I INTRODUCTION

I. 1 CUTANEOUS MELANOMA BACKGROUND

I. 1.1 Incidence and risk factors

Cutaneous melanoma arises from the malignant tranformation of melanocytes, the pigment producing cells, which are located in the basal epidermal layer of the skin. It is the most deadly form of skin cancers and its incidence among Caucasians has been doubling every 10 years for the last 3 decades (1, 2). Melanoma incidence varies by latitude and altitude, with areas closer to the equator and higher in altitude having higher rates (3). It also depends on the pigmentation of the exposed populations and on their sun exposure behavior. Yearly melanoma incidence varies from 50/100,000 inhabitants in Australia to ≤ 0.5/100,000 in Asia and Africa. Incidence in Europe varies from 5 to 15/100,000 *(Table 1)*. In the United States, the cumulative risk of developing a melanoma over lifetime is 1 in 90 (2). It is approximately 1% in France and in the Netherlands, 3% in Australia and 5% in New Zealand.

The risk of developing a melanoma is determined by the interplay between genetic factors and exposure to sunlight. For example, melanoma incidence in fair people is inversely related to latitude of residence. The independent risk factors to develop a primary cutaneous melanoma are a personal- or a family history of cutaneous melanoma, a high number of nevi (> 100) and/or the presence of atypical nevi. Immunosuppression, a fair, sun-sensitive skin (phototypes I or II) and intermittent, acute sun exposure pattern especially during childhood are additional risk factors *(Tables 2 and 3)* (4-7).

Some subjects or families, including patients with phenotypic risk factors such as fair skin and high number of nevi, are genetically susceptible to the development of cutaneous melanoma (8). It is estimated that approximately 10 to 15% of cutaneous melanomas result from an inherited predisposition and high-risk melanoma susceptibility genes are being sought actively. Although mutations in the 2 tumor-suppressor genes coding for the cyclin-dependent kinase inhibitor $p16^{INK4A}$ *(CDKN2A)* and the cyclin-dependent kinase 4 (*CDK4)*, respectively, have been shown to confer an increased risk of cutaneous melanoma, they only account for 25% of families with multiple cases of cutaneous melanoma (7). p16 is the main negative regulator of the CDK4-cyclin D1 (CCND1) complex, thus playing a crucial role in the cell cycle, at the G1-S checkpoint. In 25 to 50 % of nonfamilial melanoma, a different tumor-suppressor gene, phosphatase and tensin homologue (*PTEN*) is mutated. PTEN is a negative regulator of the phosphatidylinositol 3' kinase (PI3K) pathway that is involved in the progression of melanoma. In murine models of melanoma, mutation of either *CDKN2A* or *PTEN* alone fails to cause melanoma, but when combined which each other, melanomas do arise.

The melanocortin-1 receptor (MC1R) is a G-protein coupled receptor on melanocytes that responds to alpha-melanocyte stimulating hormone, also called alpha-melanocortin (α-MSH). α-MSH is

Table 1

	Gender[1]	Incidence[2]	Mortality[3]	Prevalence[4] 1-year	Prevalence[4] 5-year
CUTANEOUS MELANOMA					
EUROPE: Western	M	9.9	2.8	8 669	37 286
	F	15.1	2.4	13 633	61 601
Belgium	M	5.2	2.1	259	1 127
	F	9.5	2.3	487	2 217
The Netherlands	M	12.3	3.6	973	4 272
	F	16.2	2.8	1 283	5 854
Germany	M	10.3	2.8	3 955	16 441
	F	14.2	2.4	5 649	25 502
France	M	8.4	2.4	2 384	10 559
	F	15.5	2.2	46.1	20 655
Northern	M	11.9	3.4	5 346	22 429
	F	14.3	2.8	6 818	30 913
Southern	M	8.5	2.3	5 622	22 889
	F	8.4	1.9	6 076	27 074
Central and Western	M	4.1	2.0	5 142	19 578
	F	5.5	1.9	8 093	32 896
AMERICA: Northern	M	20.5	3.3	31 496	140 625
	F	15.5	1.9	24 770	114 380
USA	M	21.4	3.4	29 648	132 335
	F	16.0	2.0	23 091	106 636
Canada	M	12.1	2.9	1 848	8 290
	F	8.2	1.8	1 679	7 744
AUSTRALIA	M	49.0	7.2	5 012	23 606
	F	38.4	4.1	4 151	20 039
ASIA: Eastern	M	0.3	0.1	1 781	6 780
	F	0.2	0.1	1 471	5 687
WORLD	M	2.5	0.7	73 013	309 690
	F	2.6	0.6	75 940	332 953
OTHER CANCERS					
Breast					
World	F	37.4	13.3	1 060 042	4 406 080
More developed regions	F	103.7	30.9	624 311	2 749 763
Prostate					
World	M	21.7	7.1	604 506	2 368 659
More developed regions	M	88.4	22.4	477 997	1 967 626
Colon / Rectum					
World	M	17.6	8.9	423 416	1 515 221
	F	15.4	8.1	362 911	1 315 195
More developed regions	M	60.8	27.5	290 177	1 072 683
	F	50.9	25.1	252 367	946 179

1) M = Male. F = Female.
2) Number of new cases diagnosed per 100 000 inhabitants per year.
3) Number of cancer-related deaths per 100 000 inhabitants per year.
4) Number of patients who are alive with a given disease diagnosed within a 1-year (5-year) period of diagnosis. Prevalence data are calculated from data on incidence and on survival.

Ref: International Agency for Research on Cancer (IARC) <http://www-dep.iarc.fr/>. Globocan 2002 database
Rates are not those for the year 2002 but from the most recent data available, generally 2–5 years earlier.

Table 2 Risk Factors for the Development of Cutaneous Melanoma [1]

Factor	Approximate Relative Risk [2]
Member of melanoma-prone family *	35–70
Previous primary melanoma	8.5
Sunburns during childhood [3]	4.3
Multiple nevi and atypical nevi	2 – 12
Red hair	2.4 – 4
Family history of melanoma **	2 – 3
Freckles	2 – 3
Blue eyes	1.6
Skin phototype I	1.4

[1] After Kefford *et al., JCO* 17: 3245–51, 1999.
[2] Ratio of incidence rates in the target population/control population.
[3] Autier et al., Br. J. Cancer 76: 1521–4, 1997.
* Multiple affected relatives on the same side of the family.
** One or more affected first-degree relatives.

Table 3 Skin phototypes

Skin Phototype	Phenotype			Skin behavior upon sun exposure	Populations
	Skin color	Hair color	Freckles		
I	pale	red	yes	blistering sun burns, never tanns	Caucasians
II	pale	fair	(yes)	sun burns, then progressive tanning	(Europe, Australia
III	(pale)	brown	no	erythema, then tanns rapidly	USA…)
IV	tanned	black	no	no sun burns, immediate tanning	South America
V	yellow	black	no	no sun burns, immediate tanning	Asia
VI	black	black	no	no sun burns, immediate tanning	Africa

Variations in pigmentation and the tanning response to ultraviolet light are associated with variations in susceptibility to melanoma.

secreted in response to ultra violet radiations (UVR). The binding of α-MSH to its receptor triggers intracellular signaling and results in an increased synthesis of the enzymes involved in melanogenesis, leading to an increased synthesis of melanin. Melanin is then transferred from the melanocytes to the keratinocytes, and this tanning reaction constitutes the main defense of the skin against the UVR. The *MC1R* gene is highly polymorphic in Caucasians and germ-line mutations can generate amino-acid substitutions in the MC1R, leading to unresponsiveness to α-MSH. Melanocytes carrying such mutations are unable to proliferate and to increase melanogenesis in response to α-MSH stimulation. These loss-of-function mutations and the resulting impairement in the production of melanin have been associated with light-skin and red-hair phenotypes and increased risk of skin cancer. α-MSH also promotes the survival of human melanocytes cultures by inhibiting the apoptosis induced by UVR. This effect is absent in melanocytes with loss-of-function *MC1R* mutations (9).

Somatic mutations in the *BRAF* gene have recently been reported in 66% of primary melanoma (10) and in 82% of benign melanocytic tumors, including congenital, common acquired and dysplastic nevi (11). They occur at similar frequences in melanoma metastases as well. BRAF codes for a serine/threonine protein kinase involved in the transduction of mitogenic signals from the cell membrane to the nucleus through the mitogen-activated protein kinase pathway (MAPK). Melanocyte-specific MAPK pathways play a crucial role in both melanocyte growth and differentiation processes, including the activation of the cell surface receptor MC1R by α-MSH. Somatic mutations of N-RAS - which are associated with about 15% of melanomas - and BRAF mutations occur exclusively from each other and cause constitutive activation of the MAPK pathway.

The molecular mechanisms of the interaction between the susceptibility genes and the environment are currently being explored, which should contribute to the development of effective approaches to the prevention and treatment of melanoma. UVR cause genetic changes in the skin, impair cutaneous immune function, increase the local production of growth factors, and induce the formation of DNA-damaging reactive oxygen species that affect keratinocytes and melanocytes. Epidemiologic and molecular studies suggest that different types of human melanoma can be distinguished depending on the sun exposure pattern of the skin sites on which they arise. Somatic mutations of N-RAS - which are associated with about 15% of melanomas - and BRAF mutations occur exclusively from each other and cause constitutive activation of the MAPK pathway. Curtin and colleagues showed that *BRAF* or *N-RAS* mutations are present in 80% of melanoma arising in areas intermittently exposed to the sun whereas they are rare in melanomas developed on skin that is chronically exposed to the sun, as well as in acral sites or mucosal epithelia that are rarely or never exposed to sun (12). The latter have instead increases in the number of copie of the genes coding for CDK4 and CCND1 that are downstream elements of the RAS-BRAF pathway. This study provides genetic support for the existence of distinct molecular pathways to melanoma, each with a specific relationship to UV light

exposure and points out *CDK4* and *CCND1* as independents oncogenes in melanomas without mutations in *BRAF* or *N-RAS*. The relation between BRAF mutations in melanomas and sun exposure is complex. Although sun exposure appears necessary for the development of BRAF mutations, the latter do not show the standard "C to T" signature of mutations directly induced by UVR. This paradoxical relationship lead Landi and colleagues to hypothesize that there is an inherited susceptibility factor that predisposes individuals to develop BRAF-mutant melanomas under limited sun exposure, and that UVR would act indirectly to promote these mutations (13). They indeed found in 2 independent Caucasian populations that there was an association between MC1R variants found in germline DNA of patients and the presence of BRAF-mutations in the melanoma that they developed. BRAF mutations were 10 times as frequent in patients with at least one MC1R variant allele compared to those who were homozygous for the MC1R wild-type allele, and the association between MC1R variants and melanoma risk by BRAF mutations status was stronger in the younger subjects. These results indicate that MC1R is at least one of the components of this hypothesized sensitivity. The increased generation of reactive oxygen species in carriers of MC1R variants could directly induce the "A to T" replacement characteristic of the most common BRAF mutation in exon 15. Another susceptibility locus, 1p22, has recently been identified and there is no doubt that novel suceptibility loci remain to be characterized (14).

