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PROGNOSTIC FACTORS IN MALIGNANT MELANOMA

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1. Abstract

Background: Malignant melanoma is one of the most malignant types of skin cancer. Incidences are on the rise worldwide and in the Czech Republic an increase of 5% in diagnosed cases is noted each year. Early detection and early surgical removal are associated with reduced mortality. The strong aggressiveness of this malignant disease is caused by its local invasive growth and tendency to metastasize early.

Aim of the study: The malignant melanoma is highly metabolically active tumor that releases a number of enzymes, cytokines, growth hormones and other molecules. The aim of this work was to determine the usability of preoperative and postoperative serum and plasma levels of biomarkers in primary diagnosis of tumor activity and in the postoperative follow-up care. These findings would be of clinical relevance for the patient's prognosis, modification of multimodal treatment and follow-up of patients with malignant melanoma.

Methods: We measured circulating levels of several biomarkers in a group of 77 patients with malignant melanoma and cohort of 34 patients without cancer as a control group. Using routine immunoassays and novel multiplex xMAP technology, we measured: thymidine kinase, tissue polypeptide specific antigen, protein S100A, osteoprotegerin, osteopontin , insulin-like growth factor 1 and 3, epidermal growth factor, interleukin -2, -6, -8, -10, vascular endothelial growth factor and basic fibroblast growth factor. Samples of peripheral blood were collected preoperatively (the day of surgery), 10 days after surgery and subsequently at 3-months intervals according to clinical examinations.

Results: We found statistically significant correlation of the concentration of the protein S100A serum with the tumor load, lymph node status and clinical prognostic information such as Breslow thickness, ulceration or tumor localization. Serum levels of tissue polypeptide specific antigen also correlated with tumor load and were increased in advanced melanoma compared to preoperative levels in primary melanoma. Differences in protein S100A and tissue polypeptide specific antigen profiles were determined between melanoma patients and healthy subjects. No other proliferative markers in our study reflected any association with studied variables. As for angiogenic factors reflected in the presented study, we found no relation between serum levels of vascular endothelial factor or basic fibroblast factor and studied parameters. Increasing osteopontin expression has been identified as a powerful predictor of sentinel lymph node involvement. Serum levels

were correlated with lymph node status and higher serum levels were observed in advanced melanoma compared to preoperative levels in primary melanoma. Differences in osteopontin and osteoprotegerin profiles were found to exist between melanoma patients and healthy subjects. Dynamic studies of serum levels of interleukins have shown that serum levels of interleukin-2 were correlated with sentinel lymph node positivity/negativity in preoperative levels and preoperative serum levels of interleukin-6 were correlated with Breslow thickness or tumor localization. Interleukin-8 has been found to be elevated in melanoma group compared to the healthy control group. Insulin-like growth factor reflected tumor load and was elevated in melanoma patients compared to healthy controls in our study. As for sensitivity and specificity of studied markers - the ROC curves did not highlight any acceptable concentration.

Conclusion: According to new and promising results in immunotherapy, we should aim our attention at increasing the accuracy of patient follow-up. Using biomarkers in primary diagnosis and then during follow-up, we can determine the biological activity of the tumor.

2. Souhrn

Úvod: Maligní melanom je jedním z nejzhoubnějších kožních nádorů. Na celém světě se neustále incidence tohoto nádoru zvyšuje, v České republice je diagnostikováno o 5% více případů každý rok. Zásadní pro léčbu melanomu je včasná diagnostika a včasné chirurgické odstranění tumoru. Silná agresivita tohoto maligního onemocnění je způsobena místním invazivním růstem a tendencí k časnému metastazování.

Cíl: Maligní melanom je vysoce metabolicky aktivní nádor, který produkuje celou řadu enzymů, cytokinů, růstových hormonů a jiných molekul. Cílem této práce bylo zjistit využitelnost předoperační a pooperační sérové a plazmatické hladiny biomarkerů v diagnostice primárního nádoru a v pooperační následné péči. Tato zjištění by měla klinický význam pro prognózu, úpravu multimodální léčby a následné sledování pacientů s maligním melanomem.

Metodika: V souboru 77 pacientů s maligním melanomem a 34 pacientů bez nádorového onemocnění jako kontrolní skupiny jsme měřili hladiny dále uvedených cirkulujících biomarkerů pomocí běžných imunologických metod a multiplexové analýzy: thymidinkináza, tkáňový polypeptidový specifický antigen, protein S100A, osteoprotegerin, osteopontin, inzulinu podobný růstový faktor 1 a 3, epidermální růstový faktor, interleukin -2, -6, -8, -10, vaskulární endoteliální růstový faktor. Vzorky periferní krve byly odebrány před operací (v den operace), 10 dní po operaci a následně každé 3 měsíce v rámci klinických kontrol.

Výsledky: Zjistili jsme statisticky významnou korelaci sérové koncentrace proteinu S100A s velikostí nádoru, stavem lymfatických uzlin a s klinickými prognostickými informacemi jako je tloušťka nádoru dle Breslowa, ulcerace nebo lokalizace nádoru. Sérové hladiny tkáňového polypeptidu specifického antigenu také korelovaly s velikostí nádoru a byly zvýšeny v pokročilém stadiu melanomu ve srovnání s předoperačními hladinami u primárního nádoru. Rozdíly hladin proteinu S100A a tkáňového polypeptidového specifického antigenu byly stanoveny mezi pacienty s melanomem a zdravými jedinci bez nádorového onemocnění (kontrolní skupina). Žádné další proliferační markery v naší studii neodráží spojitost se studovanými parametry. Co se týče faktorů angiogeneze, v prezentované studii jsme nezjistili žádný vztah sérových hladin cévního endotheliálního faktoru a studovanými parametry. Zvýšená exprese osteopontinu výraz byla shledána jako významný prediktor postižení sentinelové lymfatické uzliny. Sérové

hladiny osteopontinu byly korelovány se stavem lymfatických uzlin a vyšší hladiny v séru byly pozorovány u pokročilého melanomu ve srovnání s předoperačními hodnotami u primárního melanomu. Byly zjištěny rozdíly v hladinách osteopontinu a osteoprotegerinu mezi pacienty s melanomem a kontrolní skupinou. Dynamická studie sérových hladin interleukinů ukázala statisticky signifikantní korelace mezi předoperačními sérovými hladinami interleukinu-2 a pozitivitou/negativitou sentinelové uzliny. Předoperační sérové hladiny interleukinu-6 korelovaly s tloušťkou nádoru dle Breslowa a s lokalitou tumoru. Hladina interleukinu-8 byla zvýšena u melanomové skupiny ve srovnání s kontrolní skupinou. Dynamika hladin insulinu podobného růstového faktoru reflektovala velikost nádoru a byla zvýšena u pacientů s melanomem ve srovnání s kontrolní skupinou. Co se týče citlivosti a specificity markerů a ROC křivek nebyla prokázána žádná statisticky významná koncentrace.

Závěr: Na základě nových terapeutických možností bychom měli naši pozornost zaměřit na přesné sledování nemocných a včasné odhalení recidivy onemocnění. Sledování dynamiky biomarkerů může přispět ke zlepšení péče o nemocné s maligním melanomem a zároveň nám umožňuje lepší pochopení biologického chování nádoru.

3. Author's declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of Charles University and that it has not been submitted for any other academic award. I have identified all material in this dissertation which is not my own work through appropriate referencing and acknowledgement. Where I have quoted from the work of others, I have included the source in the bibliography.

I understand that one print copy of my dissertation will be deposited in the University Library for archival and preservation purposes.

In Plzen, 1.7.2013 MUDr. Inka Třešková

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5. Abbreviations

AJCC	The American Joint Committee on Cancer
AFP	Alpha-fetoprotein
APC	Adenomatous polyposis coli
AUC	Area under the curve
bFGF	Basic fibroblast growth factor
BRCA	Gene breast cancer
βHCG	Human chorionic gonadotropin
Ca	Calcium
CA 15-3	Carcinomic antigen 15-3
CA 125	Carcinomic antigen 125
CA 19-9	Carcinomic antigen 19-9
CEA	Carcinoembryonic antigen
CM	Cutaneous melanoma
CRP	C-reactive protein
CT	Computed tomography
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DFI	Disease-free interval
DNA	Deoxyribonucleic acid
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CDK4	Cyclin-dependent kinase
ECM	Extracellular matrix
ECLIA	Enhanced Chemiluminescence assay
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FDA	The Food and Drug Administration
HMB45	Human melanoma black antibody
ICAM	Intracellular adhesion molecule
IFN- α	Interferon alpha
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IgG	Immunoglobulin G
IL-2, -6, -8, -10	Interleukin-2, -6, -8, -10
IRMA	Immunoradiometric assay
LDH	Lactate dehydrogenase
LMM	Lentigo maligna melanoma
MC1R	Melanocortin 1 receptor
MIA	Melanoma inhibitory activity
MMP	Matrix metalloproteinase
MRI	Magnetic Resonance Imaging
MSLT-1	The first Multicenter Selective Lymphadenectomy Trial
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NSCLC	Non-small-cell lung carcinoma
OPG	Osteoprotegerin
OPN	Osteopontin
OS	Overall survival
p14ARF	Alternate reading frame (ARF) product of the CDKN2A locus
PET/CT	Positron emission tomography - computed tomography
PDGF	Platelet-derived growth factor

PSA	Prostate specific antigen
RANKL	Receptor activator of nuclear factor kappa-B ligand
REA	Electrochemical immunoanalysis
ROC	Receiver operating characteristic
sICAM-1	Soluble intracellular adhesion molecule 1
SLN	Sentinel lymph node
sVCAM	Soluble vascular adhesion molecule 1
TIMP	Tissue inhibitor of metalloproteinase
TILs	Tumor-infiltrating lymphocytes
TK	Thymidine kinase
TNFRSF11B	Tumor necrosis factor receptor superfamily member 11B
TPA	Tissue polypeptide antigen
TPS	Tissue polypeptide specific antigen
TRAIL	TNF-related apoptosis-inducing ligand
USA	United States of America
VEGF	Vascular endothelial growth factor

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7. Introduction

Malignant melanoma is as old as humanity itself. References to black cancer and fatal black tumors with metastasis date to the writings of the legendary Greek physician Hippocrates in the fifth century B.C. In Bohemia, professor Eiselt was the first to describe melanoma in literature (1) (2).

Melanoma is a cancer that develops in melanocytes, which arise from the neural crest and migrate to the epidermis, uvea, meninges, and ectodermal mucosa (3). Melanoma affects relatively young population and it has a tendency to metastasize at an early stage (4).

8. List of current knowledge

a) Epidemiology

Malignant melanoma currently represents a serious medical problem worldwide (especially in the Caucasian population) (1). Over the last decades, we observed the rapid increase of melanoma in the United States, Australia and Europe. Melanoma has come to be considered an epidemic cancer in these areas. Melanoma is responsible for 80% of deaths from skin malignancies despite the fact that it is accountable only for 4% of all dermatologic cancers (4). Melanoma incidences have continuously increased over the last 30 years. In contrast, melanoma mortality rates have not increased as dramatically as the incidence rate (5) (6).

The incidence rate of melanoma has been increasing by about 5 percent per year (7). The highest incidence rate is found in areas with intensive sun irradiation (Australia, New Zealand, etc.). In the Czech Republic, cutaneous melanoma is the sixth most common malignancy in men and the fifth in women. In 2008, melanoma was identified in 8,420 men and in 10,726 women.

The dramatic change in incidence and mortality in the Czech Republic is illustrated by the following: while in 1970 the crude incidence of CM was 3.1 in men and 3.2 per 100,000 inhabitants in women, the data from 2008 showed the incidence to be 19.6 in men and 16.0 per 100,000 inhabitants in women. Mortality in 1970 in men and women was 1.8 and 1.6 per 100 000 inhabitants respectively. However, by 2008 it had risen to 3.7 per 100,000 inhabitants in men and 3.0 per 100,000 inhabitants in women. It can be seen that over the past 40 years, the incidence of CM in the Czech Republic has risen by more than 600% (8).

b) **Etiology**

There are many factors influence melanoma development. Genotype, phenotype and environmental factors play their roles in this process (1).

Sun exposure

A major environmental risk factor for melanoma is ultraviolet radiation. Sun exposure is also the only factor that significantly influences the development of melanoma. The risk of developing melanoma increases the more time a person spends outdoor. Although childhood exposure to UV radiation plays an important role in the risk of developing melanoma, more facts suggest that overall sun exposure throughout patient´s lifetime is important for melanoma risk (4) (5). Ultraviolet radiation causes genetic changes in the skin, it impairs cutaneous immune function, increases the local production of growth factors, etc. Chronic or low-grade exposures to ultraviolet light induces protection against DNA damage, whereas intermittent intensive exposure causes genetic damage (6) (9).

A number of case–control studies have investigated the potential melanoma risk associated with sunbed use (10).

Genetic factors

No specific gene has so far been discovered as being responsible for melanoma (1). But as we know, melanoma usually appears in families. Recent research identified several genotypes which are indicative of the risk for melanoma development. As a result this cancer is mostly perceived as a genetic disease (11). A family history of melanoma approximately doubles the risk of developing melanoma. Familial melanoma is also heterogeneous. Two major susceptibility genes have been identified: CDKN2A and CDK4. CDKN2A codes for two different proteins, p16 (in the retinoblastoma pathway) and p14ARF (in the p53 apoptosis pathway). CDK4 is also presented in the retinoblastoma pathway. The prevalence of mutations varied by continent, with the mutation being least common in Australia (20%); somewhat common in North America (45%); and most frequently found in Europe (57%). The frequency of mutations in CDK4 is much lower. Within melanoma-prone families, MC1R variation increases the risk of melanoma in families without CDKN2*A* mutation and modifies the risk of melanoma associated with CDKN2A mutations. The gene MC1R encodes a protein involved in the production of

eumelanin, which is responsible for dark coloring, and phaeomelanin, which is responsible for red hair and freckles. Patients with red hair have a higher presents of three MC1R variants. These variants are known as red-hair variants, and it has been discussed whether these variants also appears more frequently in non-red-haired patients with sporadic melanoma (10). Although high risk susceptibility genes CDKN2A and CDK4 have been identified, they explain less than half the occurrences of familial melanoma (5). The most important mutation in sporadic melanoma affects BRAF, a member of RAF kinase family (4). Studies show significant genetic heterogeneity among melanomas (9).

