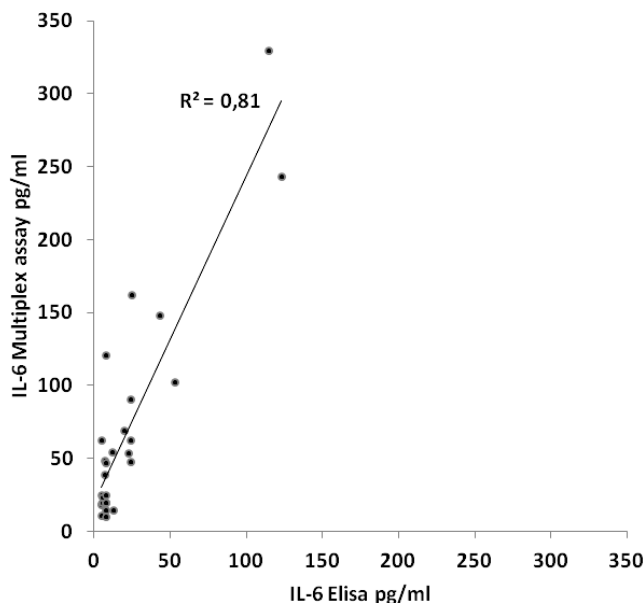
18
 19 elevated IL-6 levels was tested for 50 healthy controls, of these 3 (6.0%) tested positive
 20 with a median of 5 pg/ml, ranging 5-6 pg/ml.

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

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

34 Interleukin-1 β

35 Baseline IL-1 β levels were elevated in 4 out 32 (12.5%) of the patients in the observation
 36 arm and in 15 out of 152 (9.9%) patients who received IFN (Table 4). During follow-up, in
 37 12,3% (19/155) and in 11.5% (85/742) of the screened sera, IL-1 β was detectable for the
 38 observation and the IFN group respectively. Interestingly, 14 patients had elevated IL-1 β
 39 levels at multiple time points. From these 14 patients, a total of 96 sera were tested of



17 **Figure 1.**

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

21 **Table 4.**

		Number of screened sera	Number of IL-1 β positive sera ≥ 25 pg/ml	Median (range) pg/ml
	Controls (n=70)	80	16 (25.0%)	96 (28-5331)
	EORTC 18952			
IL-1 β 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)
IL-1 β MULTIPLEX	EORTC 18952 (78 sera)			
	Sera tested by ELISA			
	Positive	24	9	132 (63-1276)
	Negative	54	17	90 (61-390)

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

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

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

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

25

26 **Table 5.**

27 IL-1 β	Normal conditions	Median (range) pg/ml	With bovine IgG + NMS	Median (range) pg/ml
28 EORTC	104 positive IL-1 β	56 (25 – 397)	33 sera ND	-
29 18952	sera		19 sera $\bar{}$	28 (25 – 70)
			52 sera \neq /	241 (37 – 2729)
30 Controls			14/150 (9.3%)	42 (25 – 761)
31			positive IL-1 β sera	

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

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

36

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

1 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-1 β (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-1 β levels in more than half of the sera. Subsequently, the addition of anti-IL-1 β did not block IL-1 β detection in the majority of the sera. Only for serum sample #1 an undetectable IL-1 β level, and for sample #18 a clear decrease in IL-1 β level was observed after addition of anti-IL-1 β , suggesting the presence of IL-1 β 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-1 β pg/ml	2)bovine IgG + NMS	3)bovine IgG + NMS with anti- IL-1 β	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-1 β blocking assays of 20 positive sera determined by IL-1 β ELISA under default conditions. Samples were retested (2-5) under several conditions:

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

1 DISCUSSION

2

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

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

29 The aim of our study was to evaluate the prognostic value of IL-1 β in melanoma
30 patients randomized to the EORTC 18952 study. However the results of the IL-1 β ELISA
31 were largely influenced by confounding factors as demonstrated in the group of healthy
32 controls. We found an unexpected high positive rate in patients and controls, whereas
33 14 patients (and 3 controls) remained positive at all measured time points. Furthermore
34 the discrepancy with the multiplex assay suggested that these data included false posi-
35 tive test results. We performed additional analyses to clarify which factors contributed
36 to the false positive results, despite the use of our High Performance blocking buffer
37 (HPE). The addition of bovine IgG and normal mouse serum reduced the number of
38 positive test results to 50%. This blocking effect of bovine IgG and NMS suggested the
39 existence of antibodies in human sera capable of crosslinking the monoclonal antibody

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

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

23 Although ELISAs have become a standard method for analyzing cytokines, results
24 should be interpreted with caution, especially in the case of unexpected high positive
25 rates. The differences in concentrations in the cytokine assays should raise concerns at
26 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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1 ABSTRACT

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

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

14 Results: Baseline ferritin levels were comparable in the 2 treatment groups ($P =$
15 $.92$). However, ferritin ratios were significantly higher in IFN-treated patients ($N = 96$)
16 compared to untreated patients ($N = 21$), at end of induction (mean: 2.88 vs 0.75 ; $P =$
17 $.0003$) and at 6 months (mean: 3.18 vs 1.02 ; $P = .009$). In the IFN arm, higher ferritin
18 ratios at end of induction and at 6 months were not associated with improved outcome
19 (respectively $P = .66$ and $P = .86$). Concerning CRP ratios, no differences between the
20 treatment groups, neither an association with DMFS, were observed.

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

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1 INTRODUCTION

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

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

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

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

1 18952 trial).²² Ferritin levels are influenced by sex and age²³ therefore, ratios of increase/
2 decrease (compared to pre-treatment value) were calculated for both ferritin and CRP.

5 PATIENTS AND METHODS

7 Patients

8 Patients participated in the EORTC 18952 study and gave their informed consent for transla-
9 tional research for identification of prognostic factors. Between 1996 and 2000 a total of 1388
10 patients were enrolled, these were diagnosed with stage IIB melanoma (Breslow thickness \geq
11 4 mm, node negative [N0]), or had undergone curative dissection of regional lymph nodes,
12 either completion lymphadenectomy following positive sentinel node procedure (stage III,
13 N1) or therapeutic lymph node dissection for palpable nodes (stage III, N2). Randomization
14 occurred in a 2:2:1 fashion to the following treatment schedules, induction treatment of 4
15 weeks of 10 million units (MU) interferon- α 2b five days a week followed by either 10 MU
16 three times a week for 1 year (Arm A) or 5 MU three times a week for 2 years (Arm B). All were
17 given by subcutaneous injections. Patients in Arm C did not receive treatment.

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

24 Enzyme-linked immusorbent assays

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

32 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 incuba-
33 tions were at room temperature, no blocking step was used and serum samples and
34 conjugate were diluted in HPE buffer (Sanquin Reagents). The coating antibody (87/654)
35 the HRP-conjugate (87/662) as well as the recombinant ferritin standard (94/572) were
36 kindly provided by Stephen Poole, NIBSC, Blanche Lane South Mimms Potters Bar Hert-
37 fordshire EN6 3QG United Kingdom. Sera were tested at 1:5 and 1:25 dilution. Detection
38 limit at the 1:5 dilution was 5 ng ferritin/ml.

1 **Statistical analysis**

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

16

17

18 **RESULTS**

19

20 **Baseline measurements**

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

39

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)

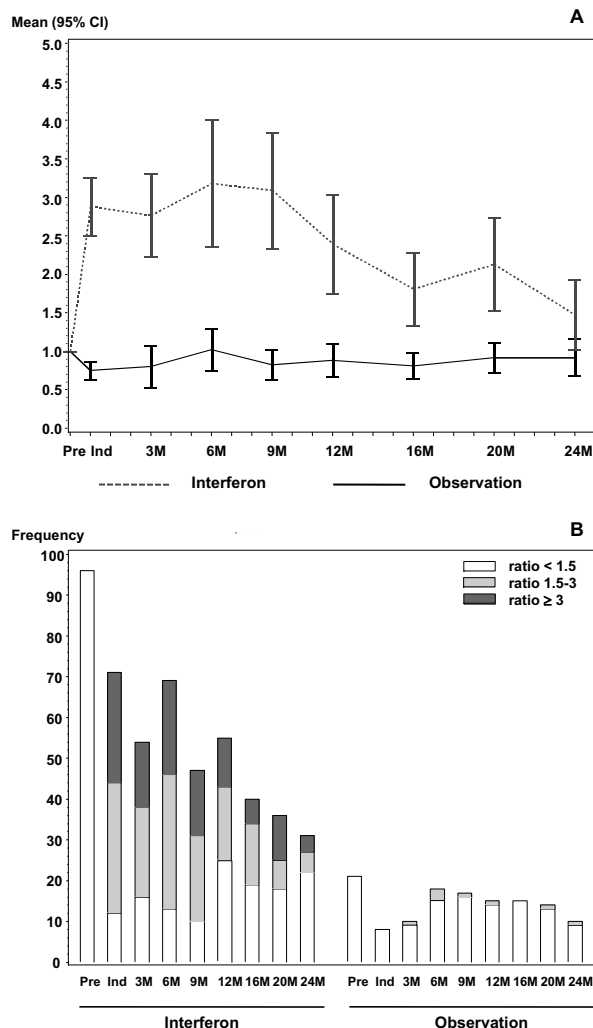
IFN = Interferon- α ; MU = million units

* Because of rounding not all percentages equal 100

† SE = Standard Error

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

2 Ferritin ratios (ferritin level at time point t compared to the baseline ferritin level)
 3 remained constant over time in the observation arm (Figure 1). However, means of
 4 ferritin ratios in the IFN-treated patients as compared to the untreated patients were
 5 significantly higher, at all time points except from the 24-month time point (at end of
 6 induction: 2.88 (SE 1.59) versus 0.75 (SE 0.17), $P = .0003$ and at 6 months; 3.18 (SE 3.41)
 7 versus 1.02 (SE 0.59), $P = .009$). CRP ratios were similar between treatment groups over
 8 time (Figure 2), at end of induction: 1.13 (SE 2.70) versus 0.95 (SE 0.55), $P = .85$ and at
 9



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

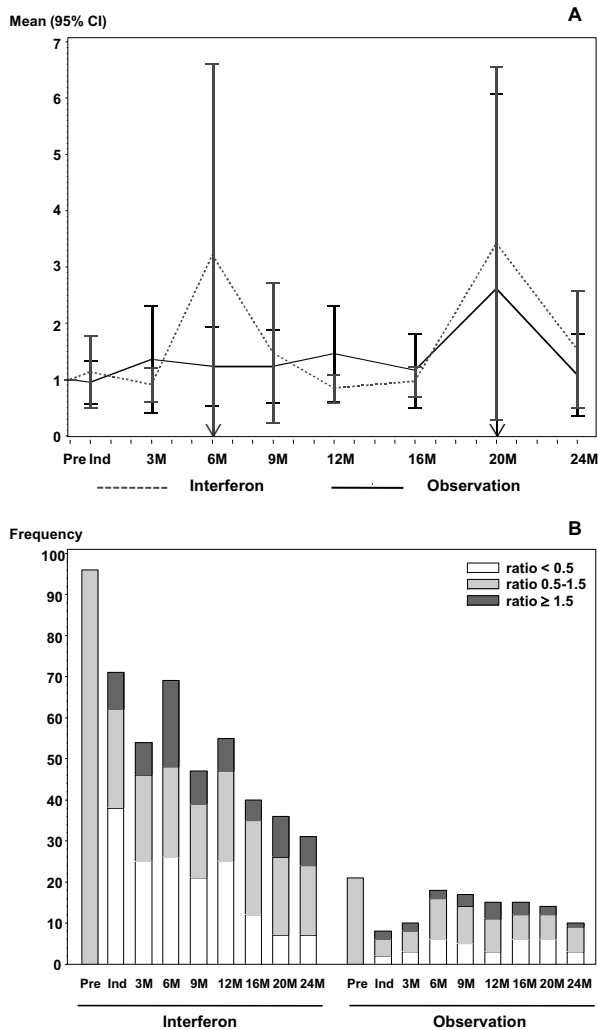


Figure 2. Mean CRP ratios for untreated patients vs IFN-treated patients at base line and up to 24 months are shown in panel (A). Frequency of the CRP ratios; <0.5 / 0.5 – 1.5 / ≥ 1.5 for both treatment arms at the different time points are depicted in panel (B). CRP ratios: CRP at time point t / CRP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

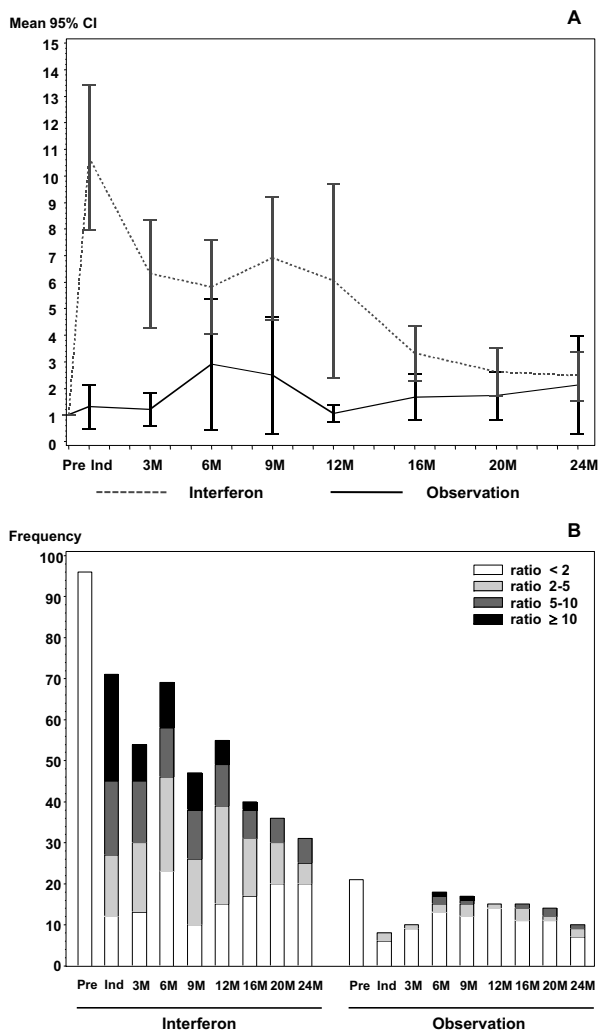
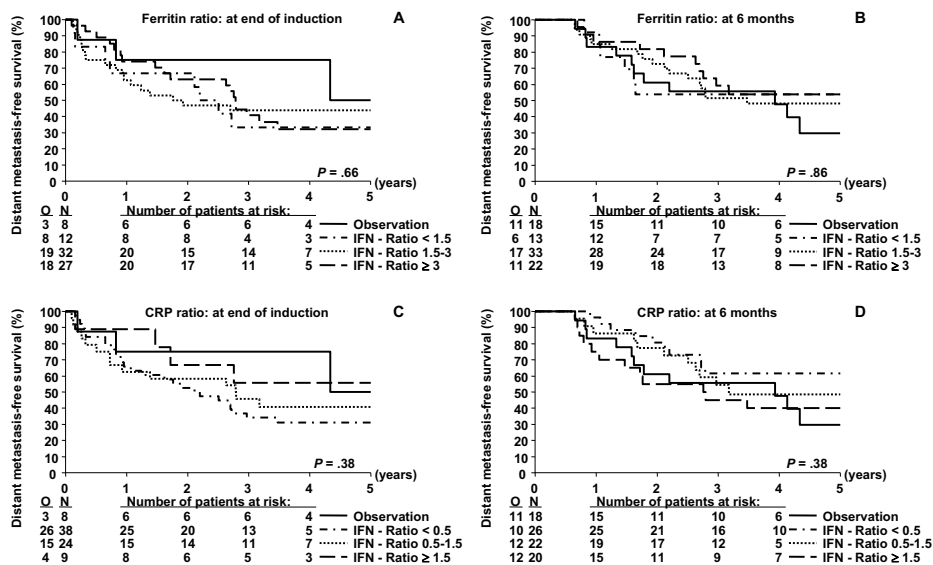