The development of new experimental models such as human skin xenografts or genetically engineered mice that develop inducible melanoma, should also improve our understanding in that field (2, 15).

Regarding prevention of cutaneous melanoma, efforts need to be sustained in public health campaigns on sun exposure and the recognition of individuals at high risk. An appropriate follow-up of these patients should allow early detection and treatment of new lesions.

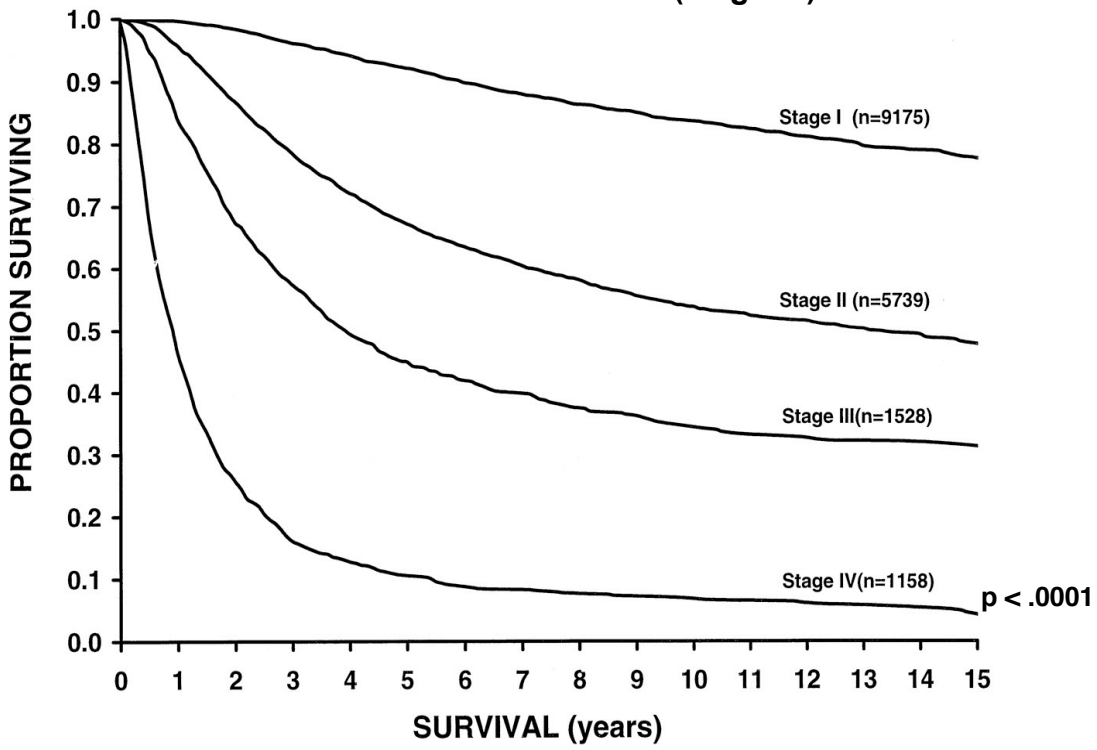
I. 1.2 Prognosis factors and tumor staging

The melanoma TNM (T, primary tumor; N, regional disease; M, distant metastasis) and stage grouping criteria have recently undergone major modifications according to the results of large retrospective studies on prognosis factors, performed by the American Joint Committee on Cancer (AJCC) on more than 15,000 patients (16-18). This new staging system reflects more accurately independent prognostic factors. It allows the selection of more homogeneous populations of patients in clinical trials, with respect to their risk of distant metastases and/or disease-related mortality. According to the last TNM and staging categories, 10-year survival rates vary from 88% for patients with a non-ulcerated primary tumor ≤ 1 mm thick (Stage IA pT1a N0 M0), to 4% in patients with distant visceral metastases (Stages IV T-any N-any M1b or c) *(Fig. 1 and Table 4)*.

The major differences between the AJCC 1992 / 1997 classification *(Table 5)* and the AJCC 2002 classification *(Table 6)* are the following:

Figure 1

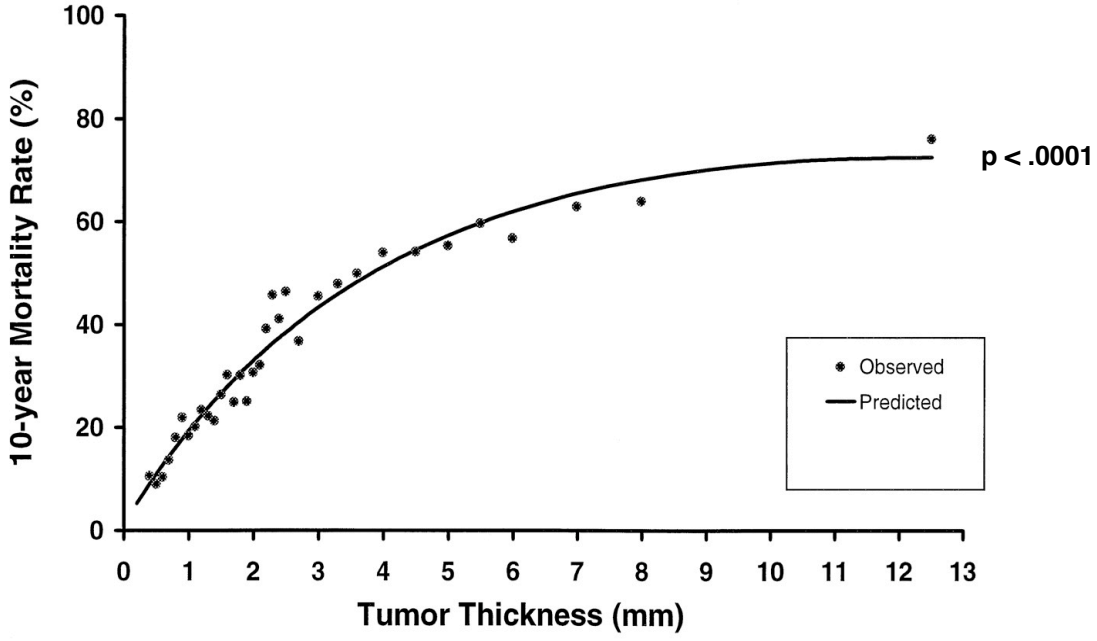
Fifteen-year survival curves comparing localized melanoma (stages I and II), regional metastases (stage III), and distant metastases (stage IV)



Balch, C. M. *et al. J Clin Oncol*; 1a9 : 3635-3648, 2001

Figure 2

Observed and predicted 10-year mortality rate of 15,320 patients with clinically localized melanoma



Balch, C. M. *et al. J Clin Oncol*; 19 : 3622-3634, 2001

- Primary tumor (T, stages I and II): thickness as defined by Breslow and ulceration become the most powerful predictors of survival in the new classification, while Clark level of invasion has a significant impact only in primaries of ≤ 1 mm thick *(Fig. 2 and Table 7)*.
- Regional disease (N, stage III): the N classification now takes into consideration the number of metastatic lymph nodes rather than their dimensions, the distinction between microscopic and macroscopic, i.e. clinically palpable metastases and the merging of satellite and in-transit metastases into a single staging entity *(Figs. 3 and 4)*.
- Distant metastases (M, stage IV): the site of distant metastases and the presence of elevated lactate dehydrogenase (LDH) are the 2 classification criteria. Cutaneous, lymph node and lung metastases have a better prognosis than other localizations *(Fig. 5)* (16). They also generally better respond to any kind of treatment, including chemotherapy and radiotherapy (19). Increased LDH were recently shown to be an independent, poor prognosis factor in patients with visceral metastasis (20, 21).

I. 1.3 Current treatments

So far no treatment - except surgery at the earliest stages of the disease - has been shown to significantly improve survival of patients suffering from a cutaneous melanoma. Routine treatments of cutaneous melanoma including surgery, biochemotherapy and radiotherapy will be briefly described here. Immunotherapy approaches will be discussed in Sections III (Results) and IV (Discussion).

I. 1.3.1 Surgery

Thorough surgical removal of the tumor is the only curative treatment for primary and regional melanoma. Two well defined procedures for the care of patients at early stages of the disease, i.e. sentinel lymph node biopsy and isolated limb perfusion, will be discussed here.

I. 1.3.1.1 Sentinel lymph node biopsy

A recent procedure, i.e. lymphatic mapping followed by sentinel lymph node biopsy, was developed in the early nineties to allow a more accurate evaluation of regional node involvement of patients with stage I and II disease (22). The sentinel lymph node is the first node on the direct lymphatic drainage pathway from a tumor. The main indications for lymphatic mapping and sentinel lymph node biopsy are tumor thickness ≥ 1 mm, ulceration of the primary tumor, Clark's level ≥ IV and histological evidence of extensive regression. Between 15% and 34% of patients undergoing sentinel lymph node biopsy will have a sentinel lymph node invaded by the tumor, depending on the inclusion criteria and on the histologic techniques used. In most solid tumors

Table 4 Survival Rates for Melanoma TNM and Staging Categories

Pathologic stage	Primary tumor		Regional lymph node metastasis		Distant metastasis	Survival rates (%) (years)			
	TNM	Thickness (mm)	Ulceration	Nbr	size	site	1	2	5	10
IA	T1a	≤ 1	No	0		-	99	99	95	88
IB	T1b	≤ 1	Yes or Clark ≥ IV	0	-	-	99	99	91	83
	T2a	1.01-2	No	0	-	-	99	97	89	79
IIA	T2b	1.01-2	Yes	0	-	-	98	93	77	64
	T3a	2.01-4	No	0	-	-	99	94	79	64
IIB	T3b	2.01-4	Yes	0	-	-	95	85	63	51
	T4a	> 4	No	0	-	-	95	89	67	54
IIC	T4b	> 4	Yes	0	-	-	90	71	45	32
IIIA	N1a	Any	No	1	Micro	-	96	88	69	63
	N2a	Any	Yes	2-3	Micro	-	93	83	63	57
IIIB	N1a	Any	Yes	1	Micro	-	93	75	53	38
	N2a	Any	Yes	2-3	Micro	-	92	81	50	36
	N1b	Any	No	1	Macro	-	88	79	59	48
	N2b	Any	No	2-3	Macro	-	77	66	46	39
IIIC	N1b	Any	Yes	1	Macro	-	78	54	29	24
	N2b	Any	Yes	2-3	Macro	-	74	44	24	15
	N3	Any	Any	≥ 4	Any	-	71	50	27	18
IV	M1a	Any	Any	Any	Any	Skin, LN	59	37	19	16
	M1b	Any	Any	Any	Any	Lung	57	23	7	3
	M1c	Any	Any	Any	Any	Other	41	24	10	6

Adapted from Balch *et al.*, JCO, **19**: 3635-3648, 2001.