Phenotype

The likely melanoma patient is a pale-skinned Caucasian with poor tanning ability, light eye and hair color, freckling (5). Atypical nevi are markers of moderately increased melanoma risk (9).

Female sex hormones

Some studies provide no evidence that prior pregnancy is a risk factor for melanoma. Similarly, according to other studies there is a lack of evidence that the usage of oral contraceptives or hormone replacement contributes to the risk of melanoma development. The prognosis is not altered by these factors in those already diagnosed with melanoma (10).

Socioeconomic status

The occurrence of melanoma in similar age groups is higher in those with a larger income. The reason for this is maybe that the higher income groups possess more resources and therefore are more likely to be exposed to ultraviolet radiation during their leisure time (10).

c) **Carcinogenesis**

Cancer is characterized by unregulated cell growth of autonomous nature with impaired regulatory mechanisms of cell proliferation, altered cell differentiation and inhibition of apoptosis.

Currently, the most common theory of oncogenesis is the theory of genetic mutations that lead to an imbalance between cell proliferation and death; that means defining cancer as a genetic disease. Cancer is a multi-stage process where an accumulation of gene mutations controlling cell proliferation, differentiation and cell death occur. Yet there are also equally important alternative theories that explain the process of oncogenesis as epigenetic alterations (heritable and reversible changes) or chromosomal abnormalities, while other theories seek to explain cancer as a metabolic disease (disorder of the cellular metabolism).

Tumor cells undergo the natural selection process (theory of clonal evolution). Cells with new genetic changes have a greater chance of surviving and they begin to multiply and soon take over; becoming dominant in a growing tumor. Tumor cells have some specific properties: self-sufficiency of growth signals, uncontrolled growth, loss of sensitivity to different signals, loss of apoptosis, immortality, support of angiogenesis, ability to invade surrounding tissues, establishment of metastases at distant sites, genomic instability and loss of ability to repair genetic errors. The course of carcinogenesis is divided into several stages. The initiation stage is a mutation in a critical gene which is an irreversible process when the cell acquires the potential of malignant transformation. At this stage the process may stop. The promotion stage, when the cells are stimulated to intensive proliferation, takes years or decades. Removing promotional factors may stop the process. The progression stage is characterized by uncontrolled cell growth, the alteration of critical points in the cell cycle and deregulation of DNA-transcription factors. Cancer is considered a genetic disease according to the most wide spread theory. The exposure to various mutagens results in damage to genes regulating cell growth. The oncogenesis starts if this damage is not corrected. Pro-oncogenes are genes that are an integral part of the genome in terms of encoding proteins that control cell proliferation, differentiation and survival. Mutations in pro-oncogenes give rise to modification of their function, increase in the amount or activity of the protein product. Pro-oncogenes become oncogenes. One of the first oncogenes that have been identified is the Ras oncogene. Mutation in the Ras

family of pro-oncogenes was reported in 30% of human tumors. The induction of oncogenes transcription by transcription factors launches malignant cell transformation. Onco-proteins are very similar (sometimes identical) to the proteins encoded by pro-oncogenes of normal cells, whose job is to manage and control the growth, proliferation, differentiation and cell death. They are classified into five classes: (a) growth factors - for example sis-oncogene, (b) growth factor receptors - such as her-2 oncogene, (c) intracellular signal transducers – e.g. ras oncogenes, and (d) nuclear transcription factors, controlling the cell cycle proteins - such as p53 or Rb. In addition, mutations can occur even in anti-oncogenes (tumor-suppressor genes). Proteins that are encoded by anti-oncogenes have an anti-proliferative effect, promote differentiation and apoptosis. One of the most important tumor suppressor genes is a p53 protein. It is a transcription factor activated by a cellular hypoxia or UV radiation, another function it has is to regulate the cell cycle, division and apoptosis. At least half of all cancers are associated with alteration of this protein. Other important tumor-suppressor genes are APC gene (adematous polyposis coli), BRCA1 and 2 gene (familial breast and ovarian cancer), CDNK2A gene (inhibitor of cyclin-dependent kinases 2A associated with malignant melanoma). Other important genes are genes for maintaining genome stability. The products of these genes are used in the correction mechanisms of damaged DNA. Their recessive mutation causes a disease called xeroderma pigmentosum and Cockayen syndrome, which are precancerous changes that increase skin susceptibility to carcinomas.

Metastasis is a complex process, which is based on the complex interactions between tumor cells, extracellular matrix and target tissue. Adhesive properties of tumor cells play a key role in this process. Metastatic cascade consists of four steps - the invasion of tumor cells into the environment, the transportation of tumor cells by lymph or blood, extravasation and nidation, the growth of metastases in the new environment. Each of these steps requires deregulation of a number of processes and the cooperation of tumor cells with the surrounding microenvironment. These processes include angiogenesis, changes in the composition of the ECM and its degradation, or changes in the repertoire of cell adhesion molecules. The ability of tumor cells to induce and sustain angiogenesis is an essential event in the process of carcinogenesis. Angiogenesis is regulated by a balance between pro-angiogenic and anti-angiogenic factors acting on the surface of endothelial cells. This balance is broken in cancer and there is an angiogenic switch presented. VEGF gene expression and PDGF, IGF2, EGF gene expression is important for the process of

angiogenesis.

Extracellular matrix does not only provide structural support for tissues, but it is also a very dynamic structure that plays an important role in normal tissue development through interaction with adhesion molecules on the cell surface. The first step is the separation of individual cells from the tumor tissue, due to loss of cellular interactions. Cadherins, and especially epithelial E-cadherins, play a crucial role in maintaining cell adhesion. Loss of E-cadherins expression has been described in connection with a variety of tumors. Loss of E-cadherins expression is accompanied by the obtained expression of N-cadherins. N-cadherins expression enhances the invasiveness, motility and migration of tumor cells, thereby positively influences tumor metastasis. The cytoplasmic domain is created through the catenin association with intracellular structures. These catenins interact with growth receptors. Loss of normal function and cadherin and catenin complex is associated with the development of an invasive phenotype. Invasion of tumor cells from the primary tumor into the submucosa and vessels depends on whether the tumor is able to activate proteolytic enzymes that dissolve the basement membrane and connective tissue. Increased activity of matrix metalloproteinases increases the chances of tumor cells to penetrate through the basement membrane. Metalloproteinases are normally regulated by specific inhibitors, such as TIMP (tissue inhibitor of metalloproteinases). Loss of expression of the inhibitor TIMP reinforces aggressive potential of the cells. After a successful invasion to the mucosa, cancer cells penetrate into lymphatic and blood vessels. Cancer cells bind on endothelial cells via adhesion molecules, such as selectins or integrins, and penetrate the target tissue. The integrins interact with ICAM-1 and ICAM-2 (intercellular adhesion molecule) on the cell membrane of endothelial cells. Tumor cells leave the vascular system by binding to the basement membrane and dissolving it. They penetrate into subendothelial tissue and form colonies of micrometastases along blood vessels. If there is a balance in proliferation and apoptosis, colonies of micrometastases do not increase. This is called a dormant metastasis (tumor dormancy). Factors derived from tumor cells or factors that are formed in the tissue surrounding the tumor are important for macrometastasis development (12).

d) **Melanoma subtypes**

Melanoma has two phases of growth, namely radial and vertical. During the radial growth phase, malignant cells grow in a radial manner in the epidermis. With time, most melanomas progress to the vertical growth phase, in which the malignant cells invade the dermis and develop the ability to metastasize (4).

Lentigo maligna melanoma = melanosis praecancerosa Dubreuilh

LMM arises from a lentigo maligna precursor lesion, especially in elderly, it is typically found on sun-exposed areas. It has an initial flat phase that can evolve into a nodular phase with a capacity to grow invasively and metastasize. Standard excision of LMM with 5 mm margins is insufficient in 50% of cases. The recurrence rate with standard excision ranges up to 20% (1) (13).

Superficial spreading melanoma

It is the most common subtype. Its characteristics is an initial flat phase that displays changes in size, shape or color. Mostly it arises in previously normal skin. Sometimes this disease evolves from a precursor lesion, usually a dysplastic nevus. A prolonged radial growth phase, where the lesion remains thin, may eventually be followed by a vertical growth phase where the lesion becomes thick and nodular (3) (14).

Nodular melanoma

It is the most aggressive form of melanoma, characterized by rapid progression and early metastasis. It often carries a poor prognosis. There is a lack of horizontal phase; from the very beginning it is symmetrical, firm, often uniformly colored and frequently non-pigmented nodule. Clinical and dermatoscopic diagnosis is difficult (1) (14) (15).

Acral lentiginous melanoma

It occurs on the acral skin of the palms and soles and subungual parts. Unlike other forms of melanoma, acral lentiginous melanoma does not appear to be linked to sun exposure. It is the most common form of melanoma diagnosed amongst Asian and Black ethnic groups. There is typically some delay in diagnosis as trophic ulceration, hyperkeratosis or subungual hematoma can imitate this type of melanoma (1) (16).

Desmoplastic melanoma

It is a rare subtype of melanoma that imitates a scar-like tissue reaction and is frequently associated with neurotropism. Often it presents as non-pigmented papule, it is associated with higher rates of local recurrence. Diagnosis is very difficult (16).

Mucosal melanoma

It is a very rare subtype of melanoma that can affect respiratory, gastrointestinal and urogenital mucosa. Always we have to exclude the metastatic origin of the lesion. It is very difficult to distinguish primary melanoma from metastasis (1).

Melanoma subtypes and incidence are summarized in Table 1.

Table 1 - Melanoma subtypes and incidence (Nestle et al. 2012)

Melanoma subtype	**% of all melanomas**
Superficial spreading melanoma	60-70%
Nodular melanoma	15-30%
Lentigo maligna melanoma	5-15%
Acral lentiginous melanoma	5-15%

e) **Diagnostics**

Skin disorders are easily recognized by simple inspection. These could lead to early detection of skin tumors. But reality is still different. There is no screening program for skin tumors so far. The precise diagnosis and early detection of melanoma significantly improves 5-year survival rates (17) (18).

The ABCD mnemonic (and later ABCDE), introduced in 1985, represents an analytical method for the evaluation of melanoma. However, the ABCDE method did not help to distinguish some dysplastic nevi from melanoma and failed to identify some melanomas at an early stage (4) (17).

- A (asymmetry) – lesion with asymmetric shape.
- B (border) – lesion with asymmetric borders.
- C (color) – lesion with dark color, often with variation in pigmentation.
- D (diameter) – greater than 6mm.
- E (evolving) – evolving over time – the change in size, borders, shape, color, surface, subjective feeling.

In clinical examination we use dermatoscopy or digital dermatoscopy, photography, etc. Dermatoscopy is a non-invasive technique which makes use of a hand-held magnifying device. These methods are not diagnostic but they significantly improve diagnostic accuracy in the hands of an experienced investigator compared to naked eye examination. The sensitivity of dermatoscopy is approximately 20% higher than when examining the skin by eye. The exact diagnosis is made by lesion biopsy (14) (19).

f) **Prognosis**

Understanding the correlations between the prognostic factors and biology of the disease is a major objective of melanoma research (20).

Age

Older patients present more frequently with thicker and ulcerated melanomas and many studies have reported age to be an independent prognostic factor. Patients greater than 65 years of age have shorter disease free interval and overall survival rates (21).

Sex

Many studies report that women have a better prognosis compared to men. Melanoma risk is not associated with age at menarche, menopausal status, use of hormone replacement therapy, parity, age at first birth, or oral contraceptives use (21).

Anatomical site

Tumors with axial localization have poorer prognosis than those on extremities (22).

Breslow thickness

In 1970 at George Washington University, pathologist Alexander Breslow, M.D. was the first one to report on the depth of invasion as a prognostic factor (4). Currently, Breslow's depth is included in the AJCC staging guidelines for melanoma as a major prognostic factor in case that sentinel lymph node is not performed. Tumor depth is measured in millimeters from the granular layer of the epidermis to the deepest tumor cell (21). The AJCC staging system uses tumor thickness cut points of 1.0, 2.0, 4.0mm to define T-category. As primary tumor thickness increases, there is a significant decrease in survival (Table 2) (23).

Table 2 - Tumor thickness and lymph node involvement corresponding to 5-year survival (Balch et al. 2001)

Stage	**5-year survival (%)**	**Clinical staging**		
		T	**N**	**M**
0		Tis	N0	M0
IA	95	T1a	N0	M0
IB	90	T1b, T2a	N0	M0
IIA	78	T2b, T3a	N0	M0
IIB	65	T3b,T4a	N0	M0
IIC	45	T4b	N0	M0
III		Any T	N1, N2, N3	M0
IIIA	66			
IIIB	52			
IIIC	26			
IV	7.5-11	Any T	Any N	Any M1

Clark level

Clark level describes the depth of invasion. For years, Clark level of invasion has been known to have prognostic significance, and has served as a criterion in several melanoma staging systems. However, it has been shown that the Clark level has a lower predictive value, is less reproducible, and is more subjective in comparison with Breslow's depth. According to the 7th edition of AJCC melanoma staging system, it was replaced by mitotic rate and is only to be used to define T1b tumors in the rare occurrence that mitotic rate cannot be determined (23).

Ulceration

Ulceration was defined by Balch et al. as the absence of an intact epidermis overlying a significant portion of the primary tumor. This factor corresponds with tumor thickness (21). Multiple studies demonstrate that the presence of ulceration represents a more aggressive tumor phenotype with a higher tendency of metastasis and worse prognosis. For patients with ulcerated melanomas, survival is significantly lower than for patients with nonulcerated tumors of the same depth. Moreover, several studies demonstrated that survival outcomes for patients with ulcerated tumors were remarkably similar to those of patients with nonulcerated tumors of the next highest T category (23).

Regression

A regressing melanoma is reacting to the body's immune system by shrinking in size. This points to the fact that melanoma is an immunogenic tumor (9). Regression is considered as an adverse negative factor (21).

Mitotic rate

The mitotic rate is measured as the number of mitoses per square millimeter. Primary tumor mitotic rate represents a fundamental change in the revised melanoma staging system. Salman and Rodgers first suggested the prognostic importance of the mitotic index of the primary tumor, identifying that it was associated with a higher rate of metastasis in patients with thin lesions. It is the second most important predictor of survival, after tumor thickness, and is mainly used among patients with T1 melanoma. The 10-year survival rate is 95% for nonulcerated T1 melanomas with a mitotic rate of $< 1/mm^2$, and drops to 88% if the mitotic rate is $\geq 1/mm^2$. Determining mitotic rate is important not only in providing prognostic information, but also in discussing and planning the extent of surgery (23).