Figure 3. The ferritin/CRP ratios, (ferritin at time point t / ferritin at baseline) / (CRP at time point t / CRP at baseline), for the observation arm and the IFN arm at the different time points are shown in panel (A) and the distributions of the ratios, < 2 / $2-5$ / $5-10$ / ≥ 10 for both treatment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

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



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

23 DISCUSSION

26 Stam and colleagues previously assessed the effects of interferon- α_2b administration on
 27 the acute-phase response in a subset of 21 melanoma patients of the EORTC 18952 trial.²¹
 28 This study showed significant increases in ferritin levels and less pronounced decreases
 29 in CRP levels at end of induction and at 6 months. The administration of IFN induced no
 30 significant changes in other acute-phase proteins such as secretory phospholipase A₂,
 31 alpha1-acid glycoprotein or albumin. Here we extended the analysis in order to explore
 32 the association of ferritin and CRP changes with clinical response to IFN therapy. In 117
 33 patients accrued in the EORTC 18952 trial, serial ferritin and CRP levels were determined
 34 from baseline, up to 24 months maximum.

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

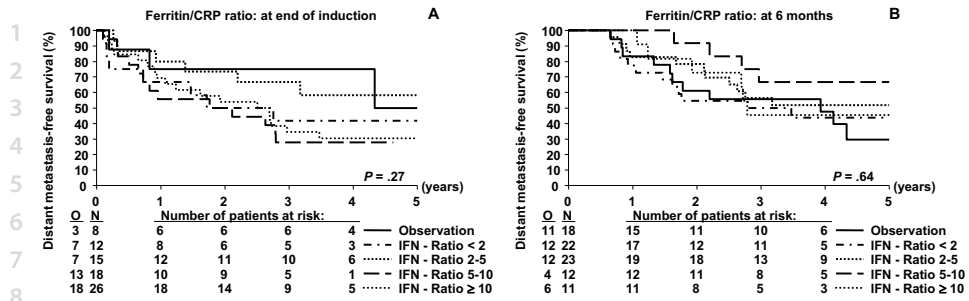


Figure 5. Landmark method for the ferritin/CRP ratio, according to the different categories for patients receiving IFN, compared to untreated patients, are shown in panel (A) for end of induction and in panel (B) for six months, regarding the DMFS.

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 half 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.

1 It has been over a decade that the E1684 trial, comparing adjuvant high-dose IFN
2 (HDI) to observation, showed an improvement in overall survival in melanoma patients.²⁶
3 Numerous randomized trials on IFN treatment followed but there are still many unsolved
4 questions. For instance; how does interferon work and what is the optimal treatment dose
5 or duration? Importantly, which patients are most likely to benefit, rectifying such a toxic
6 treatment? Results from the Hellenic Oncology Group trial of Gogas et al.²⁷ support the
7 hypothesis of an immunomodulatory mechanism of action rather than a direct cytotoxic
8 effect of IFN. This study showed improved RFS and OS in patients developing clinical and
9 serological manifestations of autoimmunity during treatment with HDI. These findings
10 could not be confirmed by studies from the EORTC and Nordic Melanoma Group, report-
11 ing no benefit for patients who became autoantibody positive during treatment with
12 adjuvant IFN or pegylated-(PEG-)IFN.^{28,29} Guarantee-time bias is an important confounder
13 when analyzing the prognostic value of any potential biomarker in serial measurements
14 and should be taken into account.^{30,31} When correction was made for guarantee-time bias,
15 autoimmunity remained a strong independent prognostic marker in the Gogas's study
16 but it lost significance in the side studies from the EORTC 18952, EORTC 18991 and Nordic
17 IFN trial. Although the same ELISAs (Quanta Lite, Inova Diagnostics) and titers were used
18 in the studies by Gogas et al. and the EORTC/Nordic Melanoma group collaboration, ma-
19 jor differences were found for the occurrence of autoantibodies at baseline: 33% and 35%
20 of the patients from the EORTC 18952 study and from the Nordic IFN trial respectively had
21 pre-existing autoantibodies, against 1.5% reported by Gogas et al. This is extremely low
22 considering antinuclear antibodies for instance, with reported prevalences ranging 4 to
23 35% in healthy individuals, and prevalences up to 40% in cancer patients.

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

35 Identification of new biomarkers and confirmatory studies of so far promising biomarkers
36 is needed because these could help us understand the mechanisms of action of interferon- α
37 and could enable tailored treatment to those most likely to benefit. Here we showed that
38 ferritin levels and ferritin/CRP ratios were significantly increased by the administration of
39 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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1 ABSTRACT

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3 Background: Appearance of autoantibodies and clinical manifestations of autoimmunity
4 in melanoma patients treated with adjuvant interferon (IFN)- α 2b was reported to be
5 associated with improved prognosis. We assessed the association of the appearance of
6 autoantibodies after initiation of treatment with recurrence-free interval in two random-
7 ized trials that compared intermediate doses of IFN to observation for the treatment of
8 melanoma patients.

9 Methods: Serum levels of anticardiolipin, antithyroglobulin, and antinuclear anti-
10 bodies were determined using enzyme-linked immunosorbent assays in 187 and 356
11 patients in the European Organization for Research and Treatment of Cancer (EORTC)
12 18952 and Nordic IFN trials, respectively, immediately before, and up to 3 years after
13 random assignment. The association of the presence of at least one of the three autoan-
14 tibodies with risk of recurrence was assessed by three Cox models in patients negative
15 for all three autoantibodies at baseline (125 from the EORTC 18952 trial and 230 from
16 the Nordic IFN trial): 1) a model that considered appearance of autoantibodies as a time-
17 independent variable, 2) one that considered a patient autoantibody positive once a
18 positive test for an autoantibody was obtained, and 3) a model in which the status of
19 the patient was defined by the most recent autoantibody test. All statistical tests were
20 two-sided.

21 Results: When treated as a time-independent variable (model 1), appearance of auto-
22 antibodies was associated with improved recurrence-free interval in both trials (EORTC
23 18952, hazard ratio [HR] = 0.41, 95% confidence interval [CI] = 0.25 to 0.68, $P < .001$;
24 and Nordic IFN, HR = 0.51, 95% CI = 0.34 to 0.76, $P < .001$). However, on correction for
25 guarantee-time bias, the association was weaker and not statistically significant (model
26 2: EORTC 18952, HR = 0.81, 95% CI = 0.46 to 1.40, $P = .44$; and Nordic IFN, HR = 0.85, 95%
27 CI = 0.55 to 1.30, $P = .45$); model 3: EORTC 18952, HR = 1.05, 95% CI = 0.59 to 1.87, $P = .88$;
28 and Nordic IFN, HR = 0.78, 95% CI = 0.49 to 1.24, $P = .30$).

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

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1 INTRODUCTION

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

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

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

33 We studied whether the occurrence of autoantibodies in melanoma patients receiv-
34 ing intermediate doses of IFN (IFN- α 2b) or no treatment was associated with response
35 to treatment. Patients for this translational study were accrued in two large randomized
36 trials: the European Organization for Research and Treatment of Cancer (EORTC) 18952
37 trial and the Nordic IFN trial. Both of these trials compared treatment with intermediate
38 doses of IFN with observation.^{11,12} Because the presence of autoantibodies varies over
39 time, it was crucial to choose a statistical approach that took time to seroconversion

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

5

6

7 **PATIENTS AND METHODS**

8

9 **Patients**

10 The EORTC 18952 study and the Nordic IFN trial were designed to evaluate efficacy and
11 toxicity of intermediate doses of IFN in high-risk melanoma patients. For EORTC 18952,
12 between 1996 and 2000 a total of 1388 patients who had been diagnosed with stage IIB
13 or III melanoma were enrolled.¹¹ These patients had tumors with Breslow thickness 4 mm
14 and greater and were lymph node-negative (N0), or had undergone curative dissection
15 of regional lymph nodes (either completion lymphadenectomy following positive sen-
16 tinel node procedure [stage III, N1] or therapeutic lymph node dissection for palpable
17 nodes [stage III, N2]). Patients were randomly assigned in relative proportions of 2:2:1 to
18 13 months of intermediate high-dose IFN, 25-months of intermediate low-dose IFN, or
19 observation only, respectively. IFN-treated patients received an induction treatment of
20 4 weeks of 10 million units (MU) IFN, delivered 5 days a week. Induction was followed by
21 either 10 MU IFN three times a week for 1 year or 5 MU IFN three times a week for 2 years,
22 with all treatments delivered subcutaneously.

23 For the Nordic IFN trial, a total of 855 patients with stage IIB or III melanoma were
24 enrolled between 1996 and 2004. Patients were randomly assigned in equal propor-
25 tions to three treatment arms: observation only, induction treatment with 10 MU IFN
26 5 days a week for 4 weeks followed by 10 MU three times a week for 1 year (as in the
27 13-month IFN arm from the EORTC 18952), or induction treatment followed by 10 MU
28 IFN three times a week for 2 years. All treatments were delivered by subcutaneous injec-
29 tion.¹² All available sera from 27 collaborating centers were collected and autoantibody
30 levels were determined centrally at the Laboratory for Experimental Surgical Oncology,
31 Erasmus University Medical Center, Daniel den Hoed Cancer Center in Rotterdam. The
32 blood samples for this study were drawn at the same time points as for regular follow-up
33 tests, that is, before treatment, at the end of the induction phase (1, 3, 6, 12, 16, 20, and
34 24 months after the start of treatment), and at 30- and 36- month follow-up. Informed
35 consent from patients for translational research in association with these trials for the
36 purposes of identification of prognostic factors was obtained.

37

38

39

1 Serum Analysis

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

14 The assays were validated for reproducibility and concordance with routine, well-
 15 standardized enzyme-linked immunosorbent assays used by the Department of Immu-
 16 nopathology, Sanquin, Amsterdam, The Netherlands.^{13,14}

17

18 Statistical Analysis

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

35 In a Cox model in which autoantibody status was considered to be a time-indepen-
 36 dent variable (Figure 1, model 1), the hazard ratio of the event intensity per time unit
 37 in autoantibody-positive patients vs autoantibody-negative patients according to the
 38 initial number of lymph nodes and sex was expressed as: $HR = e^{\beta_1 \times \text{autoantibody status}(t) + \beta_2 \times$
 39 $\text{number of positive lymph nodes} + \beta_3 \times \text{sex}.$

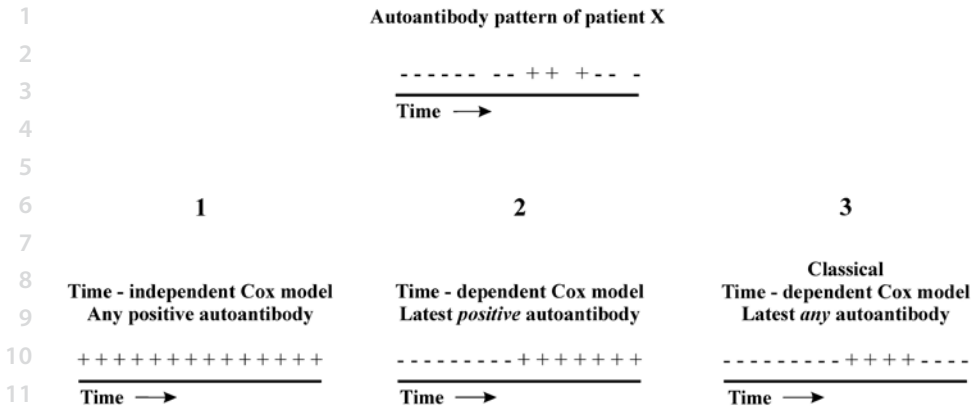


Figure 1. Three statistical approaches to evaluate the effect of autoantibodies on prognosis. The diagram at the top shows the serial results of autoantibody testing in a patient with transient seroconversion. According to model 1, if one or more autoantibody tests are positive, the patient is considered 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

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

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

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

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

31

32

33 RESULTS

34

35 Patients

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

1 baseline for at least one of three autoantibodies and thus considered to have developed
2 autoantibodies prior to treatment. No difference in recurrence-free interval between
3 patients with or without pre-existing autoantibodies was observed (Supplementary
4 Figure, Appendix). In the EORTC 18952 study, among the 125 patients who remained
5 after exclusion of those who were autoantibody positive at baseline, 54 patients were
6 randomly assigned to the 13-month IFN group, 48 to the 25-month IFN group, and 23
7 to the observation group. With a median follow-up of 4.2 years, recurrence-free interval
8 did not differ by a statistically significantly extent between treatment groups (overall P
9 = .36) (13-months IFN vs observation, HR = 0.81, 95% CI = 0.46 to 1.40; 25-months IFN vs
10 observation, HR = 0.66, 95% CI = 0.37 to 1.17). Median overall recurrence-free interval
11 among all patients who were free of autoantibodies before treatment was 2.1 years.
12 In the Nordic IFN trial, the 230 patients who did not test positive for one of the three
13 antibodies were randomly assigned as follows: 70 patients to no-adjuvant treatment,
14 79 patients to IFN treatment for 13 months, and 81 patients to IFN treatment for 25
15 months. With a median follow-up of 6.8 years, no statistically significant difference in
16 recurrence-free interval across treatment arms was observed (overall P = .43; 13-month
17 IFN vs observation, HR = 0.76, 95% CI = 0.50 to 1.17; 25-month IFN vs observation, HR
18 = 0.81, 95% CI = 0.53 to 1.24). Median recurrence-free interval for all 230 patients was
19 2.46 years.