Table 5 AJCC Rules for Staging of Cutaneous Melanoma

1992/1997

Primary Tumor (T)	
pTX	Primary tumor cannot be assessed
pT0	No evidence of primary tumor
pTis	Melanoma *in situ* (atypical melanocytic hyperplasia, severe melanocytic dysplasia), not an invasive lesion (Clark's Level I)
pT1	Tumor 0.75 mm or less in thickness and invading the papillary dermis (Clark's Level II)
pT2	Tumor more than 0.75 mm but not more than 1.5 mm in thickness and/or invades to the papillary-reticular dermal interface (Clark's Level III)
pT3	Tumor more than 1.5 mm but not more than 4 mm in thickness and/or invades the reticular dermis (Clark's Level IV)
pT3a	Tumor more than 1.5 mm but not more than 3 mm in thickness
pT3b	Tumor more than 3 mm but not more than 4 mm in thickness
pT4	Tumor more than 4 mm in thickness and/or invades the subcutaneous tissue (Clark's Level V) and/or satellite(s) within 2 cm of the primary tumor
pT4a	Tumor more than 4 mm in thickness and/or invades the subcutaneous tissue
pT4b	Satellite(s) within 2 cm of the primary tumor

Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis 3 cm or less in greatest dimension in any regional lymph node(s)
N2	Metastasis more than 3 cm in greatest dimension in any regional lymph node(s) and/or in-transit metastasis
N2a	Metastasis more than 3 cm in greatest dimension in any regional lymph node
N2b	In-transit metastasis
N2c	Both (N2a and N2b)

Distant Metastasis (M)	
MX	Presence of distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis
M1a	Metastasis in skin or subcutaneous tissue or lymph node(s) beyond the regional lymph nodes
M1b	Visceral metastasis

Note:
In-transit metastasis involves skin or subcutaneous tissue more than 2 cm from the primary tumor not beyond the regional lymph nodes

Stage Grouping				
Stage I	pT1	N0	M0	
	pT2	N0	M0	
Stage II	pT3	N0	M0	
Stage III	pT4	N0	M0	
	Any pT	N1	M0	
	Any pT	N2	M0	
Stage IV	Any pT	Any N	M1	

*American Joint Committee on Cancer, Manual for Staging of Cancer, 4th edition, 1992 and 5th edition, 1997

Table 6 AJCC Rules for Staging of Cutaneous Melanoma 2002

T	Thickness	Ulceration Status
T 1	≤ 1.0 mm	a: w/o ulceration and level II/III b: with ulceration or level IV/V
T 2	1.01-2.0 mm	a: w/o ulceration b: with ulceration
T 3	2.01-4.0 mm	a: w/o ulceration b: with ulceration
T 4	> 4.0 mm	a: w/o ulceration b: with ulceration

N	Nbr of metastatic nodes	Nodal metastatic Mass
N 1	1 node	a: micrometastasis* b: macrometastasis**
N 2	2-3 nodes	a: micrometastasis* b: macrometastasis** c: in-transit met(s) / satellite(s) *without* metastatic nodes
N 3	≥ 4 nodes or matted nodes or in-transit met(s)/satellite(s) *with* metastatic node(s)	

M	Site	Serum LDH
M 1a	Distant skin, s.c. or nodal mets	Normal
M 1b	Lung met(s)	Normal
M 1c	All other visceral mets	Normal
	Any distant met(s)	Elevated

*Micrometastases are diagnosed after sentinel or elective lymphadenectomy
**Macrometastases are defined as clinically detectable nodal metastases confirmed by therapeutic lymphadenectomy or when nodal metastasis exhibits gross extracapsular extension.

STAGE GROUPING

Clinical staging				Pathological staging			
0	Tis	N0	M0	**0**	Tis	N0	M0
IA	T1a	N0	M0	**IA**	T1a	N0	M0
IB	T1b	N0	M0	**IB**	T1b	N0	M0
	T2a	N0	M0		T2a	N0	M0
IIA	T2b	N0	M0	**IIA**	T2b	N0	M0
	T3a	N0	M0		T3a	N0	M0
IIB	T3b	N0	M0	**IIB**	T3b	N0	M0
	T4a	N0	M0		T4a	N0	M0
IIC	T4b	N0	M0	**IIC**	T4b	N0	M0
III	any T	any N	M0	**IIIA**	T1-4a	N1a	M0
					T1-4a	N2a	M0
				IIIB	T1-4b	N1a	M0
					T1-4b	N2a	M0
					T1-4a	N1b	M0
					T1-4a	N2b	M0
					T1-4a	N2c	M0
				IIIC	T1-4b	N1b	M0
					T1-4b	N2b	M0`
					T1-4b	N2c	M0
					any T	N3	M0
IV	any T	any N	M1	**IV**	any T	any N	M1

Clinical staging includes microstaging of the primary melanoma and clinical/radiological evaluation for metastases. By convention, it should be used after complete excision of the primary melanoma with clinical assessment for regional and distant metastases.

Pathological staging includes microstaging of the primary melanoma and pathological information about the regional lymph nodes after partial or complete lymphadenectomy. Pahtological Stage 0 or IA patients are the exception; they do not require pathological evaluation of their lymph nodes.

Table 7

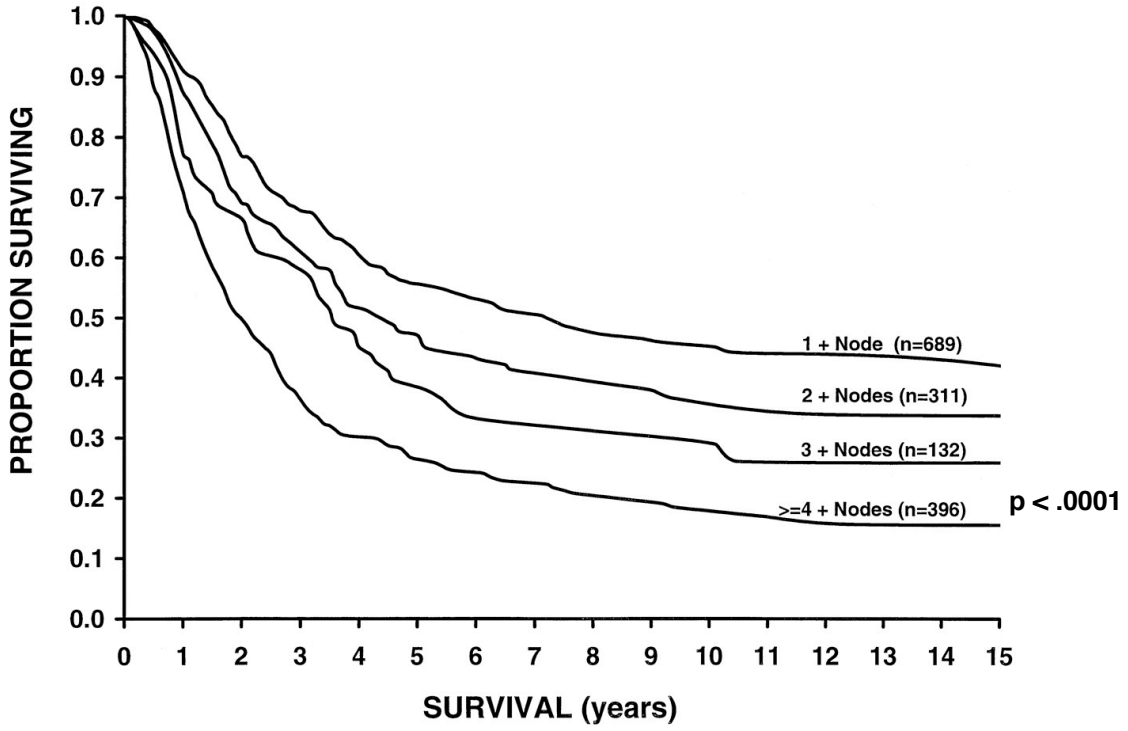
Five-year survival rates (%) of pathologically staged patients showing upstaging effect of primary melanoma ulceration

	IA	IB	IIA	IIB	IIC	IIIA	IIIB	IIIC
Nonulcerated melanoma	T1 **95**	T2 **89**	T3 **79**	T4 **67**		N1a N2a **67**	Nlb N2b **54**	N3 **28**
Ulcerated melanoma		T1 **91**	T2 **77**	T3 **63**	T4 **45**		N1a N2a **52**	N1b N2b N3 **24**

Balch, C. M. *et al., J Clin Oncol* ; 19: 3635-3648, 2001

Figure 3

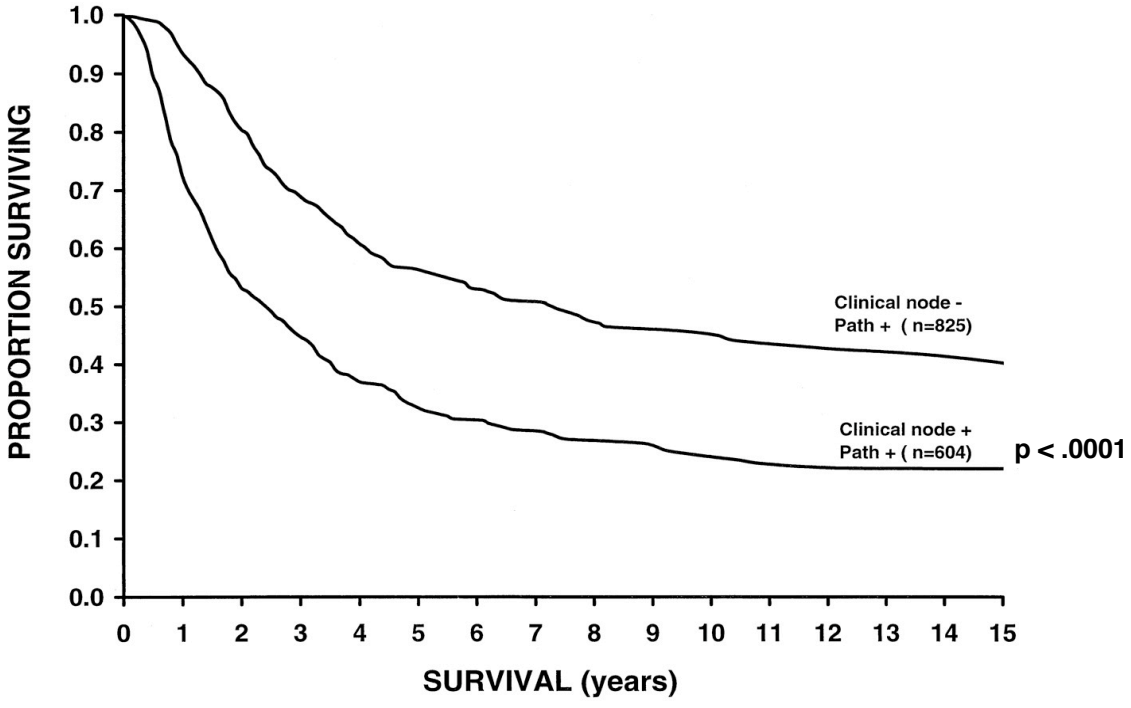
Survival curves of 1,528 melanoma patients with lymph node metastases subgrouped by the actual number of metastatic nodes