Microsatellites

Microsatellites are small tumor nests that are separated from the main body of the tumor. Several studies have proved the role of microsatellites as a prognostic factor in cutaneous melanoma (21).

Tumor-infiltrating lymphocytes

Tumor-infiltrating lymphocytes (TILs) are white blood cells that have left the bloodstream and migrated into a tumor. In melanoma, they are responsible for tumor killing and may induce spontaneous regression. Brisk TILs in the melanoma vertical growth phase is a strong, but not independent, prognostic factor associated with superior survival (24).

Lymph node involvement

The status of the sentinel lymph node is the most important prognostic factor for recurrence and survival (14). Melanoma progresses to the regional lymph nodes in 70% of patients (4).

g) **2010 AJCC staging**

Formal staging of cancer is fundamental in providing clinicians with prognostic information, developing treatment strategies, and directing and analyzing clinical trials (23).

Staging systems for melanoma continue to be refined as our understanding of the complex biology of this disease improves. According to Dickson et al., fundamental changes to the new staging system are as follows: a) localized melanoma, tumor thickness, mitotic index and ulceration are considered the most powerful prognostic parameters for patients, b) Clark level of invasion was replaced by mitotic index for patients with thin melanomas, c) all patients with microscopic melanoma metastases are classified as stage III, d) for patients with regional lymph node metastases, the number of lymph nodes involved, metastatic tumor burden and ulceration and thickness of the primary tumor were the most predictive independent factors of survival, e) for patients with distant metastases, the site and serum lactate dehydrogenase elevation defined the M category (23).

Stage I and II

The prognosis of patients with localized melanoma is generally favorable. Tumor thickness, mitotic rate and the presence of ulceration were each found to be significant independent predictors of survival in this group of patients. These three factors are used to define T categories (23).

The first Multicenter Selective Lymphadenectomy Trial (MSLT-1) confirmed the prognostic importance of sentinel lymph node status as the statistically strongest predictor of survival in patients with stage I and II melanoma (25).

Stage III

Patients with regional metastasis represent a heterogeneous group with regard to staging and prognosis. The regional lymph nodes are the most common first site of metastasis in melanoma patients. The number of nodes harboring metastatic disease is the most important predictor of survival (23). Patients with one, two to three or four or more affected lymph nodes are classified as having N1, N2 and N3 disease, respectively. In

addition, microscopic versus macroscopic lymph node involvement is further subdivided as a versus b category (26).

Stage IV

The prognosis for patients with distant metastases is generally poor, with 5-year survival rates comprising of less than 10%. Patients with an elevated serum LDH were assigned to the M1c category, regardless of site of distant metastasis. Patients with metastasis to distant skin, subcutaneous tissues, and/or lymph node basins (M1a), have the highest one-year survival rate (62%) among patients with stage IV disease. Patients with pulmonary metastasis (M1b) have an intermediate prognosis (their one-year survival rate stands at 53%). Finally, patients with non-pulmonary visceral metastases and/or an elevated serum LDH (M1c) have the worst one-year survival among stage IV patients (33%) (23).

Table 3 - Melanoma TNM classification (Nestle et al. 2012)

T classification	Thickness	Ulceration status
T1	≤1.0 mm	a: without ulceration and level II/III b: with ulceration or level IV/V
T2	1.01-2.0 mm	a: without ulceration b: with ulceration
T3	2.01-4 mm	a: without ulceration b: with ulceration
T4	>4.01 mm	a: without ulceration b: with ulceration
N classification	Number of metastatic nodes	Nodal metastatic mass
N1	1 node	a: micrometastasis b: macrometastasis
N2	2-3 nodes	a: micrometastasis b: macrometastasis c: in-transit met/satellite without metastatic node
N3	4 or more metastatic nodes, matted nodes, or in-transit met/satellite with metastatic node	
M classification	Site	Serum lactate dehydrogenase
M1a	Distant skin, subcutaneous or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases Any distant metastasis	Normal Elevated

h) **Sentinel lymph node**

Sentinel lymph node biopsy is a minimally invasive staging method performed at the same time as wide excision to identify the first (sentinel) melanoma-draining lymph node. The technique is applied to patients with moderate to high risk of nodal metastasis. Occult nodal metastasis in patients are identified by sentinel lymph node biopsy. Even patients poorer outcome could benefit from a completion of nodal dissection and evaluation for systemic adjuvant therapies (27) (28).

Although it has been adopted as a standard diagnostic technique there are many questions to be answered. One of the problems is whether to perform sentinel node biopsy in thin melanomas or not. The majority of newly detected melanomas have Breslow index of $\leq$ 1mm and a small but definite number of these patients relapse with recurrent disease (29). For this small number of patients with occult nodal metastasis, correct identification provides critical prognostic information that can only be obtained with sentinel lymph node biopsy. Patients with thin melanoma and negative sentinel lymph node can be assured that their risk of recurrence is extremely low (28). Nevertheless, some authors are against performing sentinel lymph node biopsy because of unpredictability of metastasis in melanoma. Tumor cells can quickly bypass the lymphatic system to the blood and remain undetected by this procedure. Negative sentinel lymph does not mean the lack of distant metastasis (30).

In 2006, the results of the first Multicenter Selective Lymphadenectomy trial (MSLT-I) were published. The 5-year disease free interval was higher in SLN group (78.3%) compared with the observation group (73.1%) (29). Data also indicated that immediate complete lymph-node dissection for sentinel node metastases improves disease free interval (27). The important question remaining is whether there is any benefit in the completion of a nodal dissection in case of positive sentinel node. While current data demonstrates the benefit of early removal of micrometastases, the majority of these patients will not have pathologically detected melanoma in the completion dissection specimen (31). Overall survival is comparable for those who undergo sentinel node biopsy followed by immediate completion lymphadenectomy if the sentinel node is positive, compared with those who undergo observation followed by lymphadenectomy only after presenting with clinically palpable recurrence. A large multicenter randomized trial is

currently underway to evaluate whether a completion lymphadenectomy should be performed in those with positive sentinel lymph node (MSLT-II) (29).

Although there are many controversies surrounding this topic, sentinel lymph node biopsy is an accurate, minimally invasive staging procedure and detection of the melanoma metastases in sentinel lymph node is the most important prognostic factor. We need further research for developing a more reliable system to identify patients that could benefit from sentinel lymph node biopsy the most (28).

i) **Therapy**

Surgery

Surgery is the main modality in treatment of primary melanoma. Early diagnosis combined with surgical therapy is currently the only curative treatment (32). Optimal surgical margins depend on the thickness of the primary melanoma lesion. According to large trials there is no improvement in rates of local recurrence, disease-free survival, or overall survival in patients with surgical margins greater than 3cm (33). A World Health Organization randomized trial indicated that 1 cm excisional margins are safe for melanoma with a Breslow depth <1 mm. A controversy persists about the effectiveness of 1 cm margins for melanoma 1 to 2 mm deep. Table 4 shows guidelines for surgical treatment according to American Academy of Dermatology. Table 5 shows guidelines of the German Dermatological Society. And table 6 shows Clinical practice guidelines in oncology from National Comprehensive Cancer Network (2013). A randomized trial for intermediate-thickness melanoma (1-4 mm deep) demonstrated that 2 cm margins were as effective as 4 cm margins. There is no randomized trial that shows the optimal margins for melanoma >4 mm or in situ melanoma (4) (9). In our department we follow guidelines mainly from National Comprehensive Cancer Network.

Table 4 - Surgical treatment, American Academy of Dermatology

Breslow thickness	**Excision margins (cm)**
In situ	0.5
< 1 mm	1.0
1-4 mm	2.0
>4 mm	2.0-3.0

Table 5 - Surgical treatment, German Dermatological Society

Breslow thickness	**Excision margins (cm)**
In situ	0.5
<2 mm	1.0
>2 mm	2.0

Table 6 - Surgical treatment, National Comprehensive Cancer Network

Breslow thickness	**Excision margins (cm)**
In situ	0.5
≤1.0 mm	1.0
1.01-2 mm	1-2
2.01-4 mm	2.0
>4 mm	2.0

Evidence-based data is lacking regarding the recommended depth of resection, but it is thought that deep fascia serves as a barrier to lymphatic but not local recurrence, so it is mainly left intact (34).

Surgery is also the treatment of choice for single or few local or regional metastases (35). Most local recurrences occur in first two years after diagnosis and are associated with poor overall survival rate. Local recurrence or in-transit metastases develop from residual intralymphatic disease. Surgical resection can result in prolonged survival; however, local recurrence is sometimes associated with distant systemic disease (34).

The surgical treatment of metastatic melanoma is performed in patients with single distant metastases, but only 5% with all melanoma patients with distant metastases are cured (36). Patients that are considered for complete resection of metastatic melanoma undergo a preoperative evaluation that includes a whole-body PET/CT (37). Good candidates for surgery are patients with single metastases of the lung, brain, bowel, spinal cord or liver (32). Surgery can also be used as a palliative option for carefully selected patients with symptomatic metastases (37).

Radiotherapy

Locally invasive melanomas bring risks of local and distant relapse. Regional control of the disease is very important for the quality of life of these patients. Additional treatments are therefore needed to improve the patient outcome for melanomas with a high risk of locoregional or distant recurrence (38).

The role of radiation therapy as primary or adjuvant treatment for melanoma is controversial (39). Melanoma is considered a relatively radioresistant tumor. But some clinical experience doesn´t support this point of view. Early studies demonstrated that the response rate depended on the size of the dose per fraction (40). Unfortunately, there are few randomized trials specific to melanoma to guide appropriate palliative radiotherapy (37).

Radiotherapy is not a primary treatment for invasive melanoma, but in mucosal melanomas postoperative radiotherapy appears more effective than surgery alone.

Few studies demonstrated the benefit of radiotherapy in preventing local recurrence in metastatic lymph nodes after lymphadenectomy (38). Although available data is still scarce, American and Australian guidelines recommend postoperative radiotherapy in patients with stage III at high risk of relapse (32). In contrast Nestle et al. (9) does not support the administration of adjuvant radiotherapy after resection of regional lymph node metastases.

Radiotherapy is also used for palliative purpose, most often in bone metastases. From the limited data available there is no conclusion about the effectiveness of radiotherapy for bone metastases. In patients with single brain metastases, operative resection or stereotactic single radiotherapy can be used. The local control rate was improved by applying the whole brain radiotherapy. The median survival of symptomatic patients with multiple brain lesions is only 2 months and can be extended to 4-6 months after whole-brain radiation therapy (32) (36).

Adjuvant therapy

Malignant melanoma is one of the solid malignancies most refractory to therapy. Early diagnosis and surgical removal of the primary tumor is the only curative approach currently available (41). Melanoma is an immunogenic cancer and therapeutic effect could be achieved by using immunotherapy (9). Interferon alpha is the major drug that has been

considered for adjuvant therapy and is used in various schedules in Europe, in stage II and III. High-dose interferon alpha has been considered the standard of care in USA (42). In the Czech Republic we use interferon alpha as a standard adjuvant therapy for high-risk melanoma patients (schedule of 12 months). There are some groups of oncologists that would not recommend IFN- α as standard therapy because the benefits in overall survival are relatively small, and the side effects cannot be justified in relation to these toxicities (bone marrow suppression, hepatotoxicity, fatigue, flu-like symptoms, neuropsychiatric disturbances) (33) (43). There have been several clinical trials concerning the use of IFN-α. IFN-α has shown an effect on disease free interval, however, without a clinically significant effect on overall survival (42). The identification of makers that could predict a host antitumor immune response is very important for selection of patients who would benefit the most from IFN-α therapy (42) (43).

j) **Metastatic melanoma**

Melanoma is highly curable in the early stages but the mortality is high for patients with advanced disease because of an absence of effective treatment options (44). The immunogenicity of malignant melanoma tumor cells is important. Spontaneous complete remission can be observed in patients with malignant melanoma. The interaction of the immune system with the tumor shows a promising pathway for intervention (45).

Chemotherapy

Several cytotoxic chemotherapy agents have been shown to yield tumor responses or prolonged stabilization of disease, but none have been proven to improve overall survival. Their main benefit is palliative.

Dacarbazine is the only cytotoxic agent approved by the FDA for the treatment of metastatic melanoma despite its modest efficacy (objective responses rates ranging from 5.5 to 20%, sustained objective responses have been described in 1-2% of patients). Combinations of cytotoxic agents may yield higher response rates than dacarbazine monotherapy, but is linked with them and they do not extend survival significantly (33) (46).

Immunotherapy

A number of immunotherapy trials were conducted in recent years (47). The immunogenicity of malignant melanoma tumor cells is important. Spontaneous complete remission can be observed in patients with malignant melanoma. The interaction of the immune system with tumor shows a promising pathway for intervention (45).

Nonspecific stimulation of antitumor immune responses - IFN-α, Interleukin 2.

IFN-α as discussed above, prolongs diseases-free survival, but trials failed to prove significant effect on overall survival (48). IL-2 is a potent immune modulator that stimulates activation and proliferation of T-lymphocytes. IL-2 in a high dose regiment which provides a low overall response rate and it is the only FDA-approved immunotherapeutic agent for treatment of patients with metastatic melanoma (33) (48).

Anti-cytotoxic T lymphocyte-associated antigen 4.

CTLA-4 is a T-cell surface receptor that works as an immune system checkpoint to regulate immune response. The blockade of CTLA-4 releases immune system inhibition, allowing the ability to recognize cancer cells as foreign (44). In 2011 the FDA approved ipilimumab for treatment of malignant melanoma (49). Ipilimumab is an IgG1 monoclonal antibody directed at CTLA-4. Ipilimumab is the first drug to ever show an improvement in overall survival in patients with advanced melanoma (44) (47). Treatment with anti-CTLA-4 is frequently associated with adverse immune events, most commonly involving the skin (rash, vitiligo, pruritus) and gastrointestinal tract (colitis, diarrhea) (48). Some studies proved that combining chemotherapy and anti-CTLA-4 therapy is effective for patients with advanced melanoma (44).

Active immunization (vaccines).

Development of vaccine that would show significant clinical benefit in melanoma has not been successful (50).

Novel investigational therapies.

Adoptive cell therapy, targeted pathway inhibition, etc. Trials are currently ongoing. Identification of the $BRAF^{V600}$ mutations that are found in approximately half of all melanomas is key to optimizing treatment decisions and outcomes in melanoma. A potent inhibitor of oncogenic BRAF kinase is called vemurafenib. In patients with metastatic melanoma positive for $BRAF^{V600}$ mutations, vemurafenib delivers significant improvements in response rates, progression free survival and overall survival (51).