20

21 **Seroconversion**

22 Seroconversion, the appearance of autoantibodies in patients who initially tested nega-
23 tive for all three autoantibodies, occurred in 43 (34%) of 125 and 73 (32%) of 230 patients
24 in the EORTC 18952 and Nordic IFN trials, respectively. Frequencies of the three auto-
25 antibodies were summarized (Supplementary Table, Appendix). Antinuclear antibodies
26 were the most prevalent in treated as well as untreated patients. Baseline characteristics
27 according to treatment for patients in both trials who initially tested negative for the
28 presence of autoantibodies, were comparable with those of the total patient population
29 from the EORTC 18952 and Nordic IFN trials (Table 1).^{11,12} There were no differences in the
30 crude rates of antibody appearance according to age, Breslow thickness, stage at ran-
31 dom assignment, or number of positive lymph nodes. However, differences in the crude
32 rates were seen according to treatment, sex, and the presence or absence of ulceration.

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

Characteristic	EORTC 18952†		Nordic IFN†	
	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.

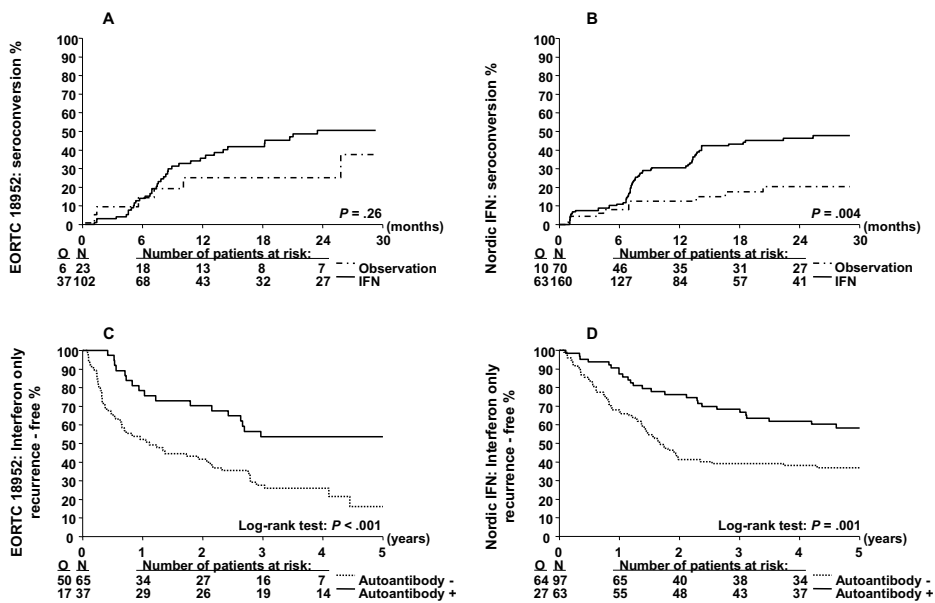


Figure 2.

Time to seroconversion for untreated patients vs patients receiving intermediate doses of IFN- α . **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.

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

5

6 **Association of Seroconversion with Recurrence-Free Interval**

7 In both trials, patients who became autoantibody positive during the study period
8 had a statistically significantly better recurrence-free interval compared with those
9 who remained autoantibody negative. In the EORTC 18952 trial, the 43 autoantibody-
10 positive patients had a statistically significantly ($P < .001$) lower relapse rate than the 82
11 autoantibody-negative patients (HR = 0.43, 95% CI = 0.27 to 0.71). In the Nordic IFN trial,
12 73 patients who became autoantibody positive during the course of the trial had a lower
13 relapse rate than the 157 patients who remained autoantibody negative (HR = 0.51, 95%
14 CI = 0.34 to 0.76; $P < .001$). Similar results were observed in IFN-treated patients only. In
15 the EORTC 18952 study (Figure 2, C), the 37 autoantibody-positive patients had statisti-
16 cally significantly better recurrence-free interval compared with the 65 autoantibody-
17 negative patients (HR = 0.39, 95% CI = 0.23 to 0.69; $P < .001$). Kaplan-Meier estimates
18 for the Nordic IFN trial (Figure 2, D) were comparable, showing a statistically significant
19 benefit in recurrence-free interval ($P = .001$) for the 63 autoantibody-positive patients
20 compared with 97 autoantibody-negative patients (HR = 0.48, 95% CI = 0.30 to 0.75).

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

31 However, these results were guarantee-time bias driven because patients with a longer
32 recurrence-free interval have a greater chance of developing autoantibodies, and, con-
33 versely, early relapses are more likely in autoantibody-negative patients because these
34 patients relapse before autoantibodies develop or can be detected.^{15,16} Thus, survival
35 estimates based on Kaplan-Meier curves are biased because they compare all patients
36 who tested positive for autoantibodies to patients who never tested positive, regardless
37 of when seroconversion occurred. To overcome the guarantee-time bias phenomenon,
38 we used two Cox models that treated autoantibody status as a time-dependent variable
39 (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*

Patient group and variable	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)	
	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).

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

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

26 To explore the effects of using higher cutoff values to dichotomize autoantibody status,
27 an analysis considering patients with a moderate test result for antibodies as negative was
28 performed. Using the higher cutoff values, only 11 of 187 (6%) and 31 of 356 (9%) patients
29 were initially strongly positive in the EORTC 18952 and Nordic IFN trials, respectively. After
30 their exclusion, there remained 176 patients in EORTC 18952 and 325 Nordic IFN patients.
31 Among these patients, 145 and 227 patients received IFN treatment in EORTC 18952 and
32 Nordic IFN, respectively. Although in the EORTC 18952 study, in the 18 (12%) IFN-treated
33 patients with strong seroconversion, there was a trend toward better recurrence-free in-
34 terval of marginal statistical significance using models 2 and 3 (model 2: HR = 0.46, $P = .07$;
35 model 3: HR = 0.38, $P = .06$), this trend was not observed in the Nordic IFN trial, in which 42
36 (18%) strongly positive patients did not have statistically significantly improved recurrence-
37 free interval (model 2: HR = 0.79, $P = .39$; model 3: HR = 0.83, $P = .53$; data not shown).

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1 DISCUSSION

2

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

21 Our results are not in accordance with the study by Gogas et al.,⁴ which demonstrated
22 a statistically significant improvement on both recurrence-free interval (HR = 0.12) and
23 OS for patients with evidence of autoimmunity when correction for guarantee-time bias
24 was applied. Furthermore, we observed that 33% of the patients in the EORTC 18952
25 study and 35% of those in the Nordic IFN trial had pre-existing autoantibodies, which is
26 in agreement with autoantibody prevalence reported in literature. Antinuclear antibod-
27 ies are commonly detected in healthy persons, with reported prevalences ranging 4 to
28 35%,⁶⁻⁹ but prevalences up to 40% in cancer patients have been reported.¹⁰ Furthermore,
29 the reported prevalence of anticardiolipin and antithyroid antibodies in healthy control
30 subjects ranges between 1 and 15%.^{6,21-26} Therefore, an autoantibody prevalence (de-
31 fined by the presence of one of the three assayed antibodies) of 33 and 35%, reported
32 for our studies at baseline, lies within the normal range. In contrast, the prevalence of
33 1.5% reported by Gogas et al.⁴ is extraordinarily low. The difference between their study
34 and our results cannot be explained by methodological differences because we used the
35 same (validated) assays and titers described by Gogas et al. Moreover, our exploration of
36 higher cutoff values did not change the overall results.

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

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

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

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

25 Our study had some limitations. The EORTC and Nordic trials were originally not
26 designed to determine whether serial serum autoantibody levels are prognostic and
27 predictive markers. It turned out that the number of patients and events reported were
28 sufficient to assess the prognostic importance in the IFN-treated patients. However in
29 the observation groups, especially in the EORTC 18952 trial, the series was quite limited
30 (23 patients), affecting the assessment of the prognostic value of autoantibody levels
31 in this subgroup and, therefore, of its predictive value (differential effect in IFN and
32 observation groups). The group of patients with prolonged recurrence-free interval is
33 relatively small, suggesting that the treatment, if active at all, is only of benefit in a minor
34 patient population. Identification of a potential biomarker is therefore only likely if this
35 marker has a strong relationship with treatment benefit.

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

1 version was more frequently observed in patients receiving treatment, yet the time to
2 seroconversion varied greatly. The findings indicate that the assessment of autoimmune
3 antibodies is not a useful tool in selecting patients who would benefit from treatment
4 with intermediate doses of IFN- α 2b.

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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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1 ABSTRACT

2

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

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

16 Results: Patients who were autoantibody negative at baseline were analyzed (n =
17 220). Occurrence of autoantibodies during follow-up was higher in the PEG-IFN-treated
18 patients (18% in the observation arm vs 52% in the PEG-IFN arm). Autoantibody appear-
19 ance was of prognostic importance by using model 1 (hazard ratio [HR] = 0.56, 95% CI =
20 0.36 to 0.87; $P = .01$). However, when guarantee-time bias was taken into account using
21 model 2 (HR = 1.19, 95% CI = 0.75 to 1.88; $P = .46$) or model 3 (HR = 1.14, 95% CI = 0.71
22 to 1.83; $P = .59$), significance was lost. Results were similar when treatment groups were
23 analyzed separately.

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

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1 INTRODUCTION

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

13 The appearance of autoantibodies and clinical manifestations of autoimmunity in
14 patients treated with adjuvant high-dose IFN was shown to strongly correlate with
15 improved RFS and OS.⁵ Likewise, in patients receiving low-dose IFN, the occurrence of
16 autoimmune thyroid disease was associated with improved RFS.⁶ These studies seemed
17 promising but could not explore the predictive value of autoimmunity on treatment
18 response because no observation arm was analyzed. Previously we assessed the impact
19 of autoantibodies on prognosis in patients with melanoma receiving intermediate doses
20 of IFN- α -2b or no treatment in a side study to the European Organisation for Research
21 and Treatment of Cancer (EORTC) 18952 trial⁷ and the Nordic IFN trial.⁸ The appearance
22 of autoimmune antibodies was neither a strong prognostic nor a strong predictive indi-
23 cator.⁹ Because the occurrence of autoantibodies varied over time, guarantee-time bias
24 affected the results of the regular log-rank test. Two time-dependent Cox models and
25 the landmark method were used to correct for guarantee-time bias.¹⁰

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

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1 PATIENTS AND METHODS

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3 Patients

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

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

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19 Serum analysis

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

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

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1 **Statistical analysis**

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

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

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

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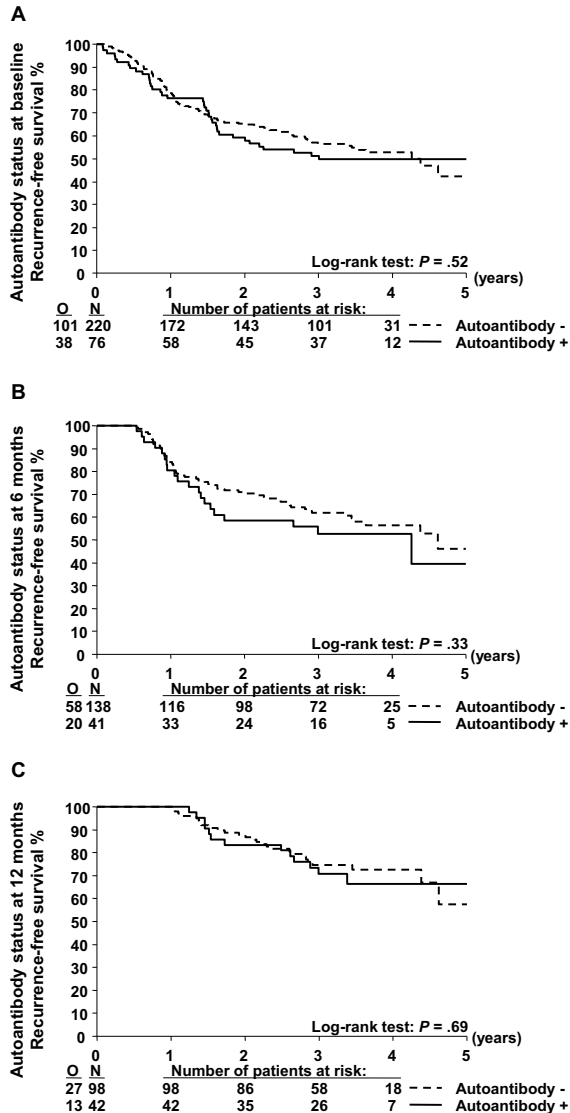
34 **RESULTS**

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36 **Patients**

37 All available serum samples from the EORTC 18991 trial were collected for this collateral
38 study. Baseline autoantibody levels were determined in 296 patients; of these, 76 patients
39 (26%) in EORTC 18991 were initially positive for at least one of the three autoantibodies.