Balch, C. M. *et al. J Clin Oncol*; 19 : 3622-3634, 2001

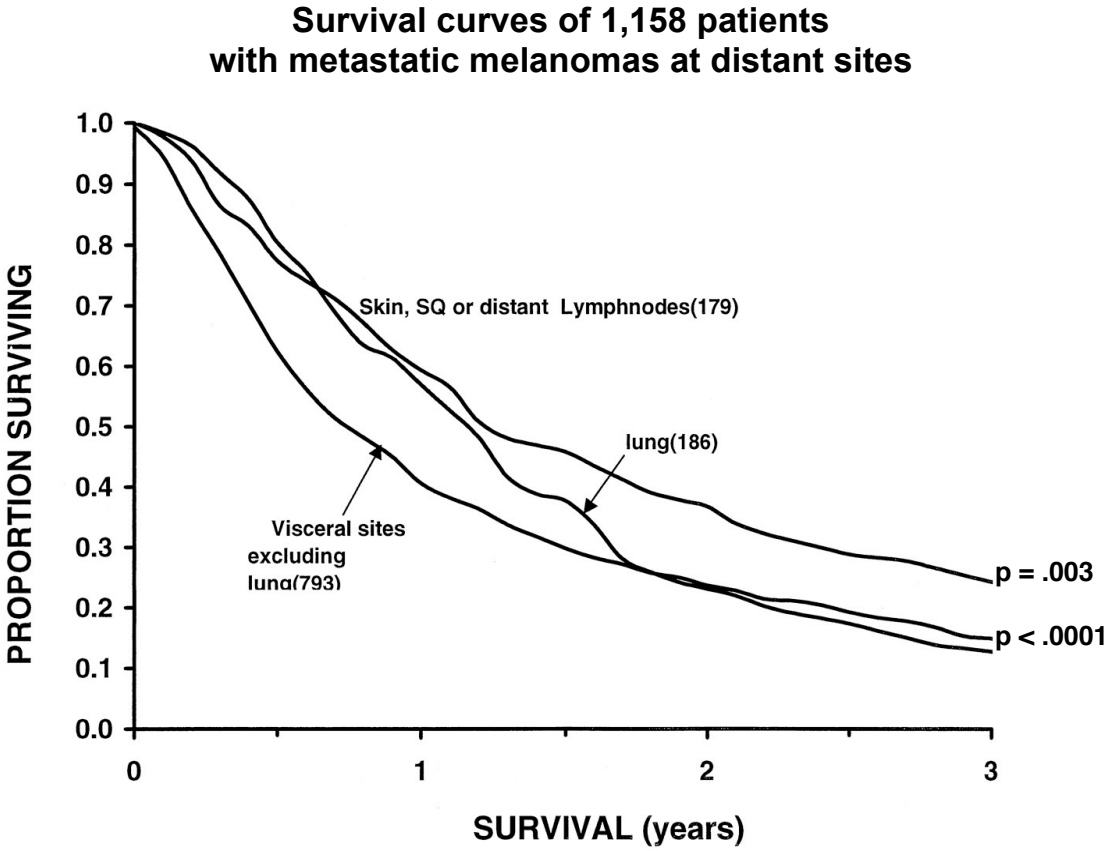
Figure 4

Survival curves of 1,429 patients with lymph node metastases subgrouped by their presenting clinical staging



Balch, C. M. *et al. J Clin Oncol;* 19 : 3622-3634, 2001

Figure 5



Balch, *C. M. et al. J Clin Oncol*; 19 : 3622-3634, 2001

Table 8 Sentinel lymph node biopsy

	Negative (66-85%)	Positive (15-34%)	References
Risk of recurrence	14%	55%	Gershenwald, JCO, 1999 P < 0.00001
5-year Disease-free survival	88%	50%	Vuylsteke, JCO, 2003 p < 0.0001
5-year Overall survival	92%	67%	Vuylsteke, JCO, 2003 p < 0.0001
Complete LN dissection	No	20% non SLN +	Dewar, JCO, 2004

including melanoma, it is acknowledged that lymphatic spread usually occurs concurrently with hematogenous metastasis. Pathologic status of the sentinel lymph node in patients with clinically negative nodes was indeed shown to be the most important independent prognostic factor for recurrence and disease-specific survival ($p < 0.00001$) *(Table 8)* (23). Patients with a negative sentinel lymph node have only 14% risk of recurrence, whereas those with positive sentinel lymph node will relapse in 55% of the cases. The 5-year disease-free survival for negative and positive sentinel lymph node patients is 88% and 50%, respectively and the 5-year overall survival is 92% and 67% for the same categories of patients ($p < 0.0001$) (24). Patients with a positive sentinel lymph node biopsy are selected for complete lymph node dissection of the involved regional basin(s) in order to remove potential occult metastasis in additional, non-sentinel nodes. Only 20% of them will display tumor involvement beyond the sentinel lymph node. Since the location of metastasis in the sentinel lymph node predicts nonsentinel lymph node involvement, complete lymph node dissection could safely be avoided in the 26% of patients who only display subcapsular metastasis *(Fig. 6)* (25). In addition to staging information and early removal of potential occult regional metastasis, another main reason for using this procedure is to select more appropriately the patients for future experimental adjuvant treatments.

Although sentinel lymph node biopsy is a part of the new AJCC staging system, there is so far no evidence that it has a significant impact in overall survival in the long term. Three major studies are investigating the effectiveness of sentinel lymph node biopsy: the Multicenter Selective Lymphadenectomy Trials I and II (26, 27) and the Sunbelt Melanoma Trial (28). The Multicenter Selective Lymphadenectomy Trial I that began in 1994 explores the potential therapeutic benefit of this procedure. This prospective, randomized Phase III trial compares sentinel lymph node biopsy immediately followed by therapeutic lymph node dissection, in patients with a positive biopsy only, with control patients who will only undergo therapeutic lymph node dissection after the appearance of palpable regional metastatic lymph nodes. Data of the third interim analysis on 2,001 patients showed a significantly ($P = 0.01$) higher disease-free survival for patients undergoing sentinel lymph node biopsy (78%) as compared with those who had only wide excision of the primary melanoma followed by observation until palpable regional metastatic lymph nodes appeared (73%). No difference was apparent in overall survival between the 2 treatment groups (29). The final analysis of this trial will be reported in 2007. The Multicenter Selective Lymphadenectomy Trial II began in 2005 and was designed for patients with microscopic or molecular involvement of the sentinel lymph node, to determine if a therapeutic benefit exists for routine completion lymph node dissection, versus sentinel lymph node biopsy only. Study accrual is ongoing. The Sunbelt Melanoma Trial is a prospective randomized study designed to examine the therapeutic value of completion lymphadenectomy and/or 1 month of intravenous high dose adjuvant interferon-α (IFN-α) in patients with molecularly involved sentinel lymph nodes based on

Figure 6 The microanatomic locations of metastatic melanoma within sentinel nodes predicts nonsentinel lymph node involvement

Subcapsular (26%)

CLND ↓

No N+

Parenchymal (11%)

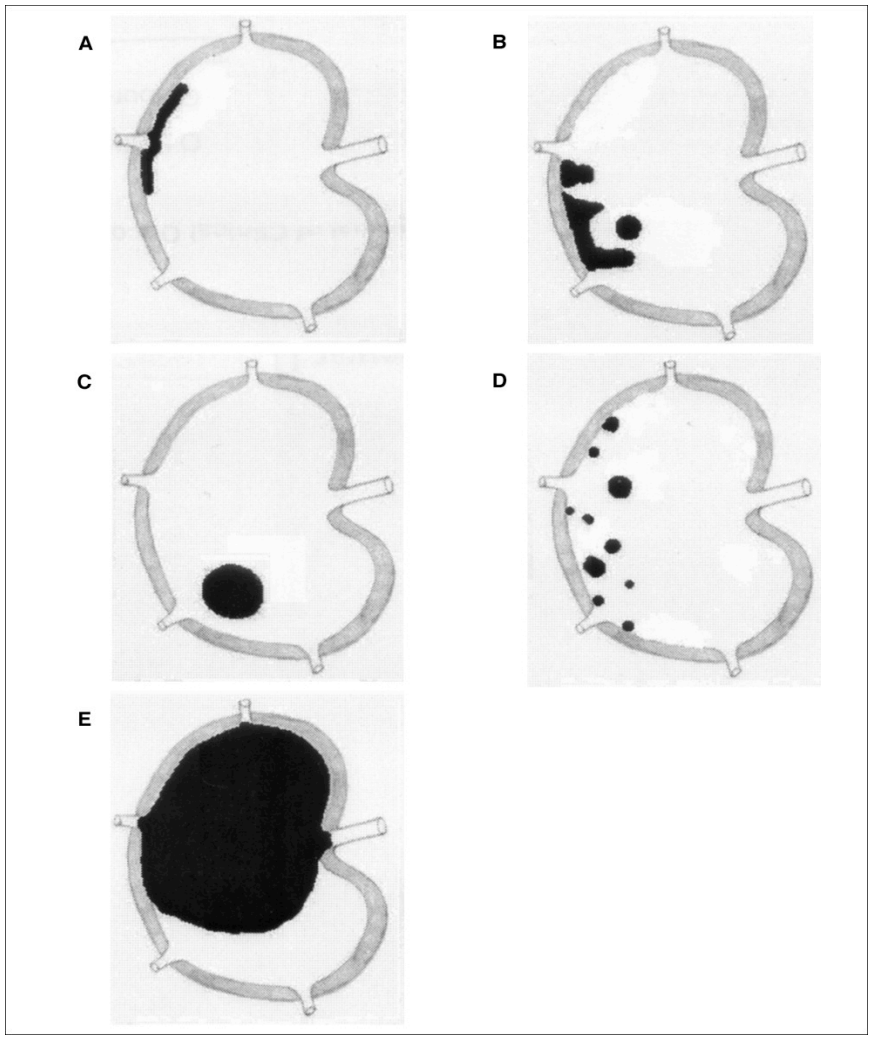
↓

19% N+

Extensive (13%)

↓

42% N+



Subcapsular and parenchymal (37%)

↓

11% N+

Multifocal (13%)

↓

37% N+

Dewar, D.J. et al. J Clin Oncol; 22:3345-3349 2004

reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. This trial should provide further insight into the therapeutic value of the sentinel lymph node biopsy procedure (28).

In stage IV disease, surgery can still be curative for solitary or few resectable lesions, although at this stage of the disease, the majority of patients is likely to have undetectable micrometastases that will finally determine survival. In case of extended distant metastatic disease, debulking surgery may also be useful in order to improve the efficacy of further treatments.