9. Tumor markers

a) Introduction

Tumor marker is a substance, a molecule or a process that is altered qualitatively or quantitatively in cancerous conditions, and whose alteration is detectable in the specimen (tissue, blood, saliva, urine, etc.) by an assay to identify the presence of cancer. It is used to assess patient prognosis, or to monitor a patient´s response to therapy with the overall goal of improving the clinical management of the patient. It is produced by tumor itself or by a surrounding tissue as a response to the tumor (52) (53).

Tumor markers make new approaches in follow up oncological diseases and optimization and monitoring of the treatment. We try to use them as tools for differentiation benign from malignant condition, determination of the stage of disease and for early detection of tumor relapse. Use of tumor markers in routine practice depends on the type of tumor and mainly on the disease stage.

Tumor markers can be classified in several ways, the most common classification combines their biochemical properties, tissue of origin, and functionality. According to the classification based on biochemical properties we distinguish:

Oncofetal proteins

Oncofetal proteins are antigens that are normally produced during the embryonic development. In adults, their production is limited or completely absent. Elevated concentration in adults is the result of the reactivation of certain genes that control cellular growth and are directly connected to malignant process.

The typical representative of this group is carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP).

Tumor-associated antigens

This is a heterogeneous group of markers compromised of various membrane structures of tumor cells. The markers of this group are more specific for the type of malignancy than the others and their serum concentrations reflect the growth or regression of the tumor mass more accurately. Carcinomic antigen 15-3 (CA 15-3), carcinomic antigen 125 (CA

125), carcinomic antigen 19-9 (CA 19-9), prostate specific antigen (PSA) belong into this group of tumor markers.

Enzymes

We can use some enzymes as tumor markers, e.g. prostatic acid phosphatase, alkaline phosphatase, neuron specific enolase, lactic dehydrogenase, thymidine kinase.

Hormones

Malignant formation can alter the synthesis and secretion of various hormones. This alteration can be the indicator of a malignant process and can be monitored as tumor marker. This group comprises hormones of malignant endocrine tumors, such as parathyroid hormone, insulin, prolactin, catecholamines. But also hormones with ectopic production, such as calcitonin or parathyroid hormone in breast cancer, etc.
From all the hormones, human chorionic gonadotropin (βHCG) is one of the most applicable tumor markers.

Special serum proteins

This group comprises e.g. ferritin, thyroglobulin, beta-2-microglobulin, S-100 protein.

Miscellaneous markers

A heterogeneous group of substances which are not specific for the type of tumor but generally indicate the presence of a malignant process. The group comprises polyamines, nucleosides and tissue polypeptide antigen (TPA) (54).

The diagnostic efficiency of tumor marker examination depends on variety of factors such as sensitivity, specificity, positive predictive value, and negative predictive value. The specificity can be determined as the percentage of healthy people who are correctly identified as not having the condition, and sensitivity as the percentage of sick people who are correctly identified as having the condition. The positive predictive value describes the probability that the disease is actually present if the test result is positive. The negative

predictive value describes the probability that the disease is not actually present if the test result is negative (55) (56).

An ideal tumor marker theoretically should have the following criteria (Malati et al.):

1. It should be highly sensitive and should have low false negatives.
2. It should be highly specific and should have low false positive.
3. It should have high positive and negative predictive value.
4. 100% accuracy in differentiating between healthy individuals and tumor patients.
5. It should be able to differentiate between neoplastic and non-neoplastic disease and show positive correlation with tumor volume and extent.
6. It should predict early recurrence and have prognostic value.
7. It should be clinically sensitive i.e. detectable at early stage of tumor.
8. Its levels should be preceding the neoplastic process, so that it should be useful for screening early cancer.
9. It should be either a universal marker for all types of malignancies or specific to one type of malignancy.
10. It should be easily assayable and be able to indicate all changes in cancer patients receiving treatment.

There is no ideal tumor marker reported to date as having these ideal characteristics (56). The determination of tumor markers is helpful in many processes: early tumor detection, differentiating benign from malignant conditions, evaluation the extent of the disease, monitoring the response to therapy, predicting or detecting the recurrence of the tumor. They are only exceptionally used in screening (prostate specific antigen), since no tumor marker with adequate sensitivity and specificity currently exist. Some tumor markers are more appropriate for the follow-up and the others for the early detection of the disease recurrence (54).

b) **Tumor markers in malignant melanoma**

Tumor markers are supposed to be a key to successful diagnosis and follow-up patients with malignant disease. Many serum markers have been evaluated in melanoma but their clinical significance remains a matter of debate. Many molecules which are involved in oncogenesis and cancer spread can be found in the serum of cancer patients, but there sensitivity and specificity is questionable. The heterogeneity of studies complicates the validation of tumor markers for malignant melanoma. Numerous potential biomarkers have been studied, but with controversial results (57) (58).

S 100
MIA (melanoma inhibitory activity)
Tyrosinase
Proangiogenic factors (VEGF, bFGF, IL-8)
Molecules involved in cell adhesion and motility
– sICAM-1 (soluble intracellular adhesion molecule 1)
- sVCAM (soluble vascular adhesion molecule 1)
- matrix metalloproteinases (MMP 1-9)
- tissue inhibitor of metalloproteinases (TIMP-1 and 2)
Cytokines and cytokine receptors (IL-6,10, sIL-2R)
Others – CRP, integrins, etc.

At the present moment, no ideal biomarker exists in the field of melanoma. Serum LDH (lactate dehydrogenase) is the only molecular marker that has been included into current melanoma staging and classification system of the American Joint Committee on Cancer. So far biomarkers specific for melanoma have not routinely been used (57) (59).

10. Tumor markers in our study

a) Protein S100A

The S100 protein family consists of twenty members (Ca2+ binding proteins). These were isolated from bovine brain by B.W.Moore in 1965 and named S100 because of their solubility in 100% saturated ammonium sulphate. S100 proteins have been implicated in many intracellular and extracellular functions such as cell growth and differentiation, cell cycle progression, transcription, inflammatory response, etc. There are diseases associated with altered expression levels of S100 proteins – neurologic disorders (traumatic brain injuries, asphyxia in newborns, chronic neurodegenerative disorders, and acute stroke), neoplastic disorders (malignant melanoma, lung cancer, gastric cancer, lymphoma, renal tumors, thyroid carcinoma, and breast carcinoma), cardiac diseases, inflammatory diseases (60).

S100 proteins are found in tissues of neuroectodermal origin, but can be expressed by cells such are chondrocytes, adipocytes and melanocytes as well. It has been investigated as a melanoma biomarker and is currently the best-studied melanoma marker that gives valuable information regarding many aspects of the clinical management of melanoma (61) (62) (63).

b) Thymidine kinase (TK)

Thymidine kinase is an enzyme of the pyridine salvage pathway, which catalyzes the phosphorylation of thymidine to thymidine monophosphate in the presence of adenosine triphosphate. In mammalian cells, it is present in two forms – TK1 and TK2. The level of TK1 rises at the G1/S boundary and increases dramatically to late S-phase/early G2-phase during the cell cycle, but is absent from quiescent cells. TK1 enzyme is of considerable interest because its level is highly dependent on the growth stage of the cell. Therefore, TK1 is a useful marker for cell proliferation and hence for malignancy (64) (65). In normal subjects, the amount of TK in serum or plasma is very low. Tumor cells release the enzyme into the circulation. The serum levels of TK therefore serves as a measure of malignant proliferation. Higher serum levels of TK correlate with a more advanced cancer stage and grade and help predict future relapse at the time of primary diagnosis (66). The most dramatic increases are seen in hematologic malignancies, but solid tumors (prostatic

carcinoma, colorectal carcinoma and breast carcinoma) give increased values of thymidine kinase as well (67).

c) Tissue polypeptide specific antigen (TPS)

All eukaryotic cells have cytoplasmic cytoskeletal structures known as intermediate filaments. Among the most important of these are cytokeratin proteins found in epithelial cells.

Tissue polypeptide antigen is a circulating complex of polypeptide fragments of cytokeratins 8,18 and 19. These three cytokeratins are characteristic of internal epithelium and are widely distributed in normal tissue and in tumors derived from them. Serum levels of TPS have correlate well with cell growth rate and tumor burden and are elevated in metastatic and disseminated disease. TPS is valuable as a prognostic marker and for monitoring treatment of patients with different carcinomas, especially with bladder carcinoma, breast carcinoma and lung cancer (68) (69).

d) Insulin-like growth factor binding proteins (IGFBP 1,2,3)

The activity of IGF1 and IGF2 is regulated by six IGF binding proteins; they form IGF/IGFBP complexes. IGF is released from IGFBP by proteolytic cleavage or dissociation (70). IGFBP3 is the most abundant member of this family, and has been shown to inhibit cell proliferation in breast, lung and prostate cancer (71). IGFBP3 regulates IGF1 signaling by acting as a competitive inhibitor for IGF1 and it also has an IGF-independent inhibitory effect on cell growth. The overexpression of IGFBPs is associated with increased, rather than decreased, IGF action and adverse effects on cancer prognosis (72) (71). A few studies have incorporated serum measurement of IGFBP3 as a biomarker of disease progression (73).

e) Vascular endothelial growth factor (VEGF)

VEGF is a cytokine that mediates numerous functions of endothelial cells including proliferation, migration, invasion, survival, and permeability. VEGF-A, -B, -C, -D, and -E are members of the large family of VEGF-related proteins (74). They bind to tyrosine kinase receptors expressed on endothelial cell surfaces with vascular endothelial growth

factor receptors (VEGFR 1,2 and 3) (75). VEGF-A has been most carefully studied. VEGF naturally occurs as a glycoprotein and is critical for vasculogenesis and angiogenesis. Angiogenesis impacts many important disease states significantly; including cancer, ischemic cardiovascular disease, wound healing and inflammation (74). Elevated levels of VEGF have been showed to correlate with tumor stage, disease progression and survival in cancer patients (76).

f) **Epidermal growth factor (EGF)**

Epidermal growth factor is a growth factor that stimulates growth, proliferation, and differentiation. EGF acts by binding to epidermal growth factor receptor on the cell surface. According to some studies, EGF has been implicated as a factor indicating tumor progression or as a prognostic factor in some cancers (77). Epidermal growth factor receptor inhibition decreases the risk of cancer. Mutations of EGFR have been identified in several types of cancer and it is the target of an expanding class of anticancer therapies. Drugs developed for this purpose are used in therapy of colorectal or lung cancer.

g) **Interleukins (IL 2, 6 , 8 and 10)**

Interleukins are a group of cytokines expressed by leukocytes. Cytokines can have either pro- or anti-inflammatory activity and immunosuppressive activity. Interleukins mediating pro-inflammatory cell mediated and humoral immunity are IL2, IL6 and IL10. IL6 and 10 are cytokines with an additional anti-inflammatory effect. A disturbed balance between pro- and anti-inflammatory mechanisms leads to chronic inflammation that plays an important role in development and progression of cancer. Increased levels of circulating cytokines (most often studied IL6) have been found in patients with malignant disease. Significant prognostic value of circulating cytokines has been found in a variety of cancers (78) (79). Increased concentrations of cytokines may serve as useful biomarkers for early diagnosis and prognosis, as well for disease and therapy monitoring (80).

h) **Osteoprotegerin (OPG)**

Osteoprotegerin is a basic glycoprotein that is encoded in humans by the TNFRSF11B gene. It is a cytokine receptor, and a member of tumor necrosis factor receptor superfamily (81). Osteoprotegerin / osteoprotegerin ligand pathway is a key regulator of bone metabolism through its effect on development and activation of osteoclasts (82). It regulates bone turnover through the binding and neutralization of the receptor activator of NFκB ligand (RANKL). OPGL is a critical factor in the immune system, from regulating development of lymph nodes to serving as an important co-stimulation molecule in optimal T cell activation and mediating dendritic cell survival. Subsequently, OPG has been found to have additional roles within the vascular systems. Several studies have demonstrated the involvement of OPG in vascular complications, including atherosclerotic plaque calcification, ischemic stroke and pulmonary arterial hypertension. It increases endothelial cell survival, proliferation and migration, as well as endothelial cell formation in angiogenesis (82) (83). There is additional evidence that OPG can promote cell survival by inhibiting TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. As a result, a number of in vitro, in vivo and clinical studies have been performed assessing the role of OPG in tumorigenesis (84). OPG acts as a decoy receptor, binding to TRAIL and neutralizing its function. TRAIL is the principle mediator of the extrinsic apoptotic pathway, which has tumor-killing activity. OPG is thought to protect against apoptosis, to be a positive regulator of tumor micro-vessel formation. OPG has an important role in tumor angiogenesis, a key process in cancer development and metastasis. It has been suggested that OPG production is a part of a tumor-cell survival strategy, and a number of different tumor cells, such as prostate, breast, and gastric cancer cells, have been found to produce OPG. Overexpression of OPG at the invasive tumor might play a crucial role in the initiation of progression and metastasis (85).

i) **Osteopontin (OPN)**

Osteopontin is an extracellular matrix phosphoglycoprotein that is biosynthesized by a variety of tissue types including fibroblasts, pre-osteoblasts, osteoblasts, osteocytes, odontoblasts, some bone marrow cells, hypertrophic chondrocytes, dendritic cells,

macrophages, smooth muscle, skeletal muscle myoblasts, endothelial cells, and extra osseous cells. Synthesis of osteopontin is stimulated by calcitriol (1.25-dihydroxy-vitamin D_3). OPN is an important factor in bone remodeling; it plays a role in anchoring osteoclasts to the mineral matrix of bones. OPN is reported to act as an immune modulator (86). OPN has also been described in the context of diverse physiological roles such as chemotaxis, cell migration, cell adhesion, angiogenesis, apoptosis, cell-extracellular matrix interactions and tumor metastasis. OPN actively promotes the tumorigenic phenotype and contributes to metastasis. Increased osteopontin expression is associated with aggressive behavior and metastasis in breast, colon, prostate, lung, liver and ovarian cancers. Elevated serum levels have been observed in patients with advanced or metastatic disease (87). High levels of OPN in several cancers are indicative of a poor prognosis. Overall and disease-free survival are inversely related to osteopontin levels according to several studies (88).