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



36 **Figure 1.**

37 (A) Kaplan-Meier estimates of recurrence-free survival for patients with or without pre-existing
 38 autoantibodies. For all patients who were autoantibody negative at baseline, landmark analyses are
 39 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

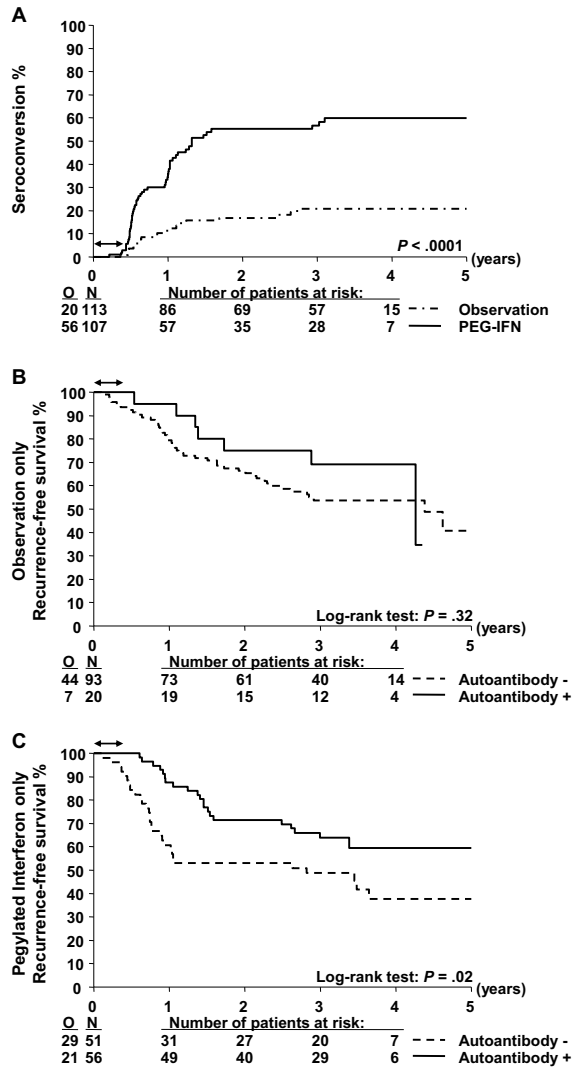


Figure 2.

(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.

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

5 6 **Prognostic impact of autoantibodies on RFS**

7 8 *Biased analysis*

9 Kaplan-Meier estimates of RFS comparing autoantibody-positive with autoantibody-
10 negative patients are depicted for observation patients in Figure 2, B and for patients
11 treated with PEG-IFN in Figure 2, C. In both treatment groups, autoantibody-positive
12 patients had a better RFS compared with autoantibody-negative patients. In the obser-
13 vation group, 20 patients who became autoantibody positive had a lower relapse rate (P
14 = .32) than 93 autoantibody-negative patients (HR = 0.67, 95% CI = 0.30 to 1.48; Figure
15 2, B). Patients treated with PEG-IFN who became autoantibody positive ($n = 56$) had
16 significantly better RFS ($P = .02$) compared with patients who remained autoantibody
17 negative ($n = 51$; HR = 0.51, 95% CI = 0.29 to 0.90; Figure 2, C).

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

25 26 *Unbiased analysis*

27 Guarantee-time bias drove the results mentioned in the previous section.¹⁰ Because
28 patients with longer survival have a higher chance for developing autoantibodies, they
29 benefit the autoantibody-positive group. Additionally, early relapses are more likely
30 in autoantibody-negative patients because these patients experience relapse before
31 autoantibodies develop or can be detected, thus disfavoring the autoantibody-negative
32 group. For instance, relapses within the first 6 months occurred in the autoantibody-
33 negative group because the first opportunity to test positive for one of the autoanti-
34 bodies was at 6 months (Figure 2, indicated by the arrows). Kaplan-Meier estimates are
35 biased since they compare patients who tested positive for autoantibodies with patients
36 who never tested positive, regardless of when seroconversion occurred. To overcome
37 guarantee-time bias, we used two different Cox models that treated the occurrence of
38 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, O=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; $P = .03$).

1 **Predictive significance of autoantibodies on RFS**

2 We evaluated the predictive value of autoantibodies, and assessed its impact on out-
3 come in both treatment groups separately. Using the time-dependent Cox models, se-
4 roconversion in PEG-IFN–treated patients was not associated with improved RFS (model
5 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
6 results were found for untreated patients (model 2: HR = 1.13, 95% CI = 0.49 to 2.59; and
7 model 3: HR = 0.83, 95% CI = 0.29 to 2.37; Table 3).

8

9

10 **DISCUSSION**

11

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

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

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

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

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

30 We assessed the prognostic value of autoimmune antibodies in 839 patients with
31 melanoma from three randomized controlled trials comparing PEG-IFN or IFN treat-
32 ment with no treatment. None of the studies showed an RFS benefit for patients who
33 developed autoantibodies, whether treated with PEG-IFN/IFN or untreated. Moreover
34 exploring higher cutoff values, the occurrence of autoantibodies was even a detrimental
35 prognostic factor in PEG-IFN-treated patients. However, the mechanism behind these
36 results remains unclear. In addition, the prognostic significance of strong seroconver-
37 sion in untreated patients is still unclear because this involved only six patients. In
38 conclusion, the results reported here suggest that determination of autoantibodies is
39 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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Organisation for Research and Treatment of Cancer (EORTC) Melanoma Group

1 ABSTRACT

2

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

6 211 patients who participated in the EORTC 18952 trial, evaluating efficacy of adju-
7 vant intermediate doses of interferon α 2b (IFN) versus observation, entered a corollary
8 study. Over a period of 36 months, 918 serum samples were collected. The Cox time-
9 dependent model was used to assess prognostic value of the latest (most recent) S100B
10 determination.

11 At first measurement, 178 patients had S100B values $< 0.2 \mu\text{g/l}$ and 33 $\geq 0.2 \mu\text{g/l}$.
12 Within the first group, 61 patients had, later on, an increased value of S100B ($\geq 0.2 \mu\text{g/l}$).
13 An initial increased value of S100B, or during follow-up, was associated with worse
14 DMFS; hazard ratio (HR) of S100B ≥ 0.2 versus S100B < 0.2 was 5.57 (95% CI = 3.81 to
15 8.16), $P < .0001$, after adjustment for stage, number of lymph nodes and sex. In stage IIB
16 patients, the HR adjusted for sex was 2.14 (95% CI = 0.71 to 6.42), whereas in stage III, the
17 HR adjusted for stage, number of lymph nodes and sex was 6.76 (95% CI = 4.50 to 10.16).
18 Similar results were observed regarding overall survival (OS).

19 Serial determination of S100B in stage IIB-III melanoma is a strong independent
20 prognostic marker, even stronger compared to stage and number of positive lymph
21 nodes. The prognostic impact of S100B $\geq 0.2 \mu\text{g/l}$ is more pronounced in stage III disease
22 compared with stage IIB.

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1 INTRODUCTION

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3 The S100B protein is an immunohistological marker for malignant melanocytes¹ that
4 was first detected in melanoma cell cultures² and is overexpressed in most cells of neu-
5 roectodermal origin.³ S100B is located in the cytoplasm and in the nucleus as a disulfide
6 cross-linked homo- or heterodimers containing primarily Ca²⁺.⁴ It has many cell func-
7 tions, mainly in cytoskeleton integrity, in cell cycle regulation⁵ and apoptosis through
8 its interaction with p53.⁶ However, the mechanism by which the protein leaks to the
9 blood is not fully understood but most probably related to cell damage or cell death.⁷
10 This is strongly supported by the fact that immunoreactive S100B is found exclusively
11 in the cytoplasm and nucleus; a limited secretion of the protein has only been reported
12 in the brain and, its substantial release has been found in brain damage and stage IV
13 melanoma.^{8,9} Additionally, serum S100B monitoring has been found useful in the latter
14 disease.

15 While many reports show that S100B blood levels provide a good indication of
16 disease progression as well of response to chemotherapy in stage IV patients,¹⁰⁻¹² it
17 remains of little or limited usefulness in early stages (II & III) where the disease is most
18 often confined to lymph nodes.¹³ Guo and colleagues¹⁴ assessed S100B serum levels in
19 126 melanoma patients and found positive levels in 1.3%, 8.7%, and 73.9% of patients
20 with stage I/II, III, and IV disease, respectively. This illustrates its poor utility in stages
21 I-II-III. However, the progression from the early stages to distant organ involvement is
22 frequently accompanied by a significant increase in S100B levels.¹⁵

23 The utility of serial determinations of S100B serum levels in patients with high-risk
24 melanomas (stages IIB-III), using a time-dependent Cox model, has not been reported on
25 until now. As early elevations in S100B levels may precede clinically detectable relapses
26 serial determinations may have a particular prognostic value in the setting of adjuvant
27 systemic therapy. This study was designed to determine the prognostic value of serial
28 serum S100B levels in stage IIB-III melanoma. Patients were enrolled in the randomized
29 phase III EORTC 18952 trial evaluating the efficacy of adjuvant intermediate doses of
30 interferon- α 2b.¹⁶ Serum S100B levels were measured during treatment and follow-up, to
31 a maximum of 36 months.

32

33

34 MATERIAL AND METHODS

35

36 Patients and sample collection

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

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

13
 14 **Table 1.** Number of patients analyzed at the different time points.

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

17 * End of induction / observation

18 19 **S100B determination**

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

24 25 **Statistical analysis**

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

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

1 landmark method was used to test the prognostic impact of S100B level at 6 months for
 2 the subsequent outcome. Since sampling was scheduled every 3 months during the first
 3 year we used a time window of 45 days not to have overlap. The serial measurements of
 4 S100B have been performed at several time points (Table 1) with the same schedule for
 5 IFN-treated as untreated patients. Since the samples were not exactly drawn at the time
 6 points according to study protocol, numbers in Table 1 reflect the closest time points. As
 7 mentioned before, for patients who developed distant metastases, generally their S100B
 8 levels have not been assessed subsequently after their relapse, therefore the sampling
 9 rate is lower towards the end of the study. To determine whether the latest (most recent)
 10 value of S100B, which was assessed during the course of the study, before or at the time
 11 of distant metastases, has a prognostic impact on the subsequent outcome, the Cox
 12 time-dependent model was used.¹⁸ In the Cox time- dependent model, for patients free
 13 of event (distant metastasis – for DMFS - or death – for OS) just before a time point t, the
 14 HR was set as; $HR = e^{\beta_1 \times S100B(t) + \beta_2 \times Stage + \beta_3 \times \text{Number of positive lymph nodes} + \beta_4 \times Sex}$
 15 with $S100B(t) = 0$ if the latest S100B determined before or at time t was $< 0.2 \mu\text{g/l}$;
 16 or $= 1$, if the latest S100B value determined before or at time t was $\geq 0.2 \mu\text{g/l}$. Thus, for
 17 purposes of risk assessment the most recent value of S100B at time t (determined at that
 18 time or earlier if not available at time t) was used. In the Cox time-dependent model, all
 19 available samples/determinations were used, regardless of a time window.

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

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

30

31

32 RESULTS

33

34 Baseline characteristics and S100B values

35 The distribution of patients according to the treatment protocol, stage of the disease,
 36 number of positive lymph nodes, sex and presence of ulcerated primary melanoma, is
 37 summarized in Table 2. These characteristics were comparable with those of patients
 38 included in the entire 18952 study, and treatment distribution was in accordance with
 39 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)

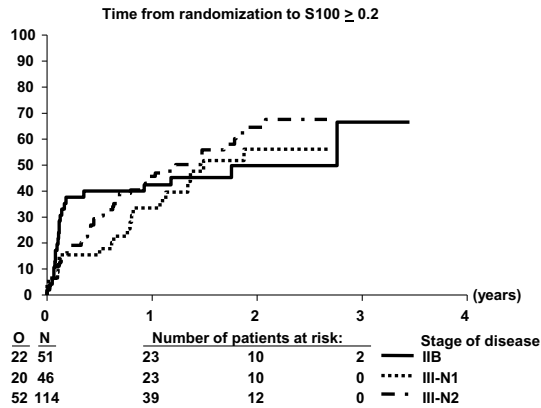
IFN group. At the initial measurement time point, corresponding generally to the end of induction/observation period (median of 28 days from randomization), 178 patients had S100B values below 0.2 µg/l and 33 above or equal 0.2 µg/l. A higher incidence of stage IIB patients has been found in those with 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

1 cumulative rate of patients with S100B level $\geq 0.2 \mu\text{g/l}$ was higher in stage IIB patients
2 than in stage III patients.



15 **Figure 1.**

16 Time to first S100B level $\geq 0.2 \mu\text{g/l}$ by initial stage of disease.

17

18 A total of 61 patients (65%) from the 94 patients reaching S100B $\geq 0.2 \mu\text{g/l}$ developed
19 distant metastases, of these, 6 patients (6%) had such a rise after the detection of the
20 metastases. For the remaining 55 patients, median time between S100B $\geq 0.2 \mu\text{g/l}$ and
21 the development of distant metastases was 94 days (range 0-1580 days). Contrary,
22 from the 117 patients with normal S100B levels, 62 (53%) patients developed distant
23 metastases.

24 The impact of the initial S100B levels on DMFS and OS was especially seen within
25 one year from randomization, but thereafter the curves converged (Figure 2). According
26 to the Wilcoxon test, a test which gives more weight to differences occurring at earlier
27 time points, there was a significant difference between initial S100B levels and DMFS
28 ($P = .03$). However, when considering the overall curve using the Log-rank test, this dif-
29 ference was not significant ($P = .21$). This temporary effect of elevated S100B levels on
30 prognosis is also illustrated in Figure 3, showing Landmark methods for DMFS and OS at
31 6 months from randomization; patients with S100B serum level $\geq 0.2 \mu\text{g/l}$ at 6 months
32 after randomization have, at a short term, a worse prognosis compared to patients with
33 levels $< 0.2 \mu\text{g/l}$.