I. 1.3.1.2 Isolated limb perfusion

Satellite and in-transit metastases are skin or subcutaneous metastases arising through lymphatic dissemination, between the site of the primary tumor and - but not beyond - the first major regional nodal basin(s). Their current incidence is approximately 17%. Most patients (>80%) with in-transit metastases will develop systemic metastases. This is the case in virtually 100% of the patients who have (had) also positive regional lymph nodes (30) (chap. 23, pp. 439-448). Patients who present with in-transit metastases (AJCC 2002 stage III N2c) have a median survival of 3.5 years with a 10-year survival rate of 24%. The presence of concomitant regional nodal metastases (AJCC 2002 stage IIIC N3) reduces the median survival time to 17 months and the 10-year survival rate to 19%.

Once the regional disease is beyond surgical control, regional chemoimmunotherapy is the main treatment available. The best modality to administer cytostatic drugs to a limb is the isolated limb perfusion procedure (ILP). Following surgical vascular isolation of the extremity, therapeutic agents can be perfused through the limb with limited or negligible systemic exposure, allowing the use of much higher and potentially more effective dosages. Several drugs have been tested, including dimethyl-triazeno-imidazole-carboxamide (DTIC or Dacarbazine), Cisplatin and melphalan. The alkylating agent melphalan has become the standard agent for ILP in advanced in-transit melanoma, because it combines a good therapeutic efficacy (80% objective response rate with an average complete remission rate of 50%) with an acceptable regional toxicity and minimal systemic side effects (30) (chap. 25, pp. 473-494). The highest response rates to ILP have been obtained with a regimen combining high-dose recombinant tumor necrosis factor-α (rTNF-α) with rIFN-γ and melphalan (31, 32). A 78% complete response rate and a 20% partial response rate were obtained. However, the survival rates were not improved, because this regional treatment has no impact on the time to distant metastasis that governs overall survival. This clearly indicates that a majority of patients with in-transit disease are susceptible to have undetectable systemic micrometastases at the time they undergo ILP. Because of its potential toxicity, the lack of survival benefit and despite the high response rates, ILP should be limited to patients with extended or invalidating regional disease. ILP should only be performed by well-trained, experienced surgical teams, in order to guarantee the lowest morbidity. ILP is the best palliative treatment to propose to

patients with severe in-transit cutaneous melanoma of the limbs. It has definitely replaced amputation, which also failed to increase the chances of survival (33, 34).

I. 1.3.2 Chemotherapy and Biochemotherapy

Melanoma is considered to be a relatively chemoresistant tumor (35, 36). Responses are observed more frequently in the skin, subcutaneous tissue, lymph node and lung-sites of metastasis, compared with other sites (19) (chap. 39, p. 499). However, a number of chemotherapeutic agents have some activity in patients with metastatic melanoma, including dacarbazine (DTIC), nitrosoureas, platinum analogs, vinca alkaloids and taxanes. They can be given either as single agents or in combination (30) (chap. 31, pp. 589-604).

I. 1.3.2.1 Single-agent chemotherapy

Dacarbazine and analogs

DTIC is an alkylating agent that requires metabolic activation in the liver, where it is converted into its active metabolite, methyl-triazen-imidazole-4-carboxamide (MTIC). It is the most active single agent for advanced melanoma. Objective response rates of 10 to 20% were initially reported. However, in recent randomized trials, DTIC produced only 6-12% objective responses (37-40). Complete responses (CR) are rare (≤ 5%) and the median response duration is only 4 to 6 months. A minority of patients has long-lasting responses and fewer than 2% live longer than 6 years. Commonly used schedules include i.v. administration of 200 to 250 mg DTIC per m^2/day (d) for 5 consecutive days, or 850 to 1,000 mg/m^2/d over 1 hour on day 1 only. DTIC cycles are administered every 3 to 4 weeks. DTIC is globally well tolerated, the major side effect being nausea and vomiting. Therefore, as many as 10 consecutive cycles can be administered, as long as no major tumor progression occurs. Although DTIC is the only cytotoxic agent approved for the treatment of advanced melanoma, no phase III trial data exist to support a survival benefit for DTIC relative to a "no treatment" control.

Temozolomide (TMZ) is an analog of DTIC that does not require metabolic activation in the liver, because it undergoes spontaneous chemical activation to MTIC at physiological pH. Contrarily to DTIC, it can therefore be administered orally. It crosses the blood brain barrier and objective tumor responses or stabilization of brain metastases were reported (41, 42). A large European, multicenter, randomized phase III trial compared the clinical activity of TMZ (200 mg/m^2/d for 5 consecutive days every 4 weeks) versus that of DTIC (250 mg/m^2/d for 5 consecutive days every 3 weeks) in 305 previously untreated patients with advanced metastatic melanoma, but without central nervous system localization (37). The results of the 2 treatments were similar in terms of overall survival, response rate and toxicity. Median progression-free

survival was significantly longer in the TMZ-treated group (1.9 months) than in the DTIC-treated group (1.5 months) (p = 0.012; hazards ratio 1.37; 95% confidence interval, 1.07 to 1.75). In addition, TMZ showed an advantage in terms of improvement in the quality of life. TMZ should therefore be considered as an oral alternative treatment to DTIC for patients with advanced metastatic melanoma.

Other drugs used as single agents

Other chemotherapy drugs also have activity in melanoma. They include nitrosourea, cispaltin (Cis-diamine-dichloro-platine or CDDP), carboplatin, vinca alkaloids and taxanes. Among nitrosourea, i.e. carmustine (Bis-chloroethyl-cyclohexyl-nitrosourea or BCNU), lomustine (CCNU), semustine (methyl CCNU) and fotemustine (FTMU), the latter has the highest capacity to cross the blood-brain barrier. The response rates to these drugs used as single agent are similar to or lower than those to DTIC.

I. 1.3.2.2 Combination chemotherapy

Because of the low response rates obtained with single chemotherapeutic agents, several attempts were made to combine several minimally active drugs, with the hope that a highly active combination would result. Several chemotherapy combinations have been described, but in phase III trials, none has had response rates superior to DTIC alone (43, 44). Among these combined regimens, the so-called "Dartmouth regimen" associating DTIC, cisplatine, carmustine and tamoxifen (an inhibitor of estrogenic receptors) was the most studied: several single-institution phase II trials have reported that the combination of DTIC 220 mg/m^2 d1-5, cisplatine (CDDP) 25 mg/m^2 d1-3, carmustine (BCNU) 150 mg/m^2 d1 every other cycle and tamoxifen 2x10 mg/d orally can induce response rates of 40 to 50% in stage IV melanoma patients. In a large phase III trial, the Dartmouth combined regimen did not improve overall survival as compared with single-agent DTIC treatment and the difference in response rates between the 2 treatments was not significant (45). The CVD regimen, which consists of DTIC, cisplatinand a vinca alkaloid (vinblastine or vindesine) induced similar response rates (46). The observation of clinical activity and an acceptable toxicity profile for the CVD regimen led to its use in the biochemotherapeutic approach in combinations with cytokines.

I. 1.3.2.3 Cytokines in stage IV melanoma

The natural history of melanoma suggests a possible modulating role of the immune system of the host. Since the 1970s, there have been multiple publications regarding the use of immunostimulating agents, both systemically and locally administered. Among the biologic response modifiers, the cytokines interleukin-2 (IL-2) and interferon-α (IFN-α) have been the most

studied molecules in the treatment of melanoma. In patients with stage IV disease, single agent treatment with high-dose IL-2 (720.000 international units (IU)/kg, every 8 hours for 5 days) or IFN-α (dose range: 10 x 10^6 Units/day 3 times a week to 20 x 10^6 Units/day 5 times per week) has resulted in objective response rates of approximately 15% (47, 48). These response rates are similar to those obtained with DTIC as single agent, but the severe toxicities of high-dose regimen of IL-2 or IFN-α limit their use in the treatment of melanoma (49-51).

I. 1.3.2.4 Combined chemoimmunotherapy

Based on the potential for additive or synergistic activity with the combination of chemotherapy and biotherapy, many investigators have evaluated such therapeutic associations in patients with advanced melanoma. Legha and colleagues developed a combination of chemotherapy (CVD regimen) and immunotherapy (IL-2 and IFN-α), which they termed biochemotherapy (52). Phase II studies of biochemotherapy using various drug combinations in metastatic melanoma patients have reported response rates of 47-63%, with CR seen in 6-23% of patients (53-59). Cytokines increase the antitumor activity of chemotherapy at the expense of considerable toxicity in patients with metastatic melanoma. Of note, long-term, durable CR persisting beyond 5 years were observed in nearly 10% of patients, which is higher than the 1-2% rates and the 4% rate of patients who achieved a similar durable remission with chemotherapy alone or high-dose IL-2 alone, respectively (56, 60-62). However, randomized phase III study comparing the combination of IFN-α/IL-2 with various chemotherapeutic agent to immunotherapy alone or to chemotherapy alone failed to demonstrate an improved survival for patients receiving biochemothearpy, as compared to those who received immunotherapy or chemotherapy alone (63-65).

I. 1.3.3 Radiotherapy

Melanoma is considered to be a relatively radioresistant tumor. Cutaneous, subcutaneous and lymph node metastases respond better to radiotherapy (65% objective response rates) than visceral localizations, with smaller lesions more likely to respond than larger ones (19) (chap 40, p 516). There are however, some indications for radiotherapy in visceral metastases, including brain localizations, malignant spinal cord compression and painful bone lesions (30) (chap 30, pp 573-586).

Symptomatic metastases to the central nervous system (CNS) develop in nearly half of the patients with advanced cutaneous melanoma and autopsy series report up to 75% CNS involvement. CNS metastases are a major cause of morbidity and mortality. The estimated median survival for patients with CNS metastases is 3 months, with 1-year survival rates of less than 15%. Most of the systemic agents used for the treatment of melanoma do not cross the blood-brain barrier and these patients rarely benefit from systemic therapy. Thus the main treatments available are surgery and/or radiotherapy. In a prospective randomized trial on 95 patients, Patchell and colleagues showed that

after surgical excision of a single brain metastasis, whole-brain irradiation reduced intracranial recurrence from 70% to 18% ($P < 0.001$). There was however no impact on overall survival (66). Patients presenting with a single brain metastasis or with a limited number of small-size brain metastases in favorable anatomic sites and with limited or no extracranial disease, can appropriately be treated with stereotactic radiotherapy. A focused high dose of radiation using multiple cobalt sources (γ-knife) or a linear accelerator (Linac) allows delivering in a single session a high dose of ionizing external beam radiation within the target lesion(s), with minimal irradiation outside the defined area. In a retrospective, nonrandomized trial on 35 patients treated with stereotactic radiosurgery for brain localization(s), a median survival of 22 months was reported in the 4 patients who had a solitary brain metastasis, compared to 4 months in the patients with multiple brain lesions (67). To date, there have been no prospective randomized trials of stereotactic radiosurgery versus whole brain radiotherapy and the optimal radiotherapeutic treatment is still unknown. Large or multiple cerebral metastases can be treated with whole-brain irradiation in conjunction with high-dose steroids, in order to control the intracranial pressure and relieve possible neurologic symptoms. Several clinical trials currently explore the association of chemotherapy, mainly temozolomide, with various radiotherapy modalities in the treatment of melanoma brain metastases.