11. The aim of study

The aim of our study was to follow selected biomarkers before surgery and during follow-up in patients with malignant melanoma and in patients with advanced disease. We followed the patients with malignant melanoma for three years.

During follow-up we wanted to evaluate:

1. Differences in serum/plasma levels of biomarkers preoperatively, during remission, during disease progression and in advanced melanoma, and to compare these levels with serum/plasma levels of biomarkers in the control group.

2. Whether the correlation of biomarkers levels with clinical-pathological features can show whether serum/plasma levels of biomarkers can predict disease prognosis and aggressiveness. We correlated serum/plasma levels of biomarkers with TNM classification, Breslow index, sentinel lymph node positivity/negativity, tumor localization, and ulceration.

3. The correlation inside the group of biomarkers and to know if there are any connections among selected biomarkers during cancer progression and if there is a clinical application of these findings.

4. Our final aim was to find new biomarkers that we could use in the early diagnosis of malignant melanoma, or in the follow-up of the disease.

We wanted to prove the ability of xMAP technology to measure serum levels of tumor markers and of tumor´s biological activity.

12. The patients and methods

We performed a prospective nonrandomized study. The study was performed at the Department of Plastic Surgery and was interdisciplinary. We have cooperated with the Immunoassay laboratory, the Department of Dermatology and the Department of Pathology.

We studied patients with malignant melanoma that had undergone radical surgery. The patients were informed about our research project and signed an agreement with clinical trial.

a) **Patients**

The patients were divided into two groups. The first group consisted of patients with malignant melanoma that have undergone surgery (n=77). The second group was the control group; it consisted of patients with no evidence of malignant disease that have undergone surgery for benign skin lesion (n=34). The average age in time of diagnosis in melanoma and control group was 57.9 and 36.8 years respectively. We performed radical surgery in all cases in the melanoma group and sentinel lymph node biopsy in some cases according to international guidelines. We performed primary operation in 51 cases, re-excision in 18 cases and operation in advanced melanoma in 8 cases.

Concerning TNM classification, 21 patients had tumor size pT1 (Figure 10-14), 19 patients pT2 (Figure 15), 17 patients had pT3 (Figure 16-18) and 14 patients had pT4 (Figure 19-25). We performed sentinel lymph node biopsy in 44 cases; in 11 patients the sentinel lymph node was positive. In the time of diagnosis, only 2 patients had distant metastases.

Concerning the tumor characteristic - Breslow index 0.1-1mm was presented in 20 cases, 1.1-2mm in 23 cases, 2.1-4 in 15 cases and >4.1mm in 15 cases. Tumor ulceration as a negative prognostic factor was described in 30 cases. Melanoma was present mostly on lower limbs and trunk.

The patients´ history demonstrated some coincidence with different tumors, in 26 cases we found positive cancer family history. Melanoma had developed de novo in 32 cases or had its origin in a pigment lesion in 38 cases.

During our study, 7 patients died because of tumor progression.

Table 7 - Characteristics of melanoma and control group

	Melanoma group	**Control group**
Patients	n	n
Total number	77	34
Female	38	23
Male	39	10
Age (years)	n	n
Minimum	11	17
Maximum	87	84
Mean	57.9	36.8

Table 8 - Different characteristics of patient group

Exitus	**n**
Exitus during study period	7
Survival	70
Co-incidation with other tumor	**n**
Breast carcinoma	1
Renal carcinoma	2
Laryngeal carcinoma	1
Basocellular carcinoma	4
Prostate carcinoma	2
Urinary bladder carcinoma	1
Thyroid gland carcinoma	2
Origin of melanoma lesion	**n**
De novo	32
In pigment lesion	38
?	7
Primary excision x reexcision	**n**
Primary excision	51
Reexcision	18
Excision of tumor recurrence	8
Family cancer history	**n**
Positive	26
Negative	51

Table 10 - Breslow thickness,ulceration, tumor localization

Breslow thickness	**n**
0.1-1	20
1.1-2	23
2.1-4	15
>4.1	15
?	4
Ulceration	**n**
Yes	30
No	45
?	2
Localization	**n**
Lower limb	24
Upper limb	12
Neck	2
Face	5
Trunk	34

b) **Surgery and follow-up**

The patients in our study underwent radical surgery at the Department of Plastic Surgery, Faculty Hospital in Plzen, in the years 2010 to 2012. The surgery was performed in accordance to the stage of disease, taking into mind the international evidence-based guidelines for the management of cutaneous melanoma. The Department of Plastic Surgery and the Department of Dermatology as well as the Department of Oncology and other medical specialties, closely cooperate in follow-ups and examining the patients as multidisciplinary care is considered to be the most desirable model. In all patients we decided the protocol taking patient history into account as well as to risk factors, the description of clinical examination, characteristics of the tumor and photography of the tumor.

The extent of surgery was discussed above. In summary, patients with thin melanomas underwent radical surgery, usually without sentinel lymph node biopsy, as is recommended. Patients with thicker melanomas underwent radical surgery with sentinel lymph node biopsy. After surgical therapy, patients are immediately followed-up at the

Department of Plastic Surgery and then at the Department of Dermatology, according to standard protocol used in our hospital. Numerous follow-up regimens have been reviewed but few are evidence based. The reason for follow-ups is to detect recurrence early when further treatment can improve prognosis and to provide support and education. Most relapses occur in the 2 years following diagnosis, but in melanoma there is significant risk of later relapse. The follow-up intervals and duration are tailored according to the stage of primary melanoma and can differ in different melanoma centers. For stage IA patients, a series of visits during up to 24 months following the operation is suggested to teach self-examination, and then they may be discharged from regular follow up. Stage IB and IIA patients should be seen every 3 months for 3 years, then every 6 month for 5 years, and then annually. Stage IIB and IIC, III and IV patients are at high risk of recurrence and further metastasis. The follow-up is the same as in the previous group; consisting of a chest X-ray, CT, MRI, PET/CT, and laboratory examination as necessary. The adjuvant therapy is used for patients with higher risk of recurrence (4) (9).

c) **Blood samples and laboratory methods**

20ml of peripheral blood were drawn from each of the subjects using standardized phlebotomy procedures. The peripheral blood was drawn by VACUETTE ® (Greiner Bio-One, Austria) with and without EDTA as an anticoagulant. Plasma was separated by centrifugation at 1300xg and all specimens were immediately aliquoted and frozen, stored at -70◦C. No more than one freeze-thaw cycle was allowed before analysis. Samples were collected preoperatively (the day of surgery), 10 days after surgery and subsequently at 3-months intervals according to clinical examinations.

The following serum marker levels were determined: TK, TPS, S100A, OPG, OPN, IGFBP1 and IGFBP3.

The following plasma marker levels were determined: EGF, IL2, 6, 8, 10, VEGF and FGF2.

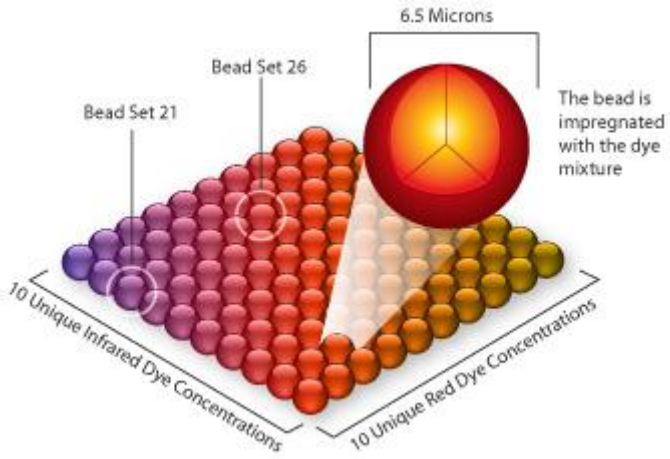
Blood samples were transported to the Immunoassay laboratory where they were analyzed. Serum TPS levels were measured by IRMA technology using commercial kits: IDL Biotech AB, Sweden. Serum TK levels were measured by REA technology using

commercial kits: Immunotech - Beckman Coulter, Czech Republic. Serum S100A levels were measured by ECLIA automated technology using commercial kits: Cobas e411, Roche, USA. The levels of cytokines and angiogenic factors: osteoprotegerin, osteopontin, IGFBP1, IGFBP3, EGF, IL2, IL6, IL8, IL10 and VEGF were determined using a multiplex immunoassay using Xmap technology. In the analysis we used a commercially available kits: Human Cytokine / Chemokine, Human Bone Panel and Human IGF Binding Protein Milliplex MAP kit (Merck-Millipore Corporation USA). Multiplex measurement was performed on the device Luminex 100: Luminex Corporation, USA.

Luminex's xMAP technology is based on existing technologies — flow cytometry, microspheres, lasers, digital signal processing and traditional chemistry — that have been combined in a unique way. The technique involves Luminex's 100 distinct sets of tiny color-coded beads, called microspheres. Each bead set can be coated with a specific capture probe or Anti Tag to allow the capture and detection of specific targets. The technology allows rapid and precise multiplexing of up to 100 unique assays within a single reaction.

Advantages of xMAP technology represent small sample volume requirements enabling study of large number of biomarkers, reduce of economy costs and time for research proceeding, enhancement of comparability of biomarker results measured in one shot compare to results measured one by one.

Figure 1 – xMAP technology (www.ebioscience.com)



Handling and processing was the same for melanoma group and for control group.

For statistical data evaluation, all results below calibration ranges were set to have the value of the lowest limit of the assay (Table 11).

Table 11 - Tumor markers in our study

Tumor marker	Abbrev.	Quantitative estimation	Commercial system	Low level of calibration	Units	Material
Thymidine kinase	TKS	REA	Immunotech	2.5	IU/L	**Serum**
Tissue polypeptide specific antigen	TPS	IRMA	IDL	10	IU/L	
Protein S100A	S100A	ECLIA	Roche	0.01	ug/L	
Osteoprotegerin	OPG	xMAP technology Luminex 100IS	MILLIPLEX MAP Human Bone Panel Merck	271	pg/mL	
Osteopontin	OPN			5.68	pg/mL	
Insulin like growth factor binding protein 1	IGFBP1		MILLIPLEX MAP Human IGF Binding Protein, Merck	0.75	ng/mL	
Insulin like growth factor binding protein 3	IGFBP3			8.5	ng/mL	
Epidermal growth factor	EGF		Milliplex MAP Human Cytokine/ Chemokine Panel Merck	3.2	pg/mL	**Plasma EDTA**
Interleukins	Il 2, 6, 8, 10			3.2	pg/mL	
Vascular endothelial growth factor	VEGF			3.2	pg/mL	
Basic fibroblast growth factor	FGF2			3.2	pg/mL	

d) **Histopathology**

The melanoma tissue is assessed by a histopathologist at the Department of Pathology. The paraffin blocks are placed in a microtome producing tissue sections that are stained with haematoxylin and eosin and histological parameters are described. Immunohistochemistry is used to identify specific types of cells and tissue elements. These techniques may help the pathologist better identify details such as the thickness of the primary melanoma, adequacy of excision or spread to lymph nodes. In current practice, the most clinically useful stains are melanoma differentiation markers (monoclonal antibodies). Most suspected melanomas were diagnosed with the most sensitive marker S100 and one or more of the more specific markers such as Melan-A, tyrosinase or HMB45.

e) **Statistical analysis**

A descriptive statistical analysis was carried out and all data were presented with basic statistical variables – mean, median, standard deviation, minimum, and maximum and quartile extent. Non-parametric tests were used for comparisons between numeric variables, a Wilcoxon two-sample test and Kruskal-Wallis tests were used for a comparison of tumor markers levels between groups. The Spearman Rank Correlation Coefficient was used because of non-Gaussian distribution of parameter values. For analyses of the sensitivity-specificity relation, receiver operating characteristic (ROC) curves were constructed and used as a tool for determination of an optimal cut-off value. The optimal cut-off value was found in the most statistically significant result (with the lowest p-value) of maximum likelihood estimates analysis. Also the area under the curve (AUC) was calculated for each of the tumor marker. A value of $p<0.05$ was considered significant.

13. Results

The melanoma follow up group with progression of the disease featured a higher median levels in comparison to the melanoma follow up group with remission in the following markers: thymidine kinase, tissue polypeptide specific antigen, protein S100A, osteoprotegerin, osteopontin, insulin-like growth factor binding protein 1 and 3, interleukin-6 and -8 (Table 12).

Concerning the comparison of the control group and advanced melanoma group, almost all biomarkers featured higher preoperative median levels in the advanced melanoma group: thymidine kinase, tissue polypeptide specific antigen, protein S100A, osteoprotegerin, osteopontin, insulin-like growth factor binding protein 1 and 3, interleukin-8 and fibroblast growth protein 2 (Table 12).

The melanoma group featured higher preoperative median levels in comparison to control group in following markers: tissue polypeptide specific antigen, protein S100A, osteoprotegerin, osteopontin, insulin-like growth factor binding protein 3 and interleukin-8 (Table 12).

Higher serum levels in advanced disease have been observed in tissue polypeptide specific antigen and osteopontin compared to preoperative levels in primary disease, $p<0.03$ and $p<0.02$ respectively (Table 13).

The patients have been followed-up during our study in determined intervals and tumor marker levels were observed during these checkups. The melanoma follow up group with progression of the disease featured a higher median levels in comparison to the melanoma follow up group with remission in the following markers: protein S100A, osteoprotegerin, insulin-like growth factor binding protein 3 and interleukin-10, $p<0.0009$, $p<0.01$, $p<0.0001$ and $p<0.01$ respectively (Table 13).

Serum levels of tumor markers from the control group have been compared to the serum levels from the melanoma group. Almost all biomarkers featured higher preoperative median levels in the melanoma group, but only these were statistically significant: tissue polypeptide specific antigen, protein S100A, osteoprotegerin, osteopontin and insulin-like growth factor binding protein 3, $p<0.0002$, $p<0.01$, $p<0.001$ and $p<0.0008$ respectively (Table 13).

The analysis also revealed that differences were obtained for tissue polypeptide specific antigen and insulin-like growth factor binding protein 3 serum levels that were

higher in higher T stage, $p<0.0001$ and $p<0.02$ respectively. These tumor markers were related to tumor size (Table 14).

Additionally, the concentrations of all tumor markers were tested in relationship to nodal status. We demonstrated higher serum levels of protein S100A and osteopontin in patients with lymph node being involved, $p<0.0008$ and $p<0.01$ respectively (Table 15).