34 In order to evaluate the prognostic value of S100B level $\geq 0.2 \mu\text{g/l}$ for the different
35 disease stages, we performed an analysis in which we selected the 94 patients with
36 S100B level $\geq 0.2 \mu\text{g/l}$, excluding the 6 patients who developed distant metastases
37 before their rise in S100B levels. In these 88 patients, outcome was very different ac-
38 cording to the initial stage: stage IIB patients had a high 3-year distant metastasis-free
39 survival (76% at 3 years), whereas stage III-N1 and especially stage III-N2 patients had a

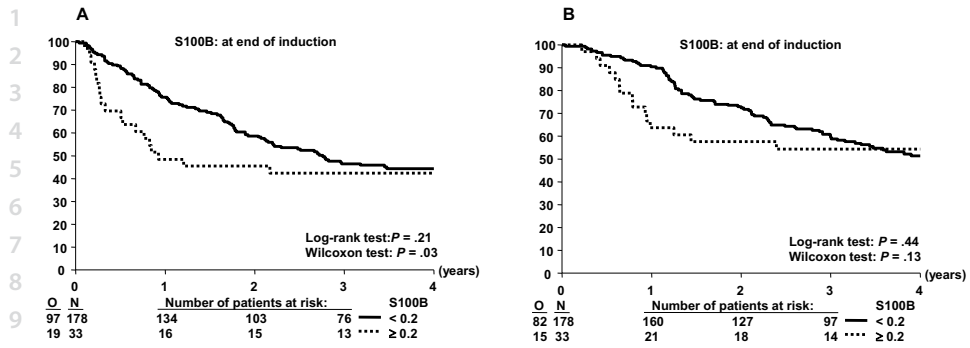


Figure 2. Kaplan-Meier curves regarding distant metastasis-free survival (A) and overall survival (B) from randomization according to initial S100B level (<math>< 0.2</math> vs ≥ 0.2 $\mu\text{g/l}</math>). Analyses were censored at 4 years. N=Number of patients at risk. O=Observed number of patients who developed distant metastases (A) or who died (B).$

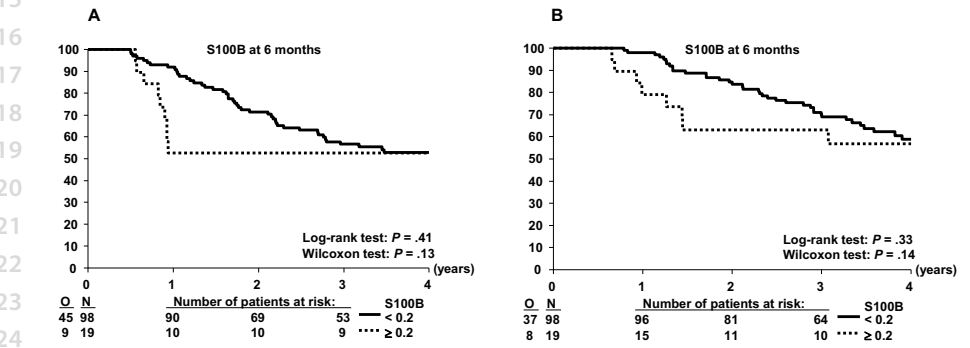


Figure 3. Landmark method: Kaplan-Meier curves regarding distant metastasis-free survival (A) and overall survival (B) from randomization according to S100B level (<math>< 0.2</math> vs ≥ 0.2 $\mu\text{g/l}</math>) assessed at 6 months post-randomization. Analyses were censored at 4 years. N=Number of patients at risk. O=Observed number of patients who developed distant metastases (A) or who died (B)$

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.

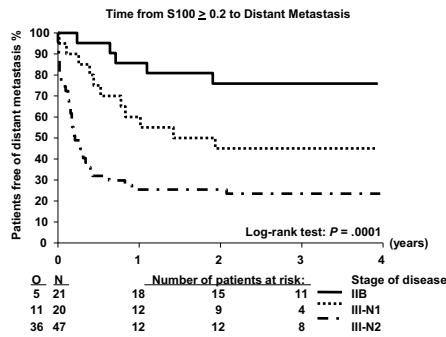


Figure 4.

Kaplan-Meier curves regarding distant metastasis-free survival from the first moment S100B level was $\geq 0.2 \mu\text{g/l}$, 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) $\geq 0.2 \mu\text{g/l}$ versus S100B(t) $< 0.2 \mu\text{g/l}$ 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,

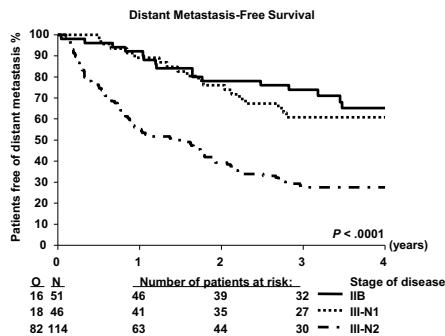


Figure 5.

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.

1 **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

24 a time variable right censored at 4 years

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

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

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

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

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

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

1 In summary, according to the time-dependent Cox model, which uses the most
2 recent S100B value (whether $< 0.2 \mu\text{g/l}$ or $\geq 0.2 \mu\text{g/l}$ at that time point), S100B is in both
3 the univariate as well as in the multivariate setting an independent prognostic factor
4 for worse DMFS and OS. This effect is most pronounced in stage III disease, and even
5 stronger than the other important prognostic factors, disease stage and number of posi-
6 tive lymph nodes.

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8

9 DISCUSSION

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

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

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

27 The prognostic impact of the baseline S100B on DMFS and OS has been observed,
28 especially within the first year after the assessment (Figure 2). Landmark analysis at 6
29 months also showed short term (6-12 months) differences between the two groups for
30 both DMFS and OS (Figure 3). These results suggest that a time-window exists within a
31 careful monitoring of S100B can be very helpful to assess the risk of distant metastasis
32 or death. This is further substantiated by the finding that while stage and the number of
33 positive lymph nodes correlated significantly and as expected with DMFS, S100B serial
34 value appeared to be in a Cox time-dependent model not only an independent prog-
35 nostic factor from these variables but also to have a much higher prognostic importance
36 than these variables. In stage III patients the estimated HR of $S100B \geq 0.20 \mu\text{g/l}$ versus
37 $S100B < 0.20 \mu\text{g/l}$, adjusted by these factors and by sex, was very high ($6.76, P < .0001$),
38 but was lower and not significant ($2.14, P = .18$, adjusted by sex only) in stage IIB patients
39 (Table 3). This finding is in accordance with previous studies that were all suggesting

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

11 Results from the EORTC 18991 trial, evaluating long-term treatment with pegylated
12 IFN- α 2b,²⁰ suggested that treatment with PEG-IFN was more beneficial in stage IIB and
13 stage III-N1 as compared to stage III-N2 patients. Our study of S100B within the frame-
14 work of the 18952 trial also raises the question whether S100B could identify patients
15 benefiting IFN treatment or not. One could argue that patients receiving IFN should
16 discontinue therapy once S100B levels of $\geq 0.2 \mu\text{g/l}$ have been reached, indicating
17 disease progression and no treatment response. Unfortunately we have no guidance
18 by biomarkers to determine use, dose or duration of adjuvant systemic therapy in
19 melanoma. Gogas and colleagues²¹ have reported that patients treated with adjuvant
20 IFN who developed autoantibodies or clinical signs of autoimmunity had a significantly
21 better outcome than patients who did not develop these signs of autoimmunity. The
22 development of markers that might predict who will mount a host immune response
23 could be extremely important. The markers could be used to determine which patients
24 to treat with IFN and for how long. An evaluation of the presence or emergence of
25 autoantibodies in patients who participated in the EORTC 18952 and Nordic IFN trial
26 did not confirm Gogas' observations.²² Nor did a subsequent similar study in the large
27 EORTC 18991 trial, showing no prognostic or predictive value of autoimmune antibodies
28 in PEG-IFN-treated patients.²³

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

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Chapter VII

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

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Minor modifications
Submitted

1 ABSTRACT

2

3 Background: To investigate the prognostic and predictive value of elevated serum YKL-
4 40 in high risk melanoma patients stage IIB-III treated with adjuvant interferon- α 2b.

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

9 Results: Univariate analysis showed no association between baseline serum YKL-40
10 and recurrence-free survival (RFS) in the Nordic Study and EORTC 18952, but in EORTC
11 18991 baseline YKL-40 was associated with RFS in the patients receiving pegylated
12 interferon (hazard ratio [HR] = 1.6, 95% confidence interval [CI] = 1.1 to 2.5; $P = .02$), but
13 not in the observation group ($P = .64$). Multivariate Cox analysis (including gender, age,
14 stage, ulceration, YKL-40) demonstrated that baseline YKL-40 was associated with overall
15 survival (OS) in the observation group from the Nordic Study and EORTC 18952 (HR =
16 1.33, 95% CI = 1.01 to 1.74; $P = .042$), but not in the interferon-treated patients (1-year
17 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;
18 $P = .64$). In EORTC 18991 multivariate analysis showed no association between baseline
19 YKL-40 and OS. During follow-up increases in YKL-40 were significantly associated with
20 short OS but not with RFS in univariate analysis.

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

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1 INTRODUCTION

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3 The incidence of melanoma has been rising in the last decade and the estimated
4 number of new cases in 2008 in USA is 62,480 and the number of deaths is 8,420.¹ The
5 prognosis of early-stage melanoma is excellent with 5-year survival of 99%, whereas the
6 prognosis of patients with thick primary melanomas (> 4 mm) or with regional lymph
7 node metastasis remains poor.² No clear-cut impact on overall survival has been found
8 using adjuvant interferon after surgery in patients with high-risk melanoma stage II and
9 III, although improved recurrence-free survival is demonstrated.³⁻⁵ No clinical or histo-
10 logical parameters with predictive value of interferon treatment have been identified. It
11 has been suggested that treatment decisions in high-risk melanoma patients should be
12 made on a case-by-case basis.⁶ Thus there is a need for new biomarkers for personalized
13 treatment of patients with high-risk melanoma stage IIB or III.

14 High serum levels of YKL-40 (Chitinase-3-like-1 protein, CHI3L1) has been suggested
15 as a new independent prognostic biomarker of poor survival in patients with melanoma
16 stage I, II and IV⁷⁻⁹ and in other types of primary and metastatic cancer with highest
17 levels in metastatic disease.¹⁰ High serum YKL-40 predicts low efficacy of anthracycline
18 therapy in patients with first recurrence of breast cancer,¹¹ and second-line chemore-
19 sistance in ovarian cancer patients.¹² Serum YKL-40 may also be useful for monitoring
20 disease progression.^{13,14}

21 YKL-40 is produced by cancer cells, including melanoma cells, and inflammatory
22 cells¹⁵⁻¹⁹ and plays a role in angiogenesis,²⁰⁻²³ tissue remodelling,¹⁰ fibroblast prolifera-
23 tion,²⁴ and protects against apoptosis.²⁵

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

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31 PATIENTS AND METHODS

32

33 Patients

34 Serum YKL-40 was determined in three prospective, independent studies of patients
35 with high risk melanoma stage IIB–III included in randomised, clinical trials on adjuvant
36 interferon, the Nordic Study,²⁶ EORTC 18952,³ and EORTC 18991.⁴ YKL-40 was analysed
37 in serum samples collected after definitive surgery, that is, also after surgery for lymph
38 node metastasis or recurrence developed during follow-up, and before treatment with
39 adjuvant interferon or observation, during treatment, and during follow-up. Patients

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

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

7 Patients included in the EORTC 18991 Study had

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

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

18 *The Nordic Study:* 855 patients were included between November 1996 and August
19 2004 from 2 Departments in Denmark, 25 in Sweden, 6 in Norway, and 5 in Finland.
20 Serum was available from 602 of these patients from 2 Departments in Denmark, 14
21 in Sweden, 2 in Norway, and 1 in Finland. Median follow-up was 92 months (range 46-
22 140 months). Patients were randomised in a 1:1:1 fashion between observation (arm
23 A), treatment with 10 MU interferon- α 2b s.c. 5 times per week for 4 weeks, followed by
24 maintenance therapy with 10 MU interferon- α 2b s.c. 3 times per week for 1 year (arm B),
25 or maintenance therapy with 10 MU interferon- α 2b s.c. 3 times per week for 2 years (arm
26 C). Serum samples were available at baseline up to 70 days after definitive surgery prior
27 to randomization, and after 3, 6, 9, 12, 16, 20, 24, 30, 36, 42, 48, 54 and 60 months, and
28 then once a year for up to 10 years of follow-up. Baseline serum samples were available
29 for YKL-40 determination in 452 patients. A total of 3971 serum samples were collected
30 and the median number of samples from each patient was 6 (range 1-21). The reasons
31 for missing samples were either that the samples were not drawn or lost during storage.

32 *EORTC 18952:* 1388 patients were included between May 1996 and June 2000. Serum
33 samples were available from 246 of these patients. Baseline samples were drawn up to
34 56 days after definitive surgery. There were 85 participating departments from France,
35 Bulgaria, United Kingdom, Switzerland, the Netherlands, and Belgium. Eight of these
36 centres contributed serum samples. Serum samples were drawn at randomisation prior
37 to treatment and after 1, 3, 6, 9, 12, 16, 20, 24 and 30 months. Patients were randomised
38 in a 1:2:2 fashion between observation (arm A), treatment with 10 MU interferon-
39 α 2b s.c. 5 times per week for 4 weeks, followed by maintenance therapy with 10 MU

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

6 *EORTC 18991*: 1256 patients were included between June 2000 and June 2004. Serum
7 samples were available from 386 of these patients. Baseline serum samples were drawn
8 up to 70 days after definitive surgery. There were 99 participating departments from 17
9 countries (mainly in Europe), of which 16 contributed serum samples. Median follow-up
10 was 43 months (range 25-68 months). Patients were randomly assigned in a 1:1 fashion
11 between observation or treatment with pegylated interferon- α 2b 6 μ g/kg/week for 8
12 weeks, followed by maintenance therapy with 3 μ g/kg/week for 5 years. Baseline serum
13 samples were available from 260 patients. Serum samples were drawn at baseline prior
14 to treatment and after 6, 12, 18, 24, 30, 36, 42, 48, 54 and 60 months. A total of 1691
15 serum samples were collected, and the median number of samples from each patient
16 was 5 (range 1-11).

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

22

23 **YKL-40 analysis**

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

33

34 **Serum YKL-40 in healthy subjects**

35 The reference interval of serum YKL-40 was determined in 245 (134 women and 111
36 men, median age 49 years, range 18-79 years) healthy subjects who did not take any
37 medicine, had no known disease or symptoms of joint, metabolic or hormonal diseases.
38 The median serum YKL-40 in these 245 healthy subjects was 43 μ g/L (range: 20-184 μ g/L;
39 5th to 95th percentile: 20-124 μ g/L) with no difference between men and women.²⁸ Since

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

4 5 **Statistical analysis**

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

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

22 23 24 **RESULTS**

25
26 Baseline clinical and histological characteristics of the patients are shown in Table 1. The
27 median serum YKL-40 and percentage of patients with elevated YKL-40 (age-corrected)
28 were similar in the three study populations ($P = .15$, Chi-square test). 10%, 18%, and 16%
29 had elevated serum YKL-40 at baseline in the Nordic Study, EORTC 18952, and EORTC
30 18991, respectively, i.e. in blood taken up to 70 days after operation. Comparison of
31 serum YKL-40 levels in the three studies demonstrated a significant difference ($P = .003$,
32 Kruskal-Wallis test), with lower levels in the Nordic Study compared with the two EORTC
33 studies (these two were not statistically different, $P = .36$). Supplementary Table 1 (Ap-
34 pendix) gives serum YKL-40 in relation to stage and ulceration. 307 (51%) patients in
35 the Nordic Study, 164 (67%) in the EORTC 18952 Study, and 203 (53%) in the EORTC
36 18991 Study had recurrence during follow-up. 265 (44%) patients in the Nordic Study,
37 121 (49%) in the EORTC 18952 Study and 139 (36%) in the EORTC 18991 Study died
38 during follow-up.