Radiotherapy should therefore be considered as an excellent palliative treatment for some unresectable melanoma metastases, considerably reducing the morbidity and increasing the patient's quality of life.

I. 1.3.4 Adjuvant systemic therapy

There have been many attempts to develop effective adjuvant therapy for patients with primary or regional melanoma with high-risk of relapse after surgical treatment. These approaches have included both chemotherapy and immunotherapeutic treatments. In prospective randomized Phase III trials, there was only little or no impact of these treatments on overall survival. Several of these trials have explored treatment with various doses and schedules of IFN-α (high dose: induction phase, 5 x 20 MU/week intravenously (i.v.) for 4 weeks, followed by a maintenance treatment of 3 x 10 MU s.c/week for 1 year or longer; intermediate dose: induction 5 x 10 MU/week i.v. for 4 weeks, followed by a maintenance treatment of 3 x 5 MU s.c/week for 1 year or longer; and low dose, 3 x 3 MU s.c/week for 1 year or longer (68). Only 2 of these trials showed a transient significant impact on both disease-free survival and overall survival at 5-year follow-up. One trial evaluated high-dose IFN in high-risk melanoma patients (Eastern Cooperative Oncology Group [ECOG] 1684 in stage IIb (resected primary tumor thickness ≥ 4 mm) or stage III disease and the other, low-dose IFN in patients with intermediate-risk melanoma (French Cooperative Group Trial in stage II disease) (69, 70). In both trials, however, the impact on overall survival was lost at 8-year follow-up. Recently, a 3-arm randomized Phase III trial performed by the European Organization for Research and Treatment of Cancer explored the distant metastasis-free interval in 1,388 patients with resected stage IIb or III

disease (EORTC study 18952) (71). Patients received intermediate doses of IFN-α 2b for 13 months (n = 553), 25 months (n = 556) or were assigned to the observation arm (n = 279). In none of these settings did IFN improve the outcome of patients. Since duration of treatment seemed more important than dose, a randomized Phase III trial is currently exploring the impact of a 5-year maintenance treatment with acceptable toxicity, as compared with an observation (no treatment) arm: EORTC study 18991 with pegylated-IFN (PEG-Intron).

New treatments are therefore clearly needed.

I. 2 T-CELL DEFINED ANTIGENS ON MELANOMA

I. 2.1 From the concept of cancer immunosurveillance to the molecular definition of tumor antigens

William B. Coley, a New York cancer surgeon, is generally considered to be the father of cancer immunotherapy. One of his patients suffering from a supposedly terminal bone cancer coincidentally developed severe skin infection caused by *Streptococcus pyogenes* and survived to his cancer. Coley wondered if the bacteria had caused the tumor to regress. In the hospital records, he found similar cases of a cancer remission after bacterial infection. Based on these few observations, he hypothesized that something in the bacteria had stimulated the immune system so it not only destroyed the bacteria, but the cancer as well. The body was not a passive receptacle for cancer, but it had the capacity to actively resist the disease. In the 1890s, he developed a vaccine of bacterial toxins that became known as Coley's toxins and began injecting patients with inoperable tumors. The first patient injected with the vaccine went into remission and died 26 years later of a heart attack. Coley treated nearly 900 patients and claimed that some 40% achieved lengthy remissions. At that time, the active ingredient was thought to be lipopolysaccharide. But many who tried to repeat his results failed. Cancer therapy shifted to chemotherapy and radiation and Coley's ideas fell into oblivion.

In 1943, Ludwik Gross induced sarcomas in mice by applying methylcholanthrene, a potent carcinogen, on their skin (72). Such tumors could be transplanted to grow progressively on mice belonging to the same inbred strain as the original tumor bearer (sygeneic mice). However, when transplanted mice were cured of their tumor by surgery and reinoculated subsequently with the same tumor, the latter almost always failed to grow, whereas skin grafts from syngeneic mice were accepted by the receivers. This observation was the first experimental evidence suggesting that tumors can carry specific transplantation antigens that cause their rejection in immunized hosts. Immunization with a given methylcholanthrene-induced tumor did not protect against another independent tumor induced by the same carcinogen, indicating that there was a great diversity among these antigens (73, 74). Similar antigens were also found on mouse tumors induced by viruses or by physical agents, notably ultraviolet radiations (75, 76). These antigens were called tumor specific transplantation antigens (TSTA) because they were not expressed by normal cells.

In the early 1970s, the theory of the "immune surveillance of cancer" was put forward by Burnet who had observed that patients with a long-term immunodepression status, such as those who had received a kidney transplantation, developed cancers more frequently than patients with a normal immune status (77). He postulated that during their development, "immunocytes" acquire the ability to distinguish "self" from "non-self" antigens. Consequently, immunocytes no longer react against self components, unless the latter are modified, as may happen during neoplastic transformation. The concept of immunosurveillance suggests that transformed cells express tumor-associated antigens that trigger an adaptive immune response aimed at rejection of the developing cancer lesions at a very

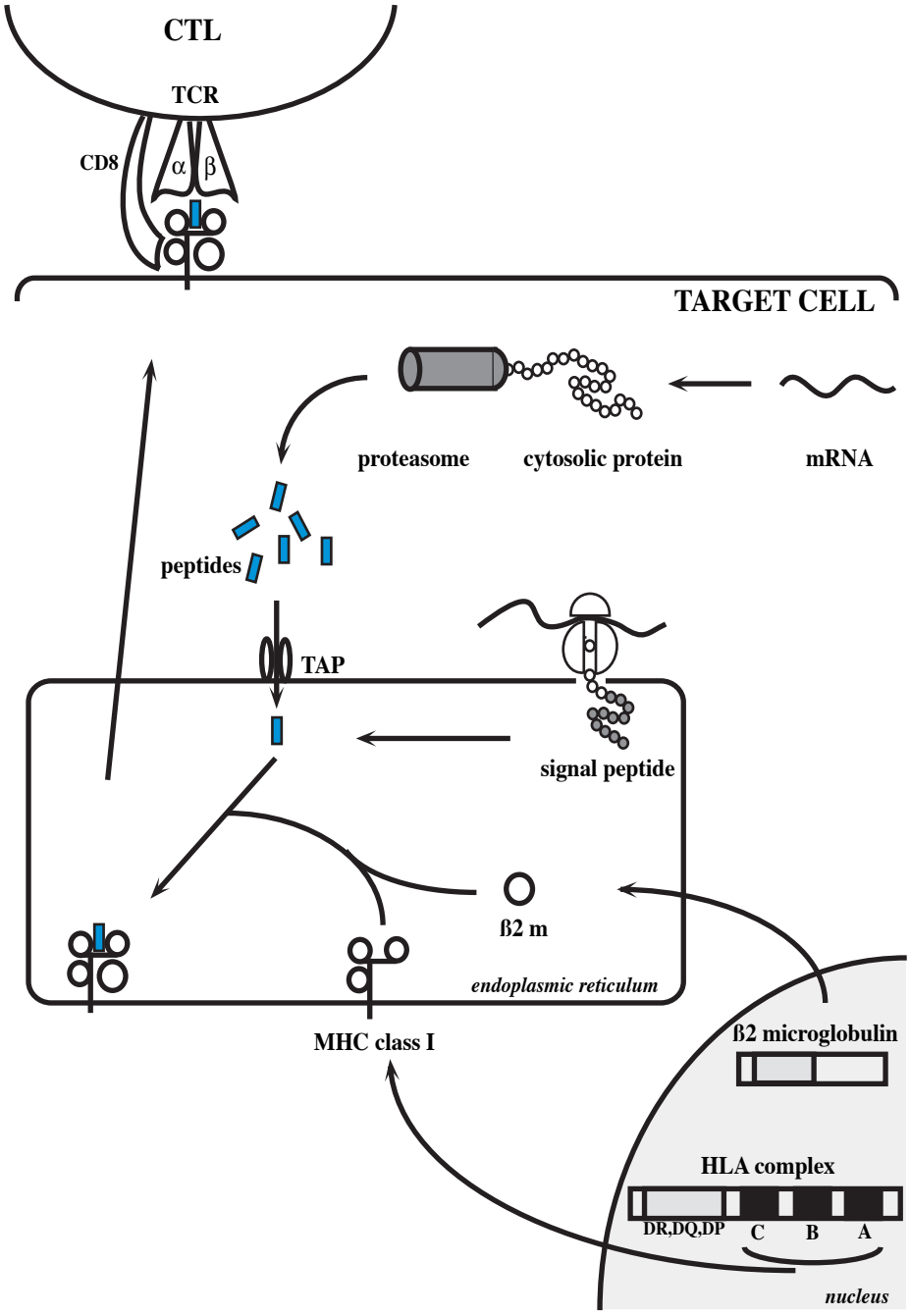
early stage, the cancerous disease being the result of an occasional failure of this surveillance. However, this hypothesis lost credibility when it became apparent that no general correlation could be made either in man or in animals between immune depression and high tumor frequency (78, 79). In 1976, Hewitt reported that tumor transplantations carried out with spontaneous tumors, i.e. tumors occurring in the absence of any experimental treatment, were unable to generate any immune protection in syngeneic mice. He suggested that TSTA were restricted to experimental tumor models induced with high doses of carcinogens and that spontaneous mouse tumors did not express TSTA, a situation which appeared to be much more comparable to the conditions of tumor formations in humans (80).

Nevertheless, further experiments on animal models by Boon and coworkers soon provided evidence that most tumors express tumor antigens that can constitute targets for rejection responses, even though these tumors often do not elicit immune responses. This was achieved by the so-called tum^- variants that were obtained by treating mouse tumor cell lines *in vitro* with a mutagen (81). These variants are unable to produce tumors in syngeneic animals because they express new very strong transplantation antigens (tum^- antigens). Tum^- variants were initially derived from a malignant teratocarcinoma cell line that appeared to lack any immunogenicity, even upon injection of living cells followed by surgical removal of the tumor. In contrast, mice that had rejected tum^- variants were significantly protected against a challenge with the original teratocarcinoma cells (82). Such a protection conferred by tum^- variants was also observed with the spontaneous tumors of Hewitt and this protection proved to be specific for the tumor from which the tum^- variants were derived (83). These results indicated that among mouse tumors that are not immunogenic, i.e. incapable of eliciting an immune rejection response, most are nevertheless antigenic, i.e. express an antigen that can be the target of an immune response generated by artificial means. Therefore, it became plausible that human tumors are antigenic and that their antigens could be rendered immunogenic by appropriate means.