Elevated interleukin-6 and -10 preoperative serum levels in primary tumor were significantly associated with increasing tumor thickness, $p<0.02$ and $p<0.05$ respectively. Elevated protein S100A serum levels were positively correlated with tumor thickness in advanced disease, $p<0.01$. None of other investigated tumor markers was found to be statistically correlated to this clinical parameter (Table 16).

In our study, only higher serum levels of osteopontin and interleukin-2 demonstrated significant correlation with the presence of lymph node metastases, $p<0.03$ and $p<0.05$ respectively (Table 17).

According to our results, only protein S100A positively correlated with presented tumor ulceration, $p<0.01$. No other interesting associations have been found (Table 18).

We have found that protein S100A serum level in advanced melanoma and interleukin-6 preoperative serum level in primary melanoma positively correlated with tumor localization, $p<0.05$ (Table 19).

The correlation analysis of investigated parameters using the Spearman correlation test showed that several biomarkers correlated with others. Using a 5% significance level and a 0.1% significance level respectively, we could distinguish significant correlations in the group of proangiogenic factors (osteoprotegerin, osteopontin or vascular endothelial growth factor) and in the group of proinflammatory factors (interleukins), as well as in the group of proliferative factors (thymidine kinase, tissue polypeptide specific antigen or protein S100). According to the Spearman Correlation Coefficient, that is not approaching value 1; being as the correlation is not very strong, we could consider using these factors as biomarkers in different clinical situations (Table 20 and 21).

The specificity and sensitivity of these tumor markers have been determined using receiver operating characteristic. The sensitivity of tissue polypeptide specific antigen, protein S100A, osteoprotegerin, osteopontin, insulin-like growth factor binding protein 3, interleukin 2 and 8 was 27.6%, 38.5%, 39.2%, 9.8%, 43.1%, 1.9%, 17.6%, respectively, at 93% specificity. All studied markers can be arranged according to the area under the curve ranging from 0.78 to 0.49 listed in decreasing manner: protein S100A, osteoprotegerin,

osteopontin, insulin-like growth factor 3, tissue polypeptide specific antigen, interleukin-8 and interleukin-2 (Table 22 and Figure 2-9).

14. Tables and diagrams associated with results

Table 12 - Serum/plasma levels of selected tumor markers in melanoma group and in control group using a descriptive statistics

Tumor marker	TK	TPS	S100A	OPG	OPN	IGFBP1	IGFBP3	EGF	IL2	IL6	IL8	IL10	VEGF
Control group													
n	33	31	31	29	29	29	29	29	29	29	29	29	29
Median	4.6	32	0.041	252.65	8659.68	3.35	529.43	23.73	3.92	3.2	4.89	3.2	129.87
Minimum	2.5	2.8	0.013	120.83	3025.61	0.75	272.89	3.2	3.2	3.2	3.2	3.2	16
Maximum	38	264	0.125	513.27	35141.76	8.41	1189.53	255.47	36.17	94.94	114.86	12	1472.92
Melanoma group													
Preoperative levels													
n	66	65	64	51	51	51	51	51	51	51	51	51	51
Median	4.2	60	0.059	355.18	15985.01	2.8	678.56	19.75	3.2	3.2	7.97	3.2	92.96
Minimum	2	3	0.024	141.92	1318.34	0.76	337.12	3.2	3.2	3.2	3.2	3.2	16
Maximum	18	565	0.507	832.72	66420.87	9.46	4686.46	293.7	35.18	179.05	503.47	35.16	5249.45
Follow up remission													
n	244	234	238	87	87	87	87	87	87	87	87	87	87
Median	5.2	55	0.045	324.81	13552.73	2.6	796.47	21.5	3.2	3.2	7.23	3.2	131.08
Minimum	2.5	4.7	0.018	162.52	578.71	0.75	355.91	3.2	3.2	3.2	3.2	3.2	16
Maximum	40	2400	1.44	1015.91	94563.27	14.51	4059.43	247.83	49.94	312.6	642.19	26.05	5223.85
Follow up progression													
n	22	22	21	16	16	15	15	16	16	16	16	16	16
Median	5.95	60.5	0.099	503.38	15652.48	2.7	638.69	16.66	3.2	3.65	8.25	3.2	81.36
Minimum	2.8	16	0.035	267.86	7546.62	1.03	409.79	3.2	3.2	3.2	3.2	3.2	16
Maximum	29.6	157	1.35	1183.45	147866.34	14.23	1314.67	352.14	24.61	361.82	454.97	135.94	10000
Advanced melanoma preop.													
n	11	11	11	9	9	9	9	9	9	9	9	9	9
Median	5.2	87	0.066	463.86	26568.66	3.7	748.4	12.69	3.2	3.2	5.47	3.2	69.5
Minimum	2.5	42	0.027	190.77	138.1	0.75	493.58	3.2	3.2	3.2	3.2	3.2	16
Maximum	16.9	251	1.21	582.64	35627.27	12.54	4922.8	71.43	7.32	66.91	40.27	7.43	725.52
Follow up stationary state													
n	24	22	23	16	16	16	16	16	16	16	16	16	16
Median	4.3	67	0.049	528.785	21128.03	3.4	648.27	3.2	3.2	3.2	3.2	3.2	16.23
Minimum	2.5	15	0.033	229.27	10504.42	0.75	530.45	3.2	3.2	3.2	3.2	3.2	16
Maximum	12.8	257	1.64	761.84	45817.68	8.93	3984.72	22.77	7.25	14.03	18.01	3.2	231.99

Table 13 - Comparison of tumor markers between groups according to clinical status

Tumor marker	TK	TPS	S100A	OPG	OPN	IGFBP1	IGFBP3	EGF	IL2	IL6	IL8	IL10	VEGF
Remission x progression	*p<0.50*	*p<0.11*	*p<0.0009*	*p<0.01*	*p<0.63*	*p<0.12*	*p<0.0001*	*p<0.67*	*p<0.14*	*p<0.76*	*p<0.36*	*p<0.01*	*p<0.27*
Primary melanoma x advanced disease	*p<0.14*	*p<0.03*	*p<0.41*	*p<0.22*	*p<0.02*	*p<0.74*	*p<0.52*	*p<0.25*	*p<0.57*	*p<0.49*	*p<0.28*	*p<0.8*	*p<0.27*
Melanoma group x control group	*p<0.48*	*p<0.0002*	*p<0.01*	*p<0.001*	*p<0.0008*	*p<0.63*	*p<0.03*	*p<0.16*	*p<0.11*	*p<0.77*	*p<0.75*	*p<0.38*	*p<0.15*

Table 14 - The analysis using the Spearman correlation test in relationship to tumor size

Tumor marker	**TK**	**TPS**	**S100A**	**OPG**	**OPN**	**IGFBP1**	**IGFBP3**	**EGF**	**IL2**	**IL6**	**IL8**	**IL10**	**VEGF**
TNM													
T	*p<0.86*	*p<0.0001*	*p<0.32*	*P<0.15*	*p<0.21*	*p<0.52*	*p<0.02*	*p<0.18*	*p<0.46*	*p<0.62*	*p<0.63*	*p<0.6*	*p<0.15*
1a													
n	17	17	17	11	11	11	11	11	11	11	11	11	11
Mean	5.15	79.53	0.064	374.77	16452.45	3.18	701.85	19.34	4.18	3.97	17.17	3.85	84.7
Minimum	2.5	12	0.024	141.92	5364.92	0.83	337.12	3.2	3.2	3.2	3.2	3.2	16
Maximum	14.3	211	0.292	718.42	28422.71	7.02	1384.4	30.8	9.99	6.86	128.68	7.43	183.92
1b													
n	3	3	2	2	2	2	2	2	2	2	2	2	2
Mean	5.03	187.66	0.11	514.95	8560.88	3.72	513.05	21.5	3.2	3.2	7.11	3.2	98.25
Minimum	2.5	134	0.08	400.58	4255.71	1.87	487.42	3.2	3.2	3.2	6.26	3.2	83.91
Maximum	6.5	221	0.15	629.33	12866.05	5.58	538.68	39.8	3.2	3.2	7.97	3.2	112.59
2a													
n	12	12	12	11	11	11	11	11	11	11	11	11	11
Mean	5.46	51.08	0.09	425.28	24085.67	3.15	1999.13	36.23	4.56	26.95	20.82	3.2	436.02
Minimum	2.8	10	0.02	211.37	1318.34	0.79	368.67	3.2	3.2	3.2	3.2	3.2	16
Maximum	16.9	161	0.43	750	66420.87	8.23	4922.8	147.2	18.24	178.06	98.27	3.2	1326.56
2b													
n	7	7	7	3	3	3	3	3	3	3	3	3	3
Mean	5.4	45.42	0.12	352.07	7603	2.87	1816,48	9.59	3.2	3.2	3.69	3.2	42.81
Minimum	2.7	10	0.038	205.63	2552.51	1.45	1132	3.2	3.2	3.2	3.2	3.2	16
Maximum	15.2	106	0.41	497.36	17455.2	4.96	2304.3	20.75	3.2	3.2	4.68	3.2	59.05
3a													
n	6	6	6	3	3	3	3	3	3	3	3	3	3
Mean	7.36	101.16	0.07	295.5	16303.92	5.83	865.59	126.14	4.2	39.26	59.01	3.2	1766.87
Minimum	2	15	0.02	264.8	10407.8	2.39	675.57	19.53	3.2	3.2	3.2	3.2	16
Maximum	18	232	0.19	320.72	23597.04	9.46	1168.7	293.7	6.23	104.46	116.89	3.2	5249.45
3b													
n	11	11	11	11	11	11	11	11	11	11	11	11	11
Mean	4.77	72.09	0.09	337.34	16329.81	3.03	941.24	24.95	3.71	12.02	19.46	5.18	151.33
Minimum	2.8	17	0.027	182.13	2022.04	0.75	493.58	3.2	3.2	3.2	3.2	3.2	16
Maximum	7.5	263	0.33	511.88	30915.59	12.54	1513.46	146.38	7.32	86.14	114.39	24.99	663.44
4a													
n	4	4	3	3	3	3	3	3	3	3	3	3	3
Mean	5.22	73.25	0.14	373.73	19053.39	3.59	597.42	17.9	5.08	4.15	6.82	4.68	96.7
Minimum	3.2	48	0.04	257.65	6797.08	1.93	480.69	5.67	3.2	3.2	3.2	3.2	69.5
Maximum	8.5	108	0.032	544.81	27439.26	6.05	780.44	40.18	8.84	6.05	9.18	7.66	148.34
4b													
n	10	9	10	10	10	10	10	10	10	10	10	10	10
Mean	4.3	240.55	0.24	525.82	26599.15	5.51	798.02	56.42	8.97	32.84	75.54	8.05	729.83
Minimum	2.5	67	0.04	308.58	13819.1	1.02	453.14	3.2	3.2	3.2	3.2	3.2	16
Maximum	7.8	565	1.21	832.72	62476.93	9.65	1960.92	280.3	35.18	179.05	503.47	28.55	3753.33

Table 15 - The analysis using the Spearman correlation test in relationship to nodal status

Tumor marker	**TK**	**TPS**	**S100A**	**OPG**	**OPN**	**IGFBP1**	**IGFBP3**	**EGF**	**IL2**	**IL6**	**IL8**	**IL10**	**VEGF**
TNM													
N	*p<0.39*	*p<0.86*	*p<0.0008*	*p<0.87*	*p<0.01*	*p<0,63*	*p<0,58*	*p<0.61*	*p<0.08*	*p<0.31*	*p<0.92*	*p<0.38*	*p<0.99*
N0													
n	32	32	31	23	23	23	23	23	23	23	23	23	23
Mean	6.14	99.47	0.09	397.97	15377.11	3.79	1289.62	35.69	4.19	9.38	23.09	4.44	350.57
Minimum	2	10	0.02	141.92	2552.39	1.17	337.12	3.2	3.2	3.2	3.2	3.2	16
Maximum	18	502	0.43	832.72	29182.93	946	4686.46	293.7	19.63	104.46	128.68	28.55	5249.45
N1													
n	5	5	4	3	3	3	3	3	3	3	3	3	3
Mean	3.92	64.4	0.07	344.78	26672.18	5.79	756.75	80.37	14.35	30.85	20.14	9.85	323.69
Minimum	2.8	17	0.04	225.22	23856	1.44	514.99	21.68	3.2	3.2	3.56	3.2	123.73
Maximum	5.2	106	0.14	429.97	30915.59	8.92	1091.46	146.38	35.18	86.14	44.16	23.14	663.44
N2													
n	5	4	5	5	5	5	5	5	5	5	5	5	5
Mean	4	102	0.14	335.27	21578.67	4.22	707.44	26.33	6.2	25.09	34.51	3.2	302.96
Minimum	2.9	35	0.04	182.13	14319.81	2.38	453.14	3.2	3.2	3.2	3.2	3.2	16
Maximum	6	245	0.33	698.65	43115.29	7.98	1336.45	66.65	18.24	87.4	114.39	3.2	1326.56
N3													
n	2	2	2	2	2	2	2	2	2	2	2	2	2
Mean	7.65	70	0.64	404.16	31097.97	5.2	660.16	37.31	7.1	35.05	22.87	3.2	370.76
Minimum	7.5	57	0.06	344.47	26568.66	0.75	571.93	3.2	6.89	3.2	5.47	3.2	16
Maximum	7.8	83	1.21	463.86	35627.27	9.65	748.4	71.43	7.32	66.91	40.27	3.2	725.52

Table 16 - The analysis using the Spearman correlation test in relationship to Breslow thickness

Tumor marker	Preoperative levels in primary tumor	Advanced melanoma
TK	*p<0.99*	*p<0.9*
TPS	*p<0.07*	*p<0.61*
S100A	*p<0.08*	*p<0.01*
OPG	*p<0.44*	*p<0.38*
OPN	*p<0.08*	*p<0.7*
IGFBP1	*p<0.71*	*p<0.65*
IGFBP3	*p<0.85*	*p<0.22*
EGF	*p<0.93*	*p<0.45*
IL2	*p<0.49*	*p<0.59*
IL6	*p<0.02*	*p<0.72*
IL8	*p<0.15*	*p<0.16*
IL10	*p<0.05*	*p<0.12*
VEGF	*p<0.96*	*p<0.48*

Table 17 - The comparison of tumor markers serum/plasma levels and positivity/negativity of sentinel lymph node using the Wilcoxon test and Kruskal-Wallis (Chi-square) test