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

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

23 Values are numbers (%).

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

26 **Breslow, <2 mm.

27 ***Breslow, 2-3.99 mm.

28 Baseline serum YKL-40 and recurrence free survival

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

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

39

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; $P = .02$).

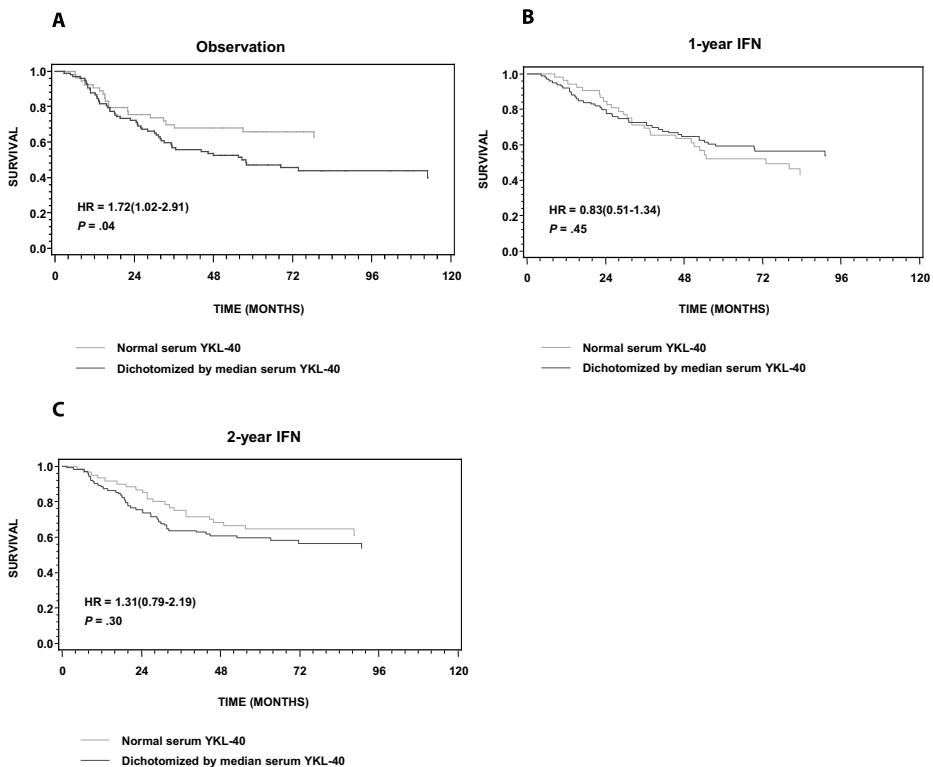
Table 2. Multivariate analyses of overall survival for gender, age, stage, ulceration, and baseline serum YKL-40 in the Nordic Study combined with the EORTC 18952 Study and in the EORTC 18991 Study.

	Nordic Study and EORTC 18952				EORTC 18991			
	Group	HR	95% CI	p-value	Group	HR	95% CI	p-value
Gender f vs m		0.71	0.44-1.13	.14		0.94	0.49-1.81	.84
Age	Obs	0.97	0.80-1.17	.74	Obs	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		0.61	0.40-0.94	.03		1.91	0.94-3.87	.07
Age	IFN 1-year	1.24	1.03-1.50	.02	Pegylated IFN	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		0.54	0.33-0.87	.01				
Age	IFN 2-year	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

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

7 Figure 1 illustrates the Kaplan-Meier curves of OS according to baseline serum YKL-40
 8 in the Nordic Study. Since only few patients had elevated (age-corrected) serum YKL-40
 9 compared to healthy subjects, the median baseline serum YKL-40 level (i.e. after operation)
 10 was used as cutoff. Serum YKL-40 above the median in the untreated patients from the
 11 Nordic Study and EORTC 18952 was associated with short OS, whereas this was not found
 12 in the patients treated with adjuvant interferon. Similar results were found for the patients
 13 included in EORTC 18991. Kaplan-Meier curves are showed for the Nordic Study (Figure 1).
 14



36 **Figure 1.** Kaplan-Meier survival curves according to baseline serum YKL-40 in the three treatment arms (**A**:
 37 observation group; **B**: patients randomized to one year of adjuvant interferon; **C**: patients randomized to
 38 two years of adjuvant interferon) in the Nordic study showing the association between serum YKL-40 above
 39 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.

1 Serum YKL-40 during follow-up

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

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

14 *EORTC 18952:* Patients with serum YKL-40 above the median level during follow-up
15 had no shorter OS or RFS, Table 3. Using the ratio of serum YKL-40 to the previous mea-
16 surement of serum YKL-40, shorter OS was demonstrated if the ratio increased (HR =
17 1.84, $P = .043$). For RFS, the results were not significant, Table 3.

18 *EORTC 18991:* Patients with serum YKL-40 above the median level during follow-up
19 had shorter OS (HR = 1.33, $P = .004$). Using the ratio of the serum YKL-40 to the previous
20 measurement of serum YKL-40 shorter OS was demonstrated if the ratio increased (HR =
21 1.39, $P = .044$). For RFS the results were not significant, Table 3.

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

		Overall survival			Recurrence-free survival		
		N	HR (95% CI)	P-value	N	HR (95% CI)	P-value
26 Nordic 27 Study	Actual value ¹	585	1.31 (1.16-1.47)	< .0001	584	1.12 (1.0-1.26)	.06
	Ratio to previous 28 measurement ²	546	1.43 (1.13-1.80)	.002	545	1.15 (0.92-1.42)	.22
29 EORTC 30 18952	Actual value ¹	138	1.15 (0.89-1.40)	.34	138	1.01 (0.82-1.24)	.94
	Ratio to previous 31 measurement ²	127	1.84 (1.02-3.31)	.04	127	1.10 (0.62-1.93)	.25
32 EORTC 33 18991	Actual value ¹	298	1.33 (1.10-1.62)	.004	298	1.17 (0.99-1.39)	.07
	Ratio to previous 34 measurement ²	298	1.39 (1.01-1.92)	.04	298	1.07 (0.82-1.39)	.61

35
36
37 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.

38 2: The ratio of the updated serum YKL-40 level to the preceding on the log scale. Only patients with at
39 least 2 levels have been included.

1 DISCUSSION

2

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

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

21 Multivariate analysis in the Nordic Study and EORTC 18952 combined showed that
22 patients with elevated baseline serum YKL-40 above the median level had shorter OS,
23 an impact that disappeared in the interferon-treated patients. This gives rise to specula-
24 tions on whether baseline serum YKL-40 has predictive value regarding adjuvant treat-
25 ment with interferon in high-risk melanoma patients. This would rest on the assumption
26 that patients operated for stage IIB-III melanoma with highest postoperative levels of
27 serum YKL-40 defines a high risk sub-group population, who do benefit from inter-
28 feron therapy, since a causative relationship between YKL-40 and effect of interferon
29 therapy has not been established. If indeed postoperative serum YKL-40 has predictive
30 value regarding adjuvant interferon therapy in stage IIB-III melanoma, an impact on RFS
31 would be expected as adjuvant interferon treatment in multiple trials^{3-5,31} with just one
32 exception³² has yielded positive impact on RFS, but not on OS. However, no impact on
33 RFS was found in our study. The question whether postoperative serum YKL-40 in stage
34 IIB-III melanoma contains predictive value therefore warrants further exploration in
35 prospective clinical studies.

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

1 to coagulate before centrifugation. In the two EORTC studies blood samples were drawn
2 into Corvac tubes, which contain silicone for the separation of serum and clot, and was
3 then allowed to coagulate for one hour at room temperature, and then centrifuged to
4 separate clot from serum. YKL-40 is contained in neutrophils and monocytes,^{15,16,19} but as
5 no enhancer of coagulation was used in any of the studies it is unlikely that differences
6 in the testing tubes can explain the higher YKL-40 levels seen in the two EORTC studies.
7 Leaving blood samples at room temperature to coagulate for up to three hours does not
8 affect the levels of serum YKL-40.³³

9 Baseline serum YKL-40 was elevated in subgroups of the patients – 10% in the Nordic
10 Study, 18% in EORTC 18952, and 16% in EORTC 18991, with median serum YKL-40 levels
11 at 47 µg/L, 54 µg/L, and 53 µg/L, respectively, well within the normal range for healthy
12 subjects. Subjects with elevated serum YKL-40 levels are at risk of dying of multiple dis-
13 eases. The patients included in this study, however, died almost exclusively of melanoma.
14 Death of other causes than cancer was 3.6% in the Nordic Study (10 of 281 deaths),
15 2.3% in EORTC 18952 (16 of 681 deaths),³ and 6.1% in EORTC 18991 (32/525).⁴ Different
16 factors might influence the levels of serum YKL-40. The baseline serum YKL-40 might be
17 a reflection of the primary and metastatic tumor cells that are excised, or a reflection of
18 residual tumor lesions. In the first case, levels would be expected to decrease, and in the
19 latter to either stay on the same level or increase in the case of active disease. Diseases
20 characterized by inflammation can influence the levels of serum YKL-40. Detailed infor-
21 mation regarding comorbidity is not available, as information on this was not prospec-
22 tively collected. Analysis of the serum samples for YKL-40 was not planned in the clinical
23 protocols. However, exclusion criteria in the three studies prevented randomization of
24 patients who used corticosteroids, NSAID, or other immunomodulatory treatment, as
25 well as patients with WHO performance status 2-4, and hereby presumably reducing the
26 number of patients with significant comorbidity. As this is a study with a large number of
27 randomized patients, patients with comorbidity are expected to be equally distributed.
28 Storage of serum samples was not under constant surveillance in the beginning, and
29 as a result a number of Norwegian serum samples were thawed unintentionally and
30 discarded. The serum YKL-40 analysis is however very robust and repetitive freezing and
31 thawing of serum samples up to 9 times had no effect on the serum YKL-40.³³⁻³⁷ Addition-
32 ally, baseline serum samples were drawn up to 70 days after surgery, carrying the risk
33 that samples drawn early after surgery give rise to falsely elevated serum YKL-40 due to
34 the trauma of surgery or infection related to surgery. However, it must be emphasized
35 that time from definitive surgery to baseline serum sample was equal in the three ran-
36 domization arms, as expected in this randomized patients population. Furthermore, an
37 increase in serum YKL-40 in connection with infection is rapidly normalized.³⁸

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

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

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

25 In conclusion, we found an association between serum YKL-40 and short OS in un-
26 treated high-risk stage IIB-III melanoma patients, but not in interferon-treated patients.
27 Increases in serum YKL-40 during treatment and follow-up were associated with short OS
28 but not with RFS. It is unknown whether serum YKL-40 will be proved useful in daily clini-
29 cal 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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1 ABSTRACT

2

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

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1 INTRODUCTION

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

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

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

1 METHODS

2

3 Patients and Controls

4 The study population consisted of 763 melanoma patients from Germany (418 male and
5 345 female), recruited by the Skin Cancer Unit Mannheim, from 2001 to 2008. Patients
6 with primary cutaneous melanoma with different disease stages that included; 10 cases
7 with in situ melanoma, 615 with stage I/II, 111 stage III, and 12 cases with stage IV of the
8 disease. For 15 patients stage was unknown. Disease staging was performed according
9 to the current AJCC criteria from 2001.¹³ Median and mean age of the melanoma cases
10 at diagnosis was 55 and 54 years, respectively. Blood samples from case subjects were
11 taken at their first presentation at the skin cancer unit. DNA was isolated from blood
12 samples using Qiagen mini-preparation kits. Informed consent was obtained from the
13 patients and the study was approved by the institutional ethical review board. Control
14 subjects included 734 healthy German individuals (367 male and 367 female) recruited
15 from blood bank Mannheim, with mean and median age of 60 and 61 years, respectively.
16 They were born in southwest Germany and were matched for ethnicity with cases. The
17 inclusion criteria for controls in the study included cancer free status. The age differ-
18 ence between the cases and controls was statistically significant (T-test; P value $< .01$),
19 whereas, the gender difference was not statistically significant (χ^2 -test; P value $> .05$).

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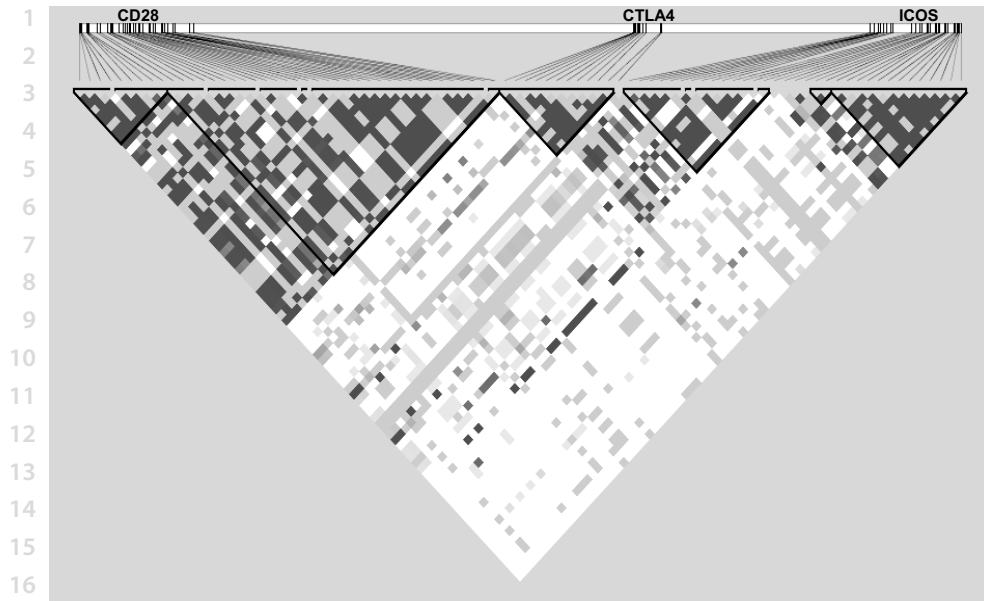
21 Genes and SNPs selection

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

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37 Validation of the SNPs by DNA sequencing

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



17 **Figure 1.**
 18 Haplotype blocks in the genomic region with CTLA-4, CD28 and ICOS genes based on HapMap data.
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20 Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Ca, USA) and the
 21 following conditions were used; initial 94°C for 1 min followed by 27 cycles at 96 °C for
 22 16 s, 56°C for 5 s and 60°C for 4 min. Reaction products were run on ABI prism 3100
 23 Genetic analyzer (Applied Biosystems). Primers used in PCR amplification and sequenc-
 24 ing reaction are listed in Supplementary Table (appendix).