Adoptive transfer of splenic lymphocytes of immunized mice have demonstrated that the cells of the immune system that constitute the specific component of tumor rejection responses and that carry long-term memory are T lymphocytes (76, 84). In the 1970s, cytolytic T lymphocytes (CTL) were identified as the effectors of tumor rejection in mice. A large body of information was obtained by studying *in vitro* CTL responses against mouse tumor cells that grow in permanent culture. Tumor specific CTL were obtained from immunized mice, after *in vitro* restimulation of spleen lymphocytes with tumor cells, called "mixed lymphocyte-tumor culture" (MLTC) (85-87). The availability of antitumor CTL clones that could be maintained indefinitely proved to be of crucial importance for the analysis of tumor rejection antigens (88, 89). Such CTL clones made it possible to select antigen-loss tumor cell variants *in vitro*. The observation that tumor cells that had escaped immune rejection in mice had become resistant to anti-tumor CTL demonstrated that tumor antigens recognized by CTL can be relevant for tumor rejection *in vivo* (90, 91). T cells recognize antigen in a way that is

Figure 7

Presentation of antigenic peptide on class I HLA molecules to CD8 cytolytic T-lymphocytes



fundamentally different from that of antibodies (92-96). In contrast to antibodies that can bind to viral particles or soluble antigens, T-cell receptors (TCR) only recognize antigens that are associated to a particular human leukocyte antigen (HLA) molecule. T-cell $CD8^+$ CTLs recognize 8-11 amino acids (AA) peptides that are derived from intracellular proteins degraded in the cytosol, such as infectious virus-encoded proteins, minor histocompatibility antigens or TSTA. Endogenous proteins are degraded by the proteasome into peptides that are transported into the endoplasmic reticulum by specific adenosine triphosphate (ATP)-dependent transporter molecules (TAP-1 and TAP-2). There, they bind to HLA class I molecules that are encoded in humans by HLA-A, -B and -C loci and to β2-microglobulin, to form antigenic complexes. These complexes then migrate through the Golgi to the cell surface where they can be recognized by the TCR of $CD8^+$ lymphocytes *(Fig. 7)*. $CD4^+$ T lymphocytes recognize longer peptides of 10-20 residues, derived from exogenous proteins that have been captured by dendritic cells (DC) or other antigen-presenting cells. Presentation of class II-restricted antigens requires endosomal degradation of the captured protein into peptides, followed by intracellular binding of the resulting antigenic peptides to major histocompatibility complex (MHC) class II molecules (HLA-DP, -DQ and –DR in humans) and transport of the peptide-MHC-complexes to the cell surface (97). A major consequence of the mode of antigen recognition of T cells is that these lymphocytes can exert surveillance not only on surface proteins but also on all proteins produced by any given cell, even if they remain inside the cell. Another major consequence of this mechanism of antigen presentation is the MHC restriction. A given peptide binds specifically to a defined HLA allele. This means that a defined peptide derived from a tumor-specific protein is only susceptible to be immunogenic in patients who carry the appropriate presenting HLA molecule.

Two types of genes coding for antigens recognized on mastocytoma P815 by syngeneic CTL clones were isolated. They were obtained by transfecting deoxyribonucleic acid (DNA) from P815 cells into antigen-loss variants selected with spectific CTL clones. The first gene, named *P1A* is a normal, non-mutated gene that is present in all cells types of syngeneic DBA/2 mice. It is unrelated to any known gene. It is expressed by several mastocytomas, but is silent in normal mouse tissues, except for testis and placenta (98). When activated in the P815 tumor, it codes for two distinct antigens, P815A and P815B recognized by CTL. The immune response to *P1A* encoded antigens was shown to be the major component of the tumor rejection response against P815 cells observed in normal mice (99). In addition to the activation of a normal gene in tumors, another genetic mechanism can lead to the generation of tum^- transplantation antigens. The P815 tum^- antigens P91A and P198 were generated by point mutations of the corresponding genes (100, 101).

The interest of immunologists for melanoma is based on particular features of this tumor. Rare spontaneous regressions have been described, which are possibly mediated by immune responses (102-105). The complete spontaneous regression of a primary cutaneous melanoma occurs with a frequency of approximately 5%, whereas spontaneous regression of a secondary localization has a much lower incidence, ranging from 0.2 to 0.3%. Cutaneous and subcutaneous metastases are those

that more frequently present a spontaneous regression, followed in order by lymphatic, pulmonary and other visceral localizations (102).

Melanoma cell lines are relatively easy to obtain, providing valuable tools for laboratory studies. Specific CTL clones recognizing autologous melanoma cell lines can be isolated from the blood or the tumor-infiltrating lymphocytes (TIL) of many patients (106, 107).

In 1982, patient MZ2 developed a cutaneous melanoma with lymph-node and multiple visceral abdominal metastases. Throughout 1982 and 1983, she was treated with chemotherapy and surgery for several abdominal relapses. In 1982, an autologous melanoma cell line MZ2-MEL derived from an adrenal metastasis was obtained by Alex Knuth. A panel of highly specific CTL clones were obtained from blood lymphocytes of this patient (106). To characterize the antigen recognized by each CTL clone, successive *in vitro* immune selection of tumor antigen-loss variants were performed. This approach demonstrated that the autologous CTL response was directed against at least 6 distinct antigens present on MZ-2-MEL cells (108). Such tumor antigen-loss variants allowed the characterization of the first gene coding for a human tumor antigen recognized by T lymphocytes on a human tumor.

In the early 1990s, using a genetic approach based on the transfection of DNA librairies into antigen-loss tumor cell variants of MZ2-MEL expressing the appropriate HLA molecule, van der Bruggen characterized the first gene coding for a human tumor antigen recognized by autologous CTL on a melanoma cell line (109). It was named *MAGE-1*, for Melanoma AntiGEn. The antigenic peptide was associated with the HLA class I molecule HLA-A1 to form the MAGE-1.A1 epitope (110). Soon after, genes coding for differentiation antigens of the melanocytic lineage were also shown to generate peptides recognized on human melanomas by specific CTL clones (111-114). A third type of mechanism, i.e. genetic point mutations, also generates CTL epitopes in humans, as it was previously shown in mice (115). Since then, many genes coding for tumor antigens recognized by HLA class I or class II restricted T lymphocytes have been characterized by our group and by others (116, 117). A complete listing of these epitopes is available on the Web at the following address: http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm (118).

According to their pattern of expression in tumor and normal tissues, antigens recognized on tumors by autologous T cells can be divided into five main categories: tumor-specific shared antigens, differentiation antigens, antigens resulting from point mutations, antigens resulting from gene overexpression and viral antigens (119).

Table 9 Percentage of tumor samples expressing MAGE-A, MAGE-C2 and NY-ESO-1 genes

Tumor type	*MAGE-A*									*MAGE-C2*		*NY-ESO-1*	
	n	1	2	3	4	6	12	*n*	10	*n*		*n*	
Melanoma													
primary lesions	*83*	**25**	**52**	**55**	**18**	**59**	**34**	*17*	**24**	*39*	**49**	*6*	**17**
metastases	*243*	**46**	**70**	**74**	**28**	**72**	**62**	*236*	**47**	*69*	**59**	*102*	**28**
Carcinomas													
Head and Neck	*85*	**31**	**38**	**51**	**53**	**58**	**27**	*23*	**30**	*20*	**20**	*18*	**22**
NSCLC													
squamous	*93*	**44**	**42**	**48**	**59**	**53**	**28**	*32*	**41**	*20*	**5**	*44*	**30**
adenocarcinoma	*43*	**49**	**47**	**44**	**35**	**49**	**33**	*14*	**50**	*15*	**20**	*42*	**36**
Esphagus	*19*	**53**	**53**	**63**	**74**	**68**	**26**	*14*	**43**	*15*	**13**	*15*	**13**
Colo-rectal	*46*	**0**	**13**	**17**	**11**	**22**	**11**	*23*	**0**	*20*	**0**	*9*	**0**
Breast	*135*	**19**	**9**	**13**	**6**	**15**	**13** *(61)**	*27*	**4**	*20*	**15**	*13*	**23**
Kidney	*44*	**5**	**0**	**0**	**2**	**0**	**5** *(21)*	*24*	**0**	*24*	**0**	*9*	**0**
Bladder													
superficial (<T2)	*70*	**14**	**11**	**16**	**23**	**19**	**10**	*69*	**20**	*15*	**40**		ND
infiltrating (≥ T2)	*53*	**32**	**43**	**57**	**45**	**57**	**34**	*53*	**38**	*15*	**20**	*15*	**47**
Prostate	*22*	**18**	**18**	**18**	**0**	**23**	**5**	*10*	**10**	*10*	**10**	*11*	**27**
Sarcomas	*15*	**7**	**13**	**13**	**33**	**13**	**7**	*12*	**0**	*15*	**13**	*17*	**35**
Leukemias	*112*	**0**	**0**	**0**	**1**	**0**	**0**	*25*	**0**	*25*	**0**	*17*	**0**
Myeloma										*3*	**1/3**		
Stages I-II	*11*	**0**	**0**	**0**	**0**	**0**	**0**	*11*	**0**				ND
Stage III	*27*	**41**	**26**	**41**	**22**	**37**	**22**	*27*	**11**				ND

F. Brasseur, S. Lucas and B. Lethé (personal communication).

n: number of tumor samples tested by RT-PCR on total mRNA, using specific primers; **(61) = n.*

ND: not done

I. 2.2 Tumor antigens used for vaccination

Tumor-specific shared antigens are expressed on several types of tumors but not on normal adult cells, which make them potentially safe targets for immunotherapy of tumors.

Many tumor-specific CTL from melanoma patients were found to recognize antigens that were present not only on the melanoma cells, but also on normal melanocytes. No other normal cells, though, carry these antigens. Vaccination with differentiation antigens of the melanocytic lineage is restricted to the treatment of melanoma.

Antigens resulting from mutations of ubiquitously expressed genes are strongly immunogenic. However, most of these mutations are individual or only found in a low percentage of tumors, which strongly limits their use for the development of cancer vaccines (115, 120). Antigens resulting from gene overexpression in tumors are often ubiquitously expressed in normal tissues, which could generate severe autoimmune side effects to vaccination. The use of viral antigens is strictly limited to the treatment of tumors bearing such antigens (121).

We will focus here on tumor-specific shared antigens and on differentiation antigens, which are the most frequently used in melanoma vaccination.

I. 2.2.1 Genes coding for widely shared tumor-specific antigens

MAGE genes are "Cancer-germ line genes" that are expressed in an important proportion of tumors of various histologic types, but that are silent in normal cells, except for male germ line cells (and placental trophoblast, for *MAGE-A3* and *MAGE-A4* genes) *(Table 9)* (122). Since the latter do not express the HLA molecules required to present these antigens to the T lymphocytes, antigens coded by cancer-germ line genes are only present on tumors, which should limit the occurrence of autoimmune diseases as a consequence of vaccination (123). In line with this hypothesis, we observed that immunization against mouse antigen P1A, which is encoded by a gene with a similar pattern of expression, did not induce any autoimmune damage in testes and did not impair male fertility (124). Therefore, these widely shared tumor specific antigens should represent good targets for the development of cancer vaccines. Moreover, their expression in many different types of tumors suggests that immunization against antigens they encode could be applied not only to melanoma but also to other cancers.