Tumor marker	**Preoperative levels primary tumor SLN positive/negative**	**Advanced melanoma SLN positive/negative**
TK	*p<0.39*	*p<0.54*
TPS	*p<0.56*	*p<0.54*
S100A	*p<0.43*	*p<1.00*
OPG	*p<0.46*	*p<1.00*
OPN	*p<0.03*	*p<1.00*
IGFBP1	*p<0.58*	*p<1.00*
IGFBP3	*p<0.29*	*p<0.54*
EGF	*p<0.27*	*p<1.00*
IL2	*p<0.05*	*p<0.54*
IL6	*p<0.57*	*p<1.00*
IL8	*p<0.61*	*p<0.54*
IL10	*p<0.19*	*p<1.00*
VEGF	*p<0.43*	*p<1.00*

Table 18 - The comparison of tumor markers serum/plasma levels and tumor ulceration using the Wilcoxon test and Kruskal-Wallis (Chi-square) test

Tumor marker	**TK**	**TPS**	**S100A**	**OPG**	**OPN**	**IGFBP1**	**IGFBP3**	**EGF**	**IL2**	**IL6**	**IL8**	**IL10**	**VEGF**
Ulceration +/-	*p<0.21*	*p<0.39*	*p<0.01*	*p<0.99*	*p<0.22*	*p<0.78*	*p<0.30*	*p<0.44*	*p<0.36*	*p<0.23*	*p<0.56*	*p<0.61*	*p<0.53*

Table 19 - The comparison of tumor markers serum/plasma levels and tumor localization using the Wilcoxon test and Kruskal-Wallis (Chi-square) test

Tumor marker	Preoperative level primary tumor	Advanced melanoma
S-TK	*p<0.94*	*p<0.21*
S-TPS	*p<0.41*	*p<0.57*
S-100A	*p<0.72*	*p<0.05*
S-OPG	*p<0.71*	*p<0.95*
S-OPN	*p<0.95*	*p<0.57*
S-IGFBP1	*p<0.20*	*p<0.18*
S-IGFBP3	*p<0.34*	*p<0.95*
P-EGF	*p<0.55*	*p<0.29*
P-IL2	*p<0.57*	*p<0.57*
P-IL6	*p<0.05*	*p<0.60*
P-IL8	*p<0.48*	*p<0.26*
P-IL10	*p<0.80*	*p<0.17*
P-VEGF	*p<0.84*	*p<0.32*

Table 20 - The correlation analysis using the Spearman correlation test with R-values and p-values ≤0.0001for biomarkers in correlation to each other

Tumor marker		TK	TPS	S100A	OPG	OPN	IGFBP1	IGFBP3	EGF	IL2	IL6	IL8	IL10	VEGF
TK	p		≤0.001	0.01	0.07	0.4	0.003	0.65	0.77	0.44	0.45	0.49	0.03	0.43
	R	1.0	0.21	0.12	0.12	0.05	-0.2	-0.03	-0.02	0.05	0.05	0.04	0.14	0.05
TPS	p	≤0.001		0,16	≤0.001	0.008	0.32	0.97	0.18	0.05	0.28	0.12	0.11	0.17
	R	0.2	1.0	0.07	0.38	0.23	0.06	0.002	-0.09	-0.13	0.07	0.1	0.11	-0.09
S100A	p	0.01	0.16		0.002	0.001	0.08	0.72	0.004	0.11	0.73	0.18	0.69	0.004
	R	0.12	0.07	1.0	0.25	0.21	0.11	0.02	-0.19	-0.11	-0.02	0.09	0.02	-0.19
OPG	p	0.07	≤0.001	0.002		0.001	0.001	0.64	≤0.001	0.002	0.55	0.96	0.1	0.001
	R	0.12	0.38	0.25	1.0	0.22	0.07	-0.03	-0.27	-0.21	-0.04	-0.003	0.11	-0.21
OPN	p	0.41	0.008	0.001	0.001		0.01	0.11	0.008	0.05	≤0.001	≤0.001	0.24	0.08
	R	0.05	0.23	0.21	0.22	1.0	0.17	0.11	0.18	0.13	0.37	0.29	0.08	0.12
IGFBP1	p	0.003	0.32	0.08	0.28	0.01		0.003	0.89	0.02	0.64	0.73	0.17	0.06
	R	-0.2	0.06	0.11	0.07	0.17	1.0	-0.14	0.009	0.15	0.03	0.02	0.09	-0.12
IGFBP3	p	0.65	0.97	0.72	0.64	0.11	0.03		0.89	0.02	0.79	0.001	0.09	0.07
	R	-0.03	0.002	0.02	-0.03	0.11	-0.14	1.0	0.008	-0.15	0.01	0.22	-0.11	0.12
EGF	p	0.77	0.18	0.004	≤0.001	0.008	0.89	0.89		≤0.001	≤0.001	0.009	≤0.001	≤0.001
	R	-0.02	-0.09	-0.19	-0.27	0.18	0.009	0.008	1.0	0.44	0.49	0.55	0.18	0.66
IL2	p	0.44	0.05	0.11	0.002	0.051	0.02	0.02	≤0.001		0.002	0.005	≤0.001	≤0.001
	R	0.05	-0.13	-0.11	-0.2	0.13	0.15	-0.15	0.44	1.0	0.25	0.19	0.39	0.36
IL6	p	0.45	0.28	0.73	0.55	≤0.001	0.64	0.79	≤0.001	0.002		≤0.0001	0.42	≤0.001
	R	0.05	0.07	-0.02	-0.04	0.37	0.03	0.01	0.49	0.25	1.0	0.63	0.05	0.52
IL8	p	0.49	0.12	0.18	0.96	≤0.001	0.73	0.001	≤0.001	0.005	≤0.001		0.01	≤0.001
	R	0.04	0.1	0.09	-0.003	0.29	0.02	0.22	0.55	0.19	0.63	1.0	0.16	0.6
IL10	p	0.03	0.11	0.69	0.10	0.24	0.17	0.09	0.009	≤0.001	0.42	0.01		0.01
	R	0.14	0.11	0.02	0.11	0.08	0.09	-0.11	0.18	0.39	0.05	0.16	1.0	0.17
VEGF	p	0.43	0.17	0.004	0.001	0.08	0.06	0.07	≤0.001	≤0.001	≤0.001	≤0.001	0.01	
	R	0.05	-0.09	-0.19	-0.2	0.12	-0.12	0.12	0.66	0.36	0.52	0.6	0.17	1.0

Table 21 - The correlation analysis using the Spearman correlation test with R-values and p-values ≤0,05 for biomarkers in correlation to each other

Tumor marker	TK	TPS	100A	OPG	OPN	IGFBP1	IGFBP3	EGF	IL2	IL6	IL8	IL10	VEGF
TK		p≤0.0001	0.01	0.07	0.41	0.003	0.65	0.77	0.44	0,45	0.49	0.03	0.43
TPS	≤0.0001		0.16	≤0.0001	0.0008	0.32	0.97	0.18	0.05	0,28	0.12	0.11	0.17
100A	0.01	0.16		0.0002	0.001	0.08	0.72	0.004	0.11	0,73	0.18	0.69	0.004
OPG	0.07	≤0.001	0.0002		0.001	0.001	0.64	≤0.0001	0.002	0,55	0.96	0.1	0.001
OPN	0.41	0.0008	0.001	0.001		0.01	0.11	0.008	0.05	≤0.0001	≤0.0001	0.24	0.08
IGFBP1	0.003	0.32	0.08	0.28	0.011		0.003	0.89	0.02	0,64	0.73	0.17	0.06
IGFBP3	0.65	0.97	0.72	0.64	0.11	0.03		0.89	0.02	0,79	0.001	0.09	0.07
EGF	0.77	0.18	0.004	≤0.0001	0.008	0.89	0.89		≤0.0001	≤0.0001	0.009	≤0.0001	≤0.0001
IL2	0.44	0.05	0.11	0.002	0.05	0.02	0.02	≤0.0001		0.0002	0.005	≤0.0001	≤0.0001
IL6	0.45	0.28	0.73	0.55	≤0.0001	0.64	0.79	≤0.0001	0.0002		≤0.0001	0.42	≤0.0001
IL8	0.49	0.12	0.18	0.96	≤0.0001	0.73	0.001	≤0.0001	0.005	≤0.0001		0.01	≤0.0001
IL10	0.03	0.11	0.69	0.10	0.24	0.17	0.09	0.009	≤0.0001	0,42	0.01		0.01
VEGF	0.43	0.17	0.004	0.001	0.08	0.06	0.07	≤0.0001	≤0.0001	≤0.0001	≤0.0001	0.01	

Table 22 - Specificity, sensitivity and ROC curves in selected tumor markers according to their significance

Tumor marker	Cut-off	Specificity %	Sensitivity %	PV+	PV-	RR	AUC
S-TK	-	-	-	-	-	-	-
S-TPS	99	93	27.69	90	38.15	1.45	0.65
S-100A	0.12	93	38.46	55.55	86.66	4.16	0.78
S-OPG	400.58	93	39.21	90.9	46.55	1.7	0.77
S-OPN	30915.59	93	9.8	71.42	36.98	1.13	0.72
S-IGFBP1	-	-	-	-	-	-	
S-IGFBP3	900.76	93	43.13	91.66	48.21	1.77	0.67
P-EGF	-	-	-	-	-	-	
P-IL2	24.08	93	1.96	33.33	35.06	0.51	0.49
P-IL6	-	-	-	-	-	-	
P-IL8	37.74	93	17.64	81.81	39.13	1.34	0.57
P-IL10	-	-	-	-	-	-	
P-VEGF	-	-	-	-	-	-	