25 26 **Genotyping**

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

34 35 **Statistical analysis**

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

1 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.

11 RESULTS

13 Case - control study

14 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).

25 **Table 1. Case Control**

26 SNP	Genotype	Cases N=763	(%)	Controls N=734	(%)	OR	95% CI	p-value	
27 CD28									
28 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	
31	G-allele	961	63	987	67				
	A-allele	565	37	481	33	1.18	1.00 – 1.38	.05	
	32 rs3181100	CC	279	37	229	31			
33	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				
35	G-allele	600	39	647	44	0.83	0.71 – 0.97	.02	
	36 rs3181101	CC	570	75	559	77			
	CG	175	23	165	23	0.98	0.76 – 1.26		
37	GG	17	2	7	10	1.97	0.78 – 4.96	.35	
	38 C-allele	1315	86	1283	88				

1 **Table 1. Continued**

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

1 **Table 1. Continued**

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

1 **Table 1. Continued**

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

1 Association between polymorphisms and prognosis

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

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21 **Table 2. CD28**

SNP	Genotype	Cases (%) N=587	Metastases-Free Survival			Overall Survival		
			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

1 **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

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20 **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

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1 DISCUSSION

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

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

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

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

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

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

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Chapter IX

General Discussion

Adapted from:
Curr Opin Oncol.2011 Mar;23:170-6
Mol Oncol.2011 Feb (In press)

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

13

14

15 **PART ONE**

16

17 **1. Cytokines**

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

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

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

9

10 **2. C-reactive protein**

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

25 Stam et al. previously assessed the effects of interferon $\alpha 2b$ on the acute-phase
26 response in a small subset of stage IIB-III melanoma patients participating in the EORTC
27 18952 trial.¹³ They found significant increases in ferritin levels and less pronounced
28 decreases in CRP levels at end of induction and at 6 months in the treated group as
29 compared to baseline levels. We recently extended the analysis in order to explore the
30 association of ferritin and CRP changes over time with clinical response to IFN therapy
31 (this thesis). Ferritin levels in the IFN-treated patients were increased from end of induc-
32 tion and remained high during the treatment period. CRP levels varied slightly during
33 the course of the study but no significant differences between treated and untreated
34 patients were observed. Some patients in the IFN group had decreased CRP levels at end
35 of induction. However, none of these changes were predictive for treatment response in
36 patients receiving IFN (*In press*).

37

38

39

1 **3. Autoimmunity**

2

3 **3.1. IL-2**

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

27

28 **3.2. IFN**

29 Gogas et al. were the first to evaluate prognostic significance of autoimmune antibod-
30 ies and or clinical signs of autoimmunity (vitiligo) in melanoma patients treated with
31 adjuvant high-dose interferon (HDI).²² Antithyroglobulin, antinuclear or anticardiolipin
32 antibodies were detected in 24% of the patients, and also no more than three patients
33 (2%) developed vitiligo without the occurrence of autoantibodies. Autoimmunity dur-
34 ing treatment was associated with a significant RFS and OS benefit according to Kaplan-
35 Meier estimates. Another study in patients treated with low-dose interferon (LDI), also
36 showed a correlation with autoimmunity and a significantly better RFS ($P = .05$) and a
37 trend towards improved OS ($P = .07$).²³ The results of abovementioned trials appeared
38 promising, however, both studies analyzed the effects of autoimmunity on outcome in

39

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

3

4 In contrast, we described the association of autoantibodies in patients receiving inter-
5 mediate-doses of interferon or no treatment with RFS (this thesis).²⁴ The analysis was
6 performed in a subset of patients participating in the EORTC 18952 or the Nordic IFN
7 trial.^{25,26} At baseline, 33% of the patients in the EORTC 18952 and 35% of the patients in
8 the Nordic IFN study tested positive for antithyroglobulin, antinuclear or anticardiolipin
9 antibodies, however, no difference in RFS between patients with or without pre-existing
10 autoantibodies was observed. During follow-up, autoantibodies were more frequently
11 detected in patients receiving interferon as compared to untreated patients, 36% (EORTC
12 18952) and 39% (Nordic IFN) versus 26% and 14% respectively in the observation arm of
13 both trials. Seroconversion in IFN-treated patients correlated with improved outcome if
14 a Cox model (model 1) in which antibody status was time independent was used (EORTC
15 18952, HR = 0.37, $P < .001$ and Nordic IFN, HR = 0.49, $P = .002$). When treating antibody
16 status as a time-dependent variable, no strong association with RFS was found. Further-
17 more, results from a comparable side study from the ECOG 2696 trial were in agreement
18 with the previous study.²⁷

19

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

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

11

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

21

22 3.3. Anti-CTLA-4 therapy

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

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

12

13 Tremelimumab is a newer agent which is generally tolerable and has demonstrated an-
14 titumor activity.^{46,47} Although ipilimumab and tremelimumab have not been compared
15 directly, it seems that response rates with tremelimumab are lower, which might be
16 explained by suboptimal dosing. Notably is the fact that these patients also developed
17 less immune-related toxicities as compared to ipilimumab-treated patients. The only
18 evidence for a correlation between treatment response and irAEs in 30 patients receiv-
19 ing tremelimumab ($P = .05$) was found by Reuben et al.⁴⁸ Five patients suffered from
20 grade 3 diarrhea and 1 patient from grade 3 dermatitis but no grade 4 adverse events
21 were observed. A more recent trial evaluating efficacy and safety in patient treated with
22 tremelimumab, 15 mg/kg, every 90 days, reported an objective response rate of 6.6%,
23 with all responses being durable⁴⁷. Grade 3-4 adverse events included diarrhea (11%),
24 fatigue (2%) and colitis (4%). Furthermore, Camacho et al. showed for the tremelimumab
25 15 mg/kg arm a treatment-related adverse event rate of 13% including; diarrhea, rash,
26 pruritus, fatigue and nausea, and a serious adverse event rate of 9%.⁴⁶ Rates for immune-
27 related adverse events were not mentioned. It is not unthinkable that if tremelimumab
28 dosing would be intensified, the number of immune-related adverse events and the
29 clinical response rates could be increased.

30

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

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

4

5 3.4. *Is it possible to uncouple tumor immunity and autoimmunity?*

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

20

21 **4. S100B**

22 While many reports show that S100B blood levels provide a good indication of disease
23 progression as well of response to chemotherapy in stage IV patients,⁵⁹⁻⁶¹ it remains
24 of little or limited usefulness in early stages (II and III) where the disease is most often
25 confined to lymph nodes.⁶² Moreover, the progression from the early stages to distant
26 organ involvement is frequently accompanied by a significant increase in S100B levels.⁶³
27 Here we evaluated the utility of serial determinations of S100B serum levels in patients
28 with high-risk melanomas, using a time-dependent Cox model. We demonstrated in
29 this corollary study to the EORTC 18952 trial in stage IIB-III melanoma patients that
30 serial determinations of S100B serum levels strongly correlate with DMFS and OS (this
31 thesis).⁶⁴ Strikingly, the hazard ratio (HR) for S100B determinations was higher and more
32 significant than the one corresponding to stage, and number of positive lymph nodes,
33 two strong prognostic factors in stage III melanoma. Median time between S100B \geq
34 0.2 $\mu\text{g/l}$ and the development of distant metastases was 94 days. Since the prognostic
35 value of S100B was not analyzed separately for the untreated and IFN-treated patients,
36 its predictive significance could not be established. However, one could argue that
37 patients receiving IFN should discontinue therapy once S100B levels of \geq 0.2 $\mu\text{g/l}$ have
38 been reached, indicating disease progression and no treatment response.

39

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-

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

7

8 **7. Absolute lymphocyte count**

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

17 Recently, in 51 patients with advanced melanoma who were treated with ipilimumab
18 10 mg/kg, clinical benefit was correlated with absolute lymphocyte count.⁴⁵ Patients
19 underwent laboratory testing before each ipilimumab administration. In order to cor-
20 relate absolute lymphocyte count (ALC) to outcome, patients were stratified based on a
21 cutoff of $\geq 1000/\mu\text{L}$ (high ALC) versus < 1000 cells/ μL (low ALC). At baseline there was a
22 borderline significant trend towards improved overall survival for the high ALC group (P
23 = .06), which remained after correction for LDH. After the first ipilimumab dose, patients
24 with high ALC had a significantly improved overall survival ($P < .01$), however this as-
25 sociation was most pronounced after the second dose of ipilimumab, with a median
26 overall survival of 11.9 months versus 1.4 months and $p < .0001$. Similar results were
27 found when corrected for LDH. Although this study comprised a relatively small number
28 of patients and no detailed multivariate analysis was performed, the results might have
29 strong implications in future trials and clinical practice. These data suggest that patients
30 with ALC < 1000 cells/ μL do not benefit from ipilimumab treatment and could be spared
31 of further toxicity.

32

33 **8. Human leukocyte antigen**

34 Serological typing for human leukocyte antigen (HLA) class I and class II antigen expres-
35 sion has previously shown to be associated with melanoma prognosis and treatment
36 response.

37 An early report by Lee et al. suggested that the risk of melanoma incidence or
38 progression could be influenced by HLA-DQB1*0301.⁷⁵ Stage I/II melanoma patients
39 with positive HLA-DQB1*0301 status were at an increased risk of developing recurrent

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

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

25

26

27 CONCLUSION

28

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

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

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

6 Only recently, two new drugs changed the whole therapeutic landscape in mela-
7 noma, ipilimumab and the highly selective BRAF inhibitor PLX4032 were the first agents
8 showing significant responses in stage IV melanoma.⁸³ And this seems just the begin-
9 ning. The first line phase trial in patients with advanced metastatic melanoma (stages
10 IIIC + IV), comparing DTIC vs ipilimumab + DTIC, was recently reported to have been
11 unblinded demonstrating a significant survival benefit for the ipilimumab containing
12 arm, will be presented at ASCO annual meeting in June 2011. Ipilimumab has already
13 been shown to improve survival in 2nd line significantly (doubling of survival at 2 and
14 3 years) in patients with advanced melanoma, and was approved in March 2011 by the
15 FDA. In first line the results may be even more spectacular. Data from phase II studies
16 with ipilimumab in first line suggest a 2 year survival rate of above 50%. If this is also
17 the case in the pivotal phase III trial to be reported at ASCO, it would probably indicate
18 that survival at 2 and 3 years in the ipilimumab containing arm may be up to three times
19 better than in the DTIC alone arm. Moreover, immune-related adverse events and abso-
20 lute lymphocyte count were associated with treatment response. Response to the BRAF
21 inhibitor PLX4032 is dependent on the BRAF mutation V600E, present in about 60%
22 of melanoma. When present, response rates up to 80% were described but in absence
23 of the mutation the drug seems not beneficial. The first interim analysis of the world-
24 wide pivotal phase III trial (BRIM3-trial) comparing PLX4032 with DTIC in patients with
25 advanced metastatic melanoma has lead to the unblinding of the study because of a
26 significant impact on both progression-free survival and overall survival in the PLX4032
27 arm. The detailed results will also be reported at the 2011 ASCO annual meeting.

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

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Chapter X

Summary

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

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

28 Changes of ferritin and CRP levels and prognostic value on treatment outcome were
29 analyzed in **chapter III**. Serial ferritin and CRP levels were determined before treatment
30 and up to 24 months in patients participating in the EORTC 18952 trial. Baseline ferritin
31 levels were comparable in the 2 treatment groups. However, ferritin ratios were signifi-
32 cantly higher in IFN-treated patients ($N = 96$) compared to untreated patients ($N = 21$),
33 at end of induction (mean: 2.88 vs 0.75; $P = .0003$) and at 6 months (mean: 3.18 vs 1.02;
34 $P = .009$). In the IFN arm, higher ferritin ratios at end of induction and at 6 months were
35 not associated with improved outcome. Concerning CRP ratios, no differences between
36 the treatment groups, neither an association with DMFS, were observed.

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

1 rather unclear, the appearance of vitiligo is considered a favorable prognostic factor.
2 Previously, Gogas et al. reported that the appearance of autoantibodies and clinical
3 manifestations of autoimmunity in melanoma patients treated with adjuvant high-dose
4 IFN was strongly associated with improved RFS and OS. In **chapter IV** the association of
5 autoantibodies and prognosis in patients from two randomized trials, the EORTC 18952
6 and the Nordic IFN trial is shown. Serum levels of anticardiolipin, antithyroglobulin, and
7 antinuclear antibodies were determined using ELISA. The association of the presence
8 of autoantibodies with risk of recurrence was assessed by three Cox models in patients
9 negative for all three autoantibodies at baseline (125 from the EORTC 18952 trial and
10 230 from the Nordic IFN trial): 1) a model that considered appearance of autoantibodies
11 as a time-independent variable, 2) one that considered a patient autoantibody positive
12 once a positive test for an autoantibody was obtained, and 3) a model in which the
13 status of the patient was defined by the most recent autoantibody test.

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

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

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

1 In **chapter VII**, YKL-40 levels were determined in patients participating in the EORTC
2 18952, the Nordic IFN or the EORTC 18991 trial. We showed that elevated baseline levels
3 of YKL-40 in observed patients from the Nordic IFN trial and the EORTC 18952 trial was
4 an independent prognostic marker for short OS ($HR = 1.33, P = .04$). However this effect
5 was not demonstrated in IFN-treated patients. Moreover, in patients receiving treat-
6 ment, IFN or PEG-IFN, we found that increases in serum YKL-40 during follow-up were
7 associated with decreased OS but not with decreased RFS.