Six subfamilies of *MAGE* genes are located on the X chromosome. *MAGE-A*, *MAGE-B* and *MAGE-C* subfamilies, include a total of 24 related genes with tumor-specific expression (125, 126). *MAGE* genes are normal, non mutated genes, that are silent in normal tissues because their promoter regions are methylated at specific CpG dinucleotide sites (Cytosine linked to a Guanine by a phosphate bond), preventing the binding of activating transcription factors (127, 128). The expression of *MAGE* genes in tumors is linked to the demethylation of their promoter (127, 129). The most frequently expressed gene in cutaneous melanoma, *MAGE-A3*, hereafter called *MAGE-3*, is found in

Table 10 Shared tumor-specific antigen encoded by *MAGE-3*

HLA	HLA frequency [1] (%)	Peptide AA [2] sequence	AA position	Antigen frequency [3] in metastatic melanoma (%)	References
A1	26	EVDPIGHLY	168–176	20	132
A2	44	FLWGPRALV	271–279	33	133
A2	44	KVAELVHFL	112–120	33	134
A24	20	TFPDLESEF	97–105	15	135
B18	6	MEVDPIGHLY	167–176	5	136
B35	20	EVDPIGHLY	168–176	15	137
B37	3	REPVTKAEML	127–136	2	138
B40	6	AELVHFLLL	114–122	5	139
B44	21	MEVDPIGHLY	167–176	16	140
B52	5	WQYFFPVIF	143–151	4	141
Cw7	41	EGDCAPEEK	212–220	31	142
DP4	75	KKLLTQHFVQENYLEY	243–258	57	143
DQ6	63	KKLLTQHFVQENYLEY	243–258	48	144
DR1	18	ACYEFLWGPRALVETS	267–282	14	145
DR4	24	VIFSKASSSLQL	149–160	18	146
DR7	25	VIFSKASSSLQL	149–160	19	146
DR11	25	GDNQIMPKAGLLIIV	191–205	19	147
DR11	25	TSYVKVLHHMVKISG	281–295	19	148
DR13	19	AELVHFLLLKYRAR	114–127	14	149
DR13	19	LLKYRAREPVTKAE	121–134	14	149

1) HLA frequency is given for the Caucasian population.

2) AA = Amino Acid.

3) Antigen frequency in a given tumor depends both on the frequency of expression of the gene by the tumor (MAGE-3 is expressed in 76% of metastatic melanoma samples), and on the frequency of expression of the presenting HLA allele in the relevant population. For example, MAGE-3.A1 antigen is expressed in [76% x 26%] = 20% of Caucasian patients with a metastatic melanoma.

After Van den Eynde B and van der Bruggen P., Cancer Immunity, Peptide Database, T-cell Epitopes, last update: March 2006, <http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm>.

36% of primary lesions and 76% of metastatic lesions (130). It codes for a protein of 314 amino acids with a molecular weight of approximately 35 kDa (131). *MAGE-A3* codes for many peptides that are recognized by T lymphocytes on HLA class I or class II molecules *(Table 10)* (132-149). Likewise, other *MAGE-A*, *MAGE-B* and *MAGE-C* genes also code for several antigenic peptides (116). In some cases, a given peptide is shared by several MAGE proteins (138, 149, 150), or the same peptide can be presented by two different HLA class I molecules (137, 151). This increases the proportion of patients liable to benefit from this immunotherapy. Six other *MAGE* subfamilies have been identified in the human genome. They are distant relatives of the 3 previously identified *MAGE* subfamilies. The genes belonging to these subfamilies are ubiquitously expressed in normal tissues. They do not code for known MAGE antigens recognized by T cells (126, 152). Little is known about the normal intracellular role of *MAGE* genes. MAGE proteins are located in the cytosolic compartment (153, 154). The only *MAGE* family members that have been functionally characterized so far are *Necdin* and *NRAGE*. The proteins encoded by these genes are involved in the cell cycle regulation of neurons (155, 156). MAGE-A1 protein present in tumor cells could act as a potent transcriptional repressor (154).

Other genes expressed only in tumors and male germ cells have been identified, notably the *BAGE, GAGE* and *NY-ESO/LAGE* families and the *SSX-2* and *SCP1* genes (116, 157-160). These genes have no homology with the *MAGE* genes, but share the same pattern of expression. *SCP1*, one of the rare cancer-germ-line-genes with a known function, codes for a synaptonemal complex protein involved in chromosome reduction during meiosis. Three other genes with tumor-specific expression, called *XAGE, HAGE* and *SAGE* have been identified in human sarcomas (161, 162). They are expressed in tumors of various histological types, including melanoma.

Besides demethylation leading to aberrant expression of cancer-germ line genes, other genetic mechanisms can lead to the expression of shared tumor antigens. It was found in melanoma that the *GnT-V* gene, coding for N-acetylglucosaminyltransferase V - an enzyme involved in protein glycosylation - and the *TRP-2* gene encoding a melanocytic protein, generate abnormal transcripts with retained intron sequences (163, 164). Translation of these abnormal transcripts generates tumor-specific peptides. Why such transcripts occur in melanoma but not in normal melanocytes is not known.

I. 2.2.2 Genes coding for differentiation antigens of the melanocytic lineage

So far, five nonmutated genes that are selectively expressed in melanocytes and melanoma were shown to code for antigenic peptides presented by HLA class I or class II molecules (117).

The first gene of this category codes for tyrosinase, a key enzyme involved in melanin synthesis (111). The first characterized epitopes were shown to be presented by HLA-A2 (112). So far, the *tyrosinase* gene has been found to encode 12 distinct epitopes, presented by HLA class I (A1, A24, B35 and B44) or class II molecules (DR4 and DR15) (165-168).

The *Melan-A/MART-1* gene codes for a 118-amino acid protein containing a transmembrane domain. CTL restricted by HLA-A2 recognize an immunodominant epitope, the nonapeptide AAGIGILTV (113, 114). Other peptides presented by HLA-A2, B35, B45 and DR4 are encoded by the same gene (169, 170).

The glycoprotein gp100 is a melanosomal matrix protein involved in the synthesis of melanin. It generates 22 distinct epitopes recognized by T lymphocytes restricted by HLA-A2, A3, A11, A24, A32, A68, B35, Cw8 and DR7 (118).

Two proteins with homology to tyrosinase, the tyrosine-related proteins 1 and 2 (TRP-1 and TRP-2) also contain several epitopes presented by HLA class I or class II molecules.

As opposed to the strictly specific tumor antigens such as MAGE, the use of differentiation antigens as melanoma vaccine can theoretically cause autoimmune destruction of normal melanocytes, leading to vitiligo. $CD8^+$ T cells recognizing melanoma differentiation antigens have been isolated from vitiligo lesions (171). It is interesting to note that the occurrence of vitiligo in melanoma patients has been associated with a better prognosis for the malignant disease (172-174). It should also be kept in mind that severe autoimmune disease has been observed in animal models when immunotherapy against tumor antigens shared with normal host cells was used (175, 176). Differentiation antigens are however widely used in clinical trials because of their high frequency of expression in cutaneous melanoma.

I. 2.2.3 Improving the immunogenicity of peptides used for vaccination

The half-life of the TCR-peptide-MHC complex depends on the quality and quantity of receptor ligand, which determine the balance between TCR triggering and degradation after the formation of the "immune synapse". Prolongation of signaling through the immune synapse could enhances the response to weakly immunogenic peptides. A correlation has been demonstrated between immunogenicity and peptide-binding affinity to class-I MHC molecules for epitopes from viral antigens (177). Binding motifs have been defined that correlate high peptide-binding affinity to the predominance of certain amino acids at particular positions in peptide sequences (178). For example, for peptides binding to HLA-A*0201, leucine (L) and methionine (M) are most frequently found at the second position from the N-amino acid terminus and Valine (V) is predominant at position 9. Thus, it seemed possible to enhance the immunogenicity of tumor antigenic peptides by introducing selective modifications at binding anchor positions, that increase HLA-A2*0201-binding affinity. Such modifications have been applied to peptides presented by HLA-A*0201 and encoded by *gp100, Melan-A* and *NY-ESO-1*, respectively (179-182). The structural modifications of the HLA-analog peptide complexes improve *in vitro* TCR affinity of specific CTL and elicit stronger T cell responses.

However, T cells activated by such altered analog peptides may not necessarily be tumor specific, i.e. they may or may not recognize a tumor cell line expressing the corresponding gene and adequately processing the native peptide (183-187). Whether CTL raised against analog peptides with increased

immunogenicity are tumor-specific or not is an important issue that should be addressed for each peptide analog for a rational optimization of superagonist peptides for clinical trials (188).

The following analog peptides with enhanced binding affinity to HLA-A2 are currently used in cancer vaccine clinical trials (187, 189, 190).

Parental (native) peptide		**Modified (analog) peptide**
gp100	I <u>T</u> D Q V P F S $V_{209-217}$	I **M** D Q V P F S V (gp100 209-2M)
Melan-A	E <u>A</u> A G I G I L T V_{26-35}	E **L** A G I G I L T V
NY-ESO-1	S L L M W I T Q <u>C</u>$_{157-165}$	S L L M W I T Q **V**

The molecular characterization of antigens recognized on human tumors by autologous T lymphocytes has opened the way for new vaccination strategies involving defined tumor antigens in order to stimulate the immune system against these antigens. Most importantly, it also makes possible to monitor the relevant specific immune responses.

Immunotherapy may be active, using antigens to stimulate an immune response *in vivo*, or passive, generating the appropriate effector cells *in vitro* and transferring them back into the patient. Both forms of immunotherapy can use undefined antigens, such as those that are present on tumor cells, or defined antigens, which can be administered under a wide variety of modalities. They will be discussed in Section IV.

In mice, the mode of immunization as well as the type of immunogen used were shown to play a crucial role to prime or to tolerize $CD8^+$ T cells *in vivo* (191, 192). Although those animal models provide valuable guidelines for the development of therapeutic vaccines in man, various parameters should be carefully re-explored in clinical trials as some of the observations made in animal models may not be reproducible in man.

We report here our clinical observations in melanoma patients who were immunized with *MAGE*-encoded peptides or with a recombinant protein encoded by gene *MAGE-A3*.

I. 3 OBJECTIVES OF OUR CLINICAL TRIALS

Our clinical trials involve therapeutic vaccinations, i. e. they include patients with a previously diagnosed cancer, most of them with a cutaneous melanoma. They have two main objectives.

First, to assess the safety and the effectiveness of various vaccination modalities.

Secondly, in selected patients, a detailed analysis of the T lymphocytes responses to the vaccine antigen(s) and of the tumor samples collected at different time points during vaccination was performed in order to improve our understanding of what happens in the patients who experienced regression of metastatic lesions and why such regression does not happen in the majority of patients.

Determining whether clinical and immunological responses are correlated is also an important issue for our understanding of the tumor rejection process. This knowledge can then be used to design new vaccination modalities.

This work mainly focuses on the clinical results, i.e. safety and tumor evolution, obtained by immunizing melanoma patients with tumor antigens recognized by T lymphocytes, using *MAGE*-encoded peptides or a recombinant protein encoded by gene *MAGE-A3*.