Figure 2 - **ROC - S_TPS**

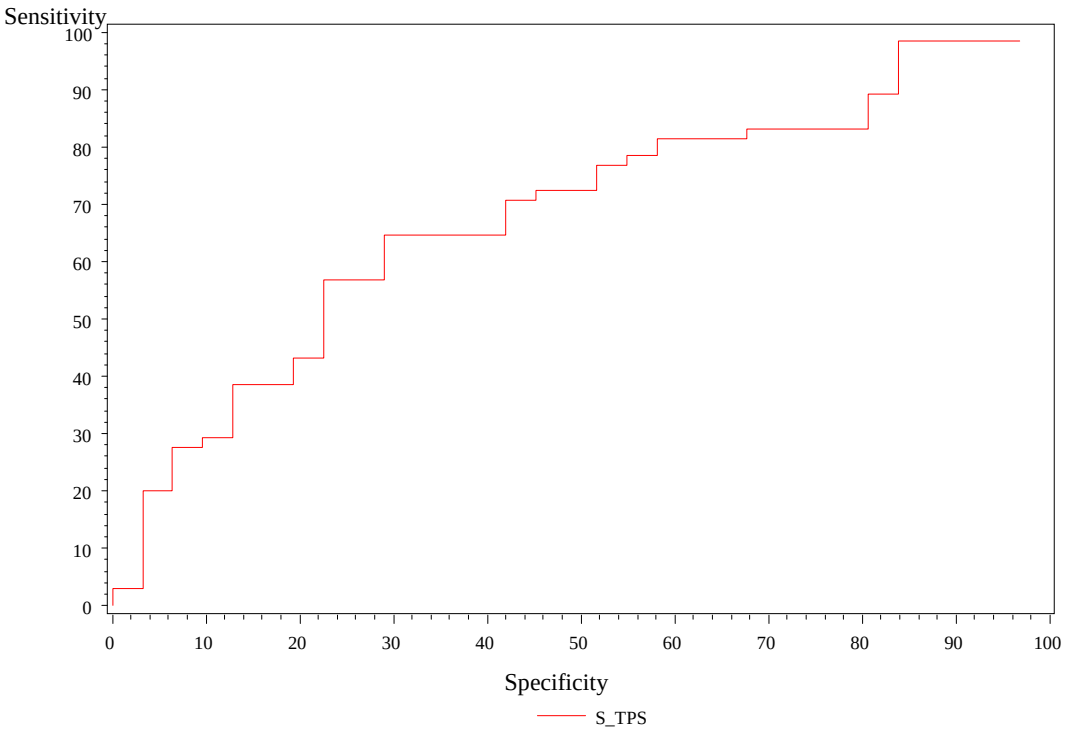


Figure 3 - **ROC - S_S100A**

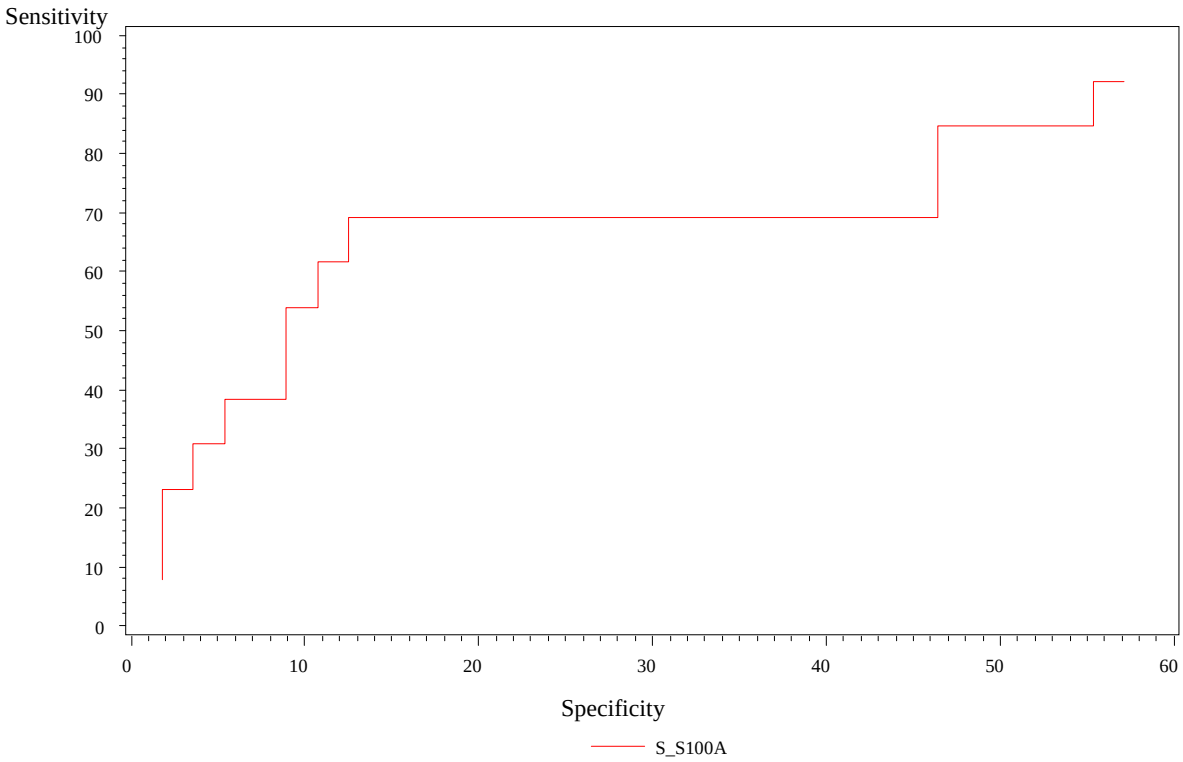


Figure 4 – **ROC - S_OPG**

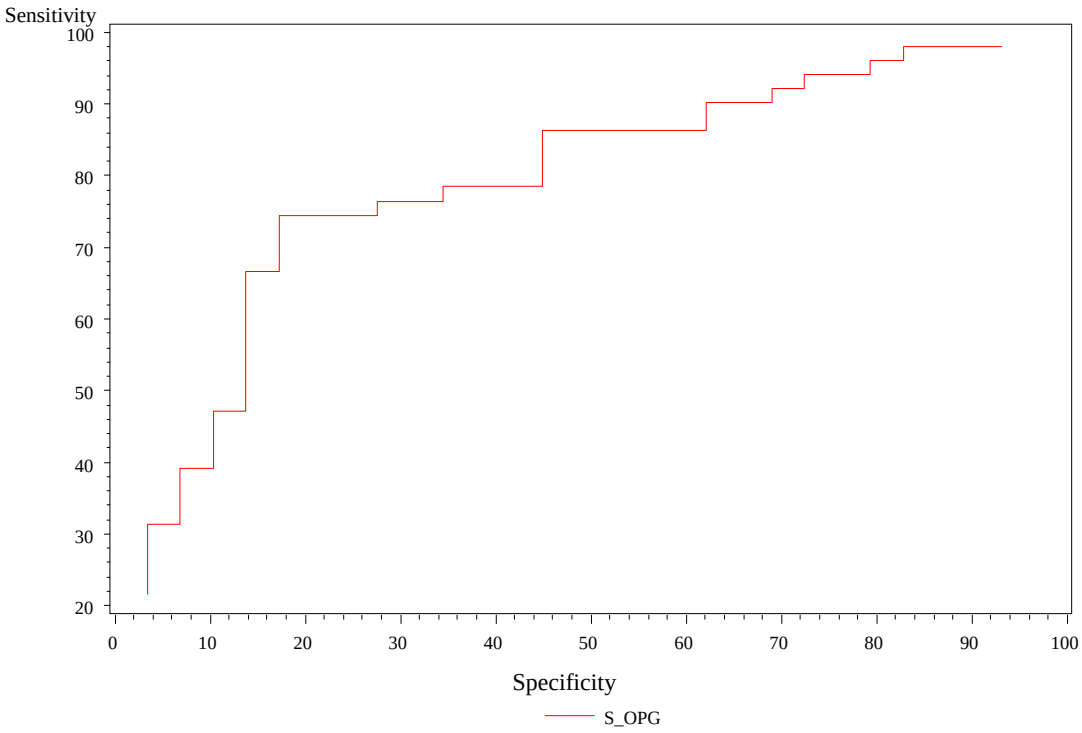


Figure 5 - **ROC - S_OPN**

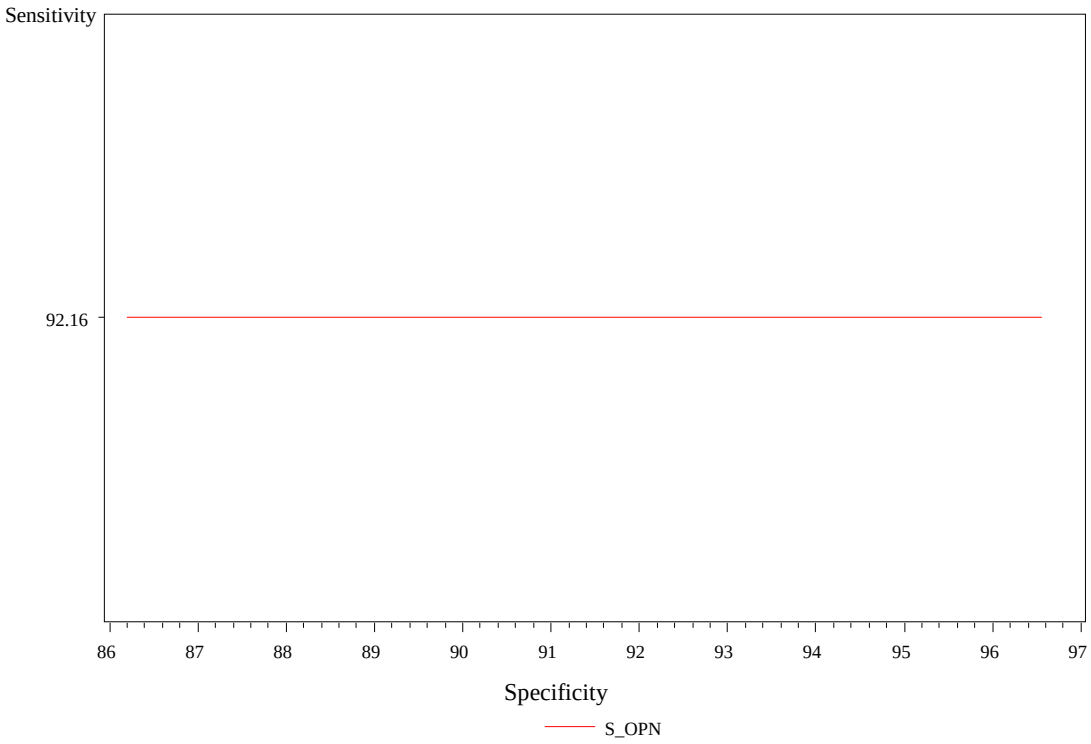


Figure 6 - **ROC – S_IGFBP3**

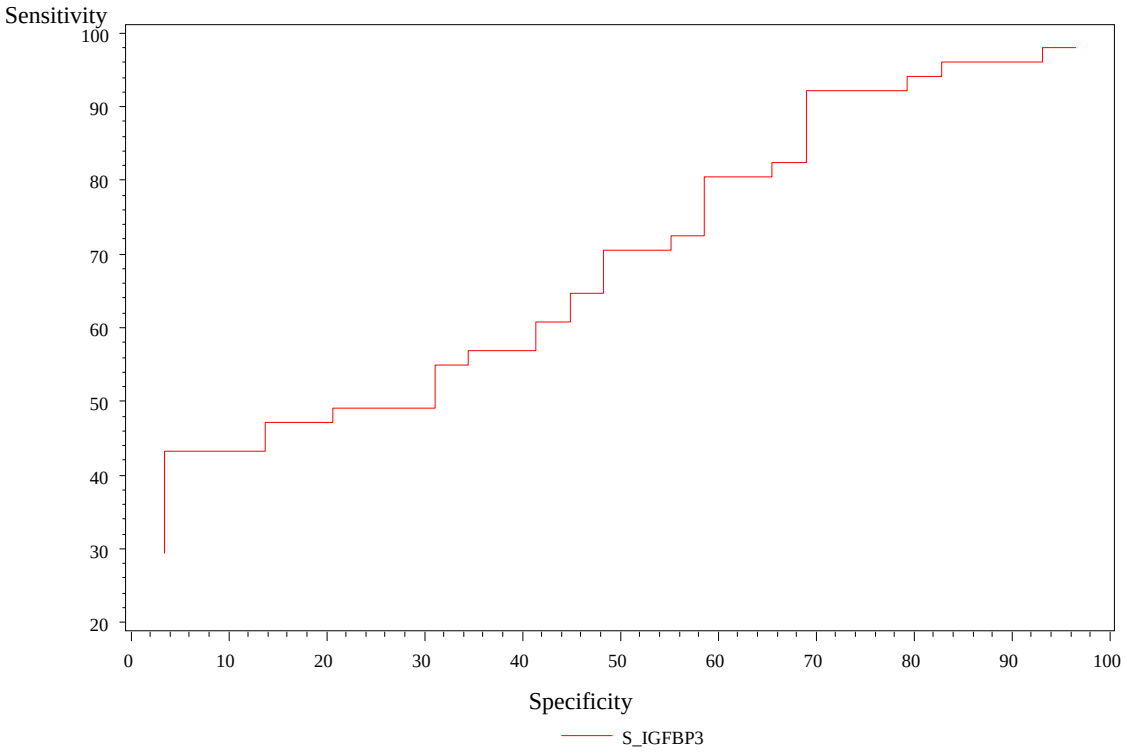


Figure 7 - **ROC - P_IL2**

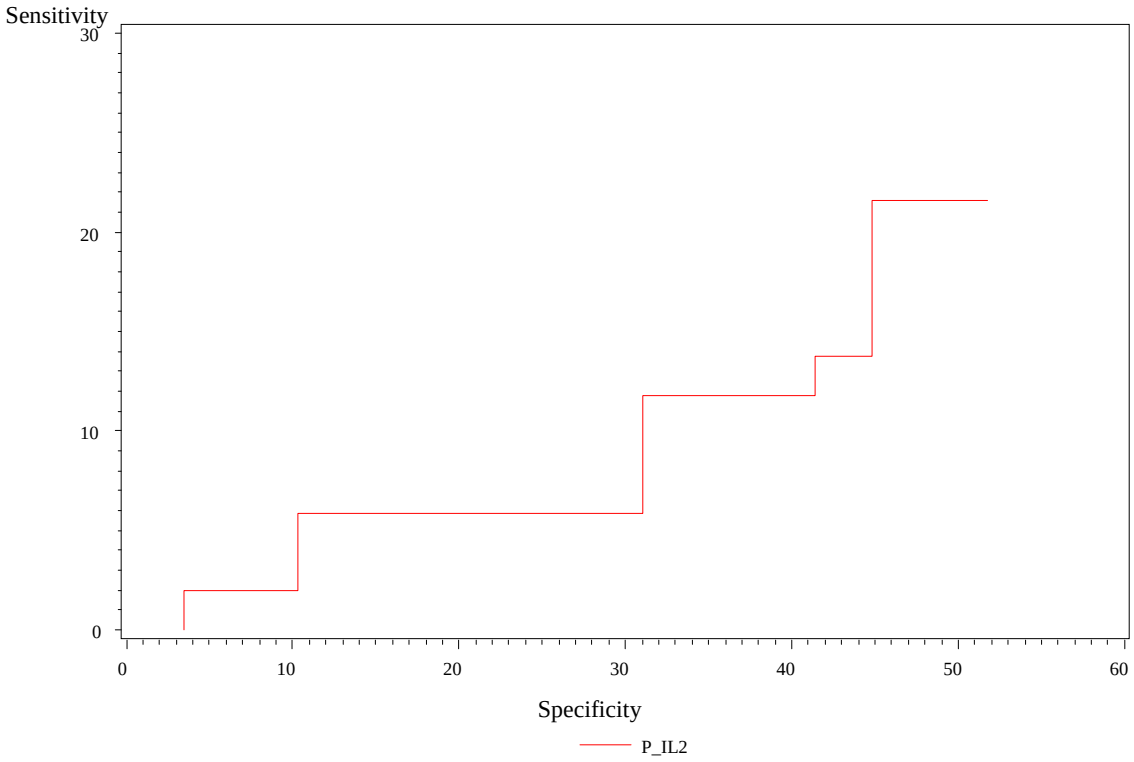


Figure 8 - **ROC - P_IL8**

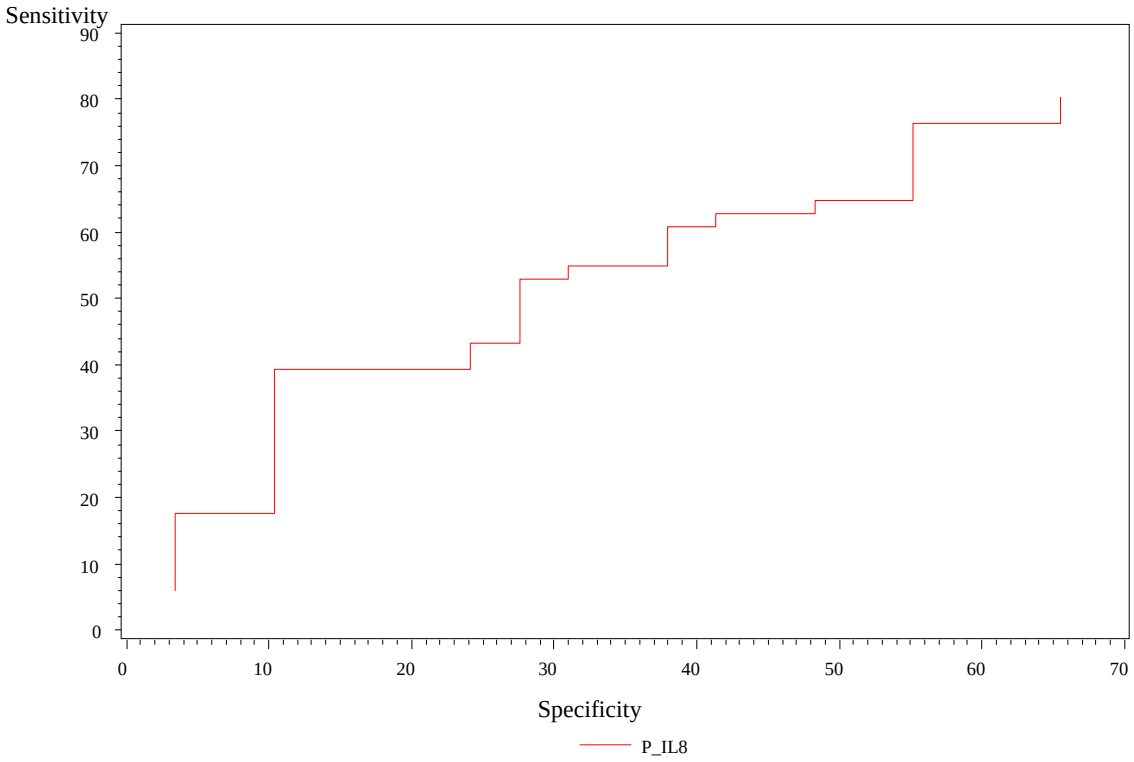