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

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

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Chapter XI

SAMENVATTING

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

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

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

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

5 In **DEEL TWEE** werd de prognostische factor auto-immuniteit geëvalueerd. Het me-
6 lanoom heeft een immunogeen karakter welke zich klinisch manifesteert door spontane
7 regressie en het ontstaan van vitiligo. Auto-immuniteit wordt gezien als een gunstige
8 prognostische factor. Eerder rapporteerden Gogas et al. dat het ontstaan van autoantili-
9 chamen en klinische manifestaties van auto-immuniteit in melanoompatiënten, die be-
10 handeld werden met hoge dosering adjuvant IFN, sterk geassocieerd was met verbeterde
11 ziekte-vrije en totale overleving. In **hoofdstuk IV** wordt de relatie tussen autoantilichamen
12 en prognose getoond bij patiënten van twee studies, de EORTC 18952 studie en de Nordic
13 IFN studie. Anticardiolipine, antithyroglobuline en antinucleaire antilichamen werden in
14 het serum bepaald middels ELISA. De associatie van de aanwezigheid van autoantilicha-
15 men met het risico op recidief werd bepaald bij patiënten met negatieve uitgangswaarden
16 voor alle drie de autoantilichamen (125 van de EORTC 18952 studie en 230 van de Nordic
17 IFN studie), waarvoor gebruik werd gemaakt van drie Cox modellen: 1) een model dat de
18 aanwezigheid van antilichamen beschouwt als een tijds-onafhankelijke variabele, 2) een
19 model dat een patiënt positief beschouwt op het moment dat een positieve testuitslag
20 werd verkregen, en 3) een model waarin de status van de patiënt werd bepaald aan de hand
21 van de meest recente autoantilichaamtest. Indien behandeld als een tijds-onafhankelijke
22 variabele (model 1), was het ontstaan van autoantilichamen geassocieerd met verbeterde
23 ziekte-vrije overleving in beide studies (EORTC 18952, HR = 0.41, $P < .001$; en Nordic IFN, HR
24 = 0.51, $P < .001$). Echter, in modellen 2 en 3 werd gecorrigeerd voor "garantie-time bias",
25 waaruit bleek dat de associatie zwakker en niet statistisch significant was.

26 We valideerden deze resultaten in **hoofdstuk V** voor patiënten die deelnamen aan
27 de EORTC 18991 studie en behandeld werden met adjuvant gepegyleerd IFN enerzijds
28 of geen therapie anderzijds. Patiënten die een negatieve uitgangswaarde voor autoan-
29 tilichamen hadden werden geanalyseerd ($n = 220$). Het ontstaan van autoantilichamen
30 tijdens follow-up gebeurde vaker bij patiënten die behandeld werden met gepegyleerd
31 IFN (52%) dan bij onbehandelde patiënten (18%). Uit model 1 bleek dat het ontstaan van
32 autoantilichamen een van prognostische waarde heeft (HR = 0.56, $P = .01$). Echter, wanneer
33 gecorrigeerd werd voor "garantie-time bias" was het niet meer significant (model 2: HR
34 = 1.19, $P = .46$, model 3: HR = 1.14, $P = .59$). De resultaten waren vergelijkbaar wanneer de
35 behandelingsgroepen separaat werden geanalyseerd, waardoor gesuggereerd kan wor-
36 den dat het ontstaan van autoantilichamen noch een prognostische noch een predictieve
37 factor is voor verbeterde overleving bij patiënten die behandeld zijn met gepegyleerd IFN.

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

1 de prognostische waarde van opeenvolgende S100B bepalingen geanalyseerd bij 211
2 patiënten die participeerden in de EORTC 18952 trial. Het Cox tijds-afhankelijke model
3 (model 3) werd gebruikt om de prognostische waarde van de laatste (meest recente)
4 S100B test te bepalen. Bij de eerste meting hadden 178 patiënten S100B waarden <
5 0.2 µg/l en 33 ≥ 0.2 µg/l. Van de eerste groep hadden 61 patiënten later een verhoogde
6 S100B (≥ 0.2 µg/l) waarde. Een initieel verhoogde S100B waarde, of tijdens follow-up,
7 was geassocieerd met verslechterde afstandsmetastasen-vrije overleving. Zo was na
8 correctie voor ziektestadium, aantal positieve lymfklieren en geslacht de HR 5.57 ($P <$
9 .0001) van S100B ≥ 0.2 versus S100B < 0.2. Vergelijkbare resultaten werden gezien voor
10 totale overleving. We toonden tevens aan dat de prognostische invloed van S100B ≥ 0.2
11 µg/l meer uitgesproken was voor ziektestadium III dan stadium IIB.

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

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


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

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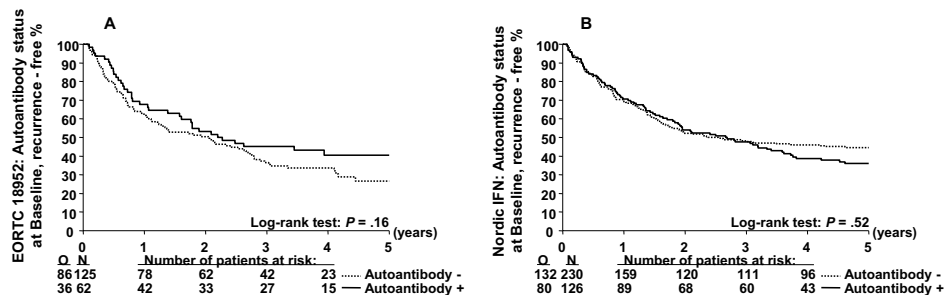




APPENDIX

1 DATA COMPLEMENT, CHAPTER IV

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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)

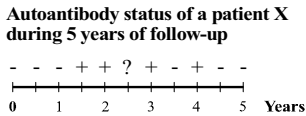
*IFN = interferon-α.

1 DATA COMPLEMENT, CHAPTER V

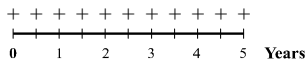
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4 **Supplementary information**

5
6 **Background information Cox models**

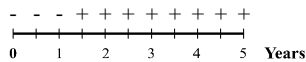
7 The prognostic value of serial measurements of
8 autoantibody status (positive vs negative)
9 observed during the study on the RFS, adjusted
10 for the initial number of positive lymph nodes
11 before randomization (0 vs 1 vs 2-4 vs ≥5),
12 considered as an ordered categorical variable,
13 have been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



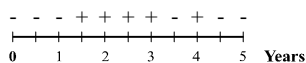
18 **1 Time - independent Cox model**
19 **Any positive autoantibody**



21
22 **2 Time - dependent Cox model**
23 **Latest positive autoantibody**



25
26 **3 Classical**
27 **Time - dependent Cox model**
28 **Latest any autoantibody**



30
31
32 **Adapted from Bouwhuis et al. JNCI 2009**

33
34 **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:
35 $HR = e^{\beta_1 \times \text{autoantibody status}(t) + \beta_2 \times \text{Number of positive lymph nodes}}$

36 where, autoantibody status (t) = 0, if a patient
37 always tested negative for autoantibodies or (t)
38 = 1 when a patient was at least once positive.

39 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 survival¹⁰. 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, O=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)

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1 **DATA COMPLEMENT, CHAPTER VI**
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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	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

1 DATA COMPLEMENT, CHAPTER VII

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4 **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
8 Stage	N0	94	45 (20-259)	.51	26	68 (20-281)	.06	0	0	.90
	N+ sentinel	105	44 (20-1416)		41	50 (21-206)		149	47 (22-285)	
	N+ palpable	267	50 (20-288)		61	54 (20-204)		111	52 (20-438)	
10 Ulceration	No	248	46 (20-259)	.61	64	52 (22-281)	.88	143	57 (11-285)	.51
	Yes	132	47 (20-288)		37	58 (14-216)		59	50 (19-438)	
	Unknown	81	48 (20-1416)		27	53 (21-197)		58	49 (19-438)	
13 Breslow thickness	<1.5 mm	106	46 (14-231)	.61	31	51 (14-204)	.29	52	50 (11-285)	.71
	1.5-3.99 mm	170	49 (12-1416)		37	60 (22-115)		129	53 (11-262)	
	≥4.0 mm	150	47 (11-426)		45	58 (20-281)		55	49 (13-438)	
	Unknown	28	45 (13-228)		15	46 (24-206)		24	51 (20-227)	
15 Clark's level	I	1	21	.66			.57			
	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)				
	V	38	47 (20-426)		13	56 (14-281)				
	Unknown	80	51 (13-228)		24	65 (24-197)				
	Missing	3	69 (48-106)							

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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
25	Gender f vs. m		0.76	0.48-1.21	.25		1.12	0.66-1.89	.67
26	Age	Obs	0.98	0.82-1.16	.77	Obs	1.11	0.88-1.40	.40
27	N0 vs. N+palp.		0.64	0.39-1.04	.003				
28	N+sent. vs. N+palp.		0.34	0.19-0.62			0.97	0.58-1.63	.92
29	YKL-40		1.13	0.89-1.44	.32		0.93	0.69-1.26	.66
30	Gender		0.67	0.41-1.10	.11		0.63	0.35-1.12	.11
31	Age 10 years	IFN 1-year	1.14	0.97-1.35	.12	Pegylated IFN	1.22	0.92-1.61	.16
32	N0 vs. N+palp.		0.57	0.35-0.94	.009				
33	N+sent. vs. N+palp.		0.51	0.30-0.86			0.54	0.30-0.95	.034
34	YKL-40		0.96	0.77-1.21	.76		1.17	0.84-1.64	.35
35	Gender		0.95	0.59-1.53	.83				
36	Age	IFN 2-year	0.98	0.82-1.17	.82				
37	N0 vs. N+palp.		0.76	0.46-1.26	.13				
38	N+sent. vs. N+palp.		0.63	0.40-1.01					
39	YKL-40		1.01	0.80-1.26	.97				

Obs = Observation, IFN = interferon

1 DATA COMPLEMENT, CHAPTER VIII

4 Supplementary table.

5 SNP ID	Region type	Position on Gene	SNP	Forward primer	Reverse primer	Taq Man assay
6 rs3181098	CD28 promotor	-1042	G/A	tgagacaccaagggctttt	ataggatggggacaggttg	C_27467172_10
7 rs3181100	CD28 intron 1	587	C/G	tgtaagttagattggctctgga	cttcgcatgattatctcat	C_2821000_10
8 rs3181101	CD28 intron 1	615	C/G	idem rs3181100	idem rs3181100	C_27469464_10
9 rs1181390	CD28 intron 1	1258	G/T	aaattaactcgaaaaatcac	ctctgtactgccaaaataaga	C_8806607_10
10 rs1181388	CD28 intron 1	4532	G/A	tggtgtgtttgtgactg	taaggagcagctcaagta	C_2821002_10
11 rs17533594	CD28 intron 1	9776	A/G	cttatggcccagctaaatg	ttcctacacaggcagacacta	C_32900233_10
12 rs3116494	CD28 intron 2	20602	A/G	ctgacattgagcgggagagta	ttctgagtcttaaccattagac	C_27464981_10
13 rs3181107	CD28 intron 2	22307	A/G	cctagtccagccctgaga	gaatcaaaaggggagagaag	C_27467173_10
14 rs3116496 (IVS3 +17)	CD28 intron 3	23093	T/C	gtggagtctggtctg	aatgcctctgggaaatctaa	C_25922478_10
15 rs16840252	CTLA4 promotor	-1147	C/T	gaggcatttggtagtatt	agacaggaccaatgactaac	C_32900355_10
16 rs5742909 (CT44)	CTLA4 promotor	-319	C/T	caagggctcagaaagttag	gaagccgtgggttag	C_27834180_10
17 rs231775 (CT42)	CTLA4 exon 1	49	A/G	gaacaccgctccataaag	gccaccagccagatt	C_2415786_20
18 rs231777	CTLA4 intron 1	923	C/T	tacacggctaaatgatgag	cactaaatgcggtcacactc	C_2415784_10
19 rs3087243 (CT60)	CTLA4 3'	6254	G/A	tcagtatctggtgagctct	caactgtaatgcctgtgat	C_3296043_10
20 rs11571319 (CT61)	CTLA4 3'	6273	G/A	idem rs3087243	idem rs3087243	C_30981401_10
21 rs11571302 (JO31)	CTLA4 intergenic	10269	G/T	ccatccatctccatccaagt	tttgcgatccccctgaat	by design
22 rs7665213 (JO30)	CTLA4 intergenic	10744	G/A	ttttctctgctgtgctatt	aaaggtgctcacagagagta	by design
23 rs11571297 (JO27)	CTLA4 intergenic	12338	T/C	ctggtcagccgagattgtgat	ccccccagcattcagagtg	C_3296036_10
24 rs10932029 (+173)	ICOS intron 1	231	T/C	attacgcacccaaaagacagt	ctatggtgccttgacatt	C_430013_10
25 rs4335928	ICOS intron 1	443	T/C	idem rs10932029	idem rs10932029	C_26263275_10
26 rs4675374	ICOS intron 1	1041	C/T	ctcttcacactgagccctat	catacaccatctggcacag	C_29934539_10
27 rs7602383	ICOS intron 1	4915	A/G	tctctgtaaatggcatctca	aagttcatatccccatttct	C_29052409_10
28 rs4521021	ICOS intron 1	15038	T/C	aatgggggaaaaatctg	gagaacaggtgcagctcat	C_377746_10
29 rs11571323	ICOS intron 1	17307	G/A	ctaataattttccaacaat	ctgcctcagcctctta	C_30981464_10
30 rs12466129	ICOS intron 1	17444	T/A	idem rs11571323	idem rs11571323	C_8166476_30
31 rs10172036	ICOS intron 4	22746	G/T	aaggcaatggagagggaaag	tcggcagtaaccaaggcagtc	C_26263283_10
32 rs10183087	ICOS 3'UTR	22787	A/C	idem rs10172036	idem rs10172036	C_30421029_10





DANKWOORD

1 Promoveren doe je niet alleen. Tijdens deze periode ben ik door veel mensen geholpen
2 en een aantal wil ik graag in het bijzonder bedanken.

3

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5 om mensen te motiveren zijn bijzonder en maken het heel prettig om met je samen te
6 werken. Ik ben je zeer dankbaar voor alle kansen die je me hebt gegeven en het vertrou-
7 wen dat je in me hebt gesteld.

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10 Amsterdam, Heidelberg of op ons lab in Rotterdam, altijd kon ik bij je terecht. Veel dank
11 voor je steun, zeker ook de laatste maanden tijdens het afronden van dit proefschrift.

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14 fessor Haanen, dank voor uw bereidheid mijn proefschrift door te nemen en voor uw
15 aanwezigheid op deze voor mij zo belangrijke dag.

16

17 Stefan Suciu, statistician, dear Stefan, we both know that without you I could never have
18 done this. You taught me the critical attitude towards statistics. Thank you for all your
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20

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23

24 Rajiv Kumar, I really enjoyed my stay in Heidelberg and working with you at the DKFZ.
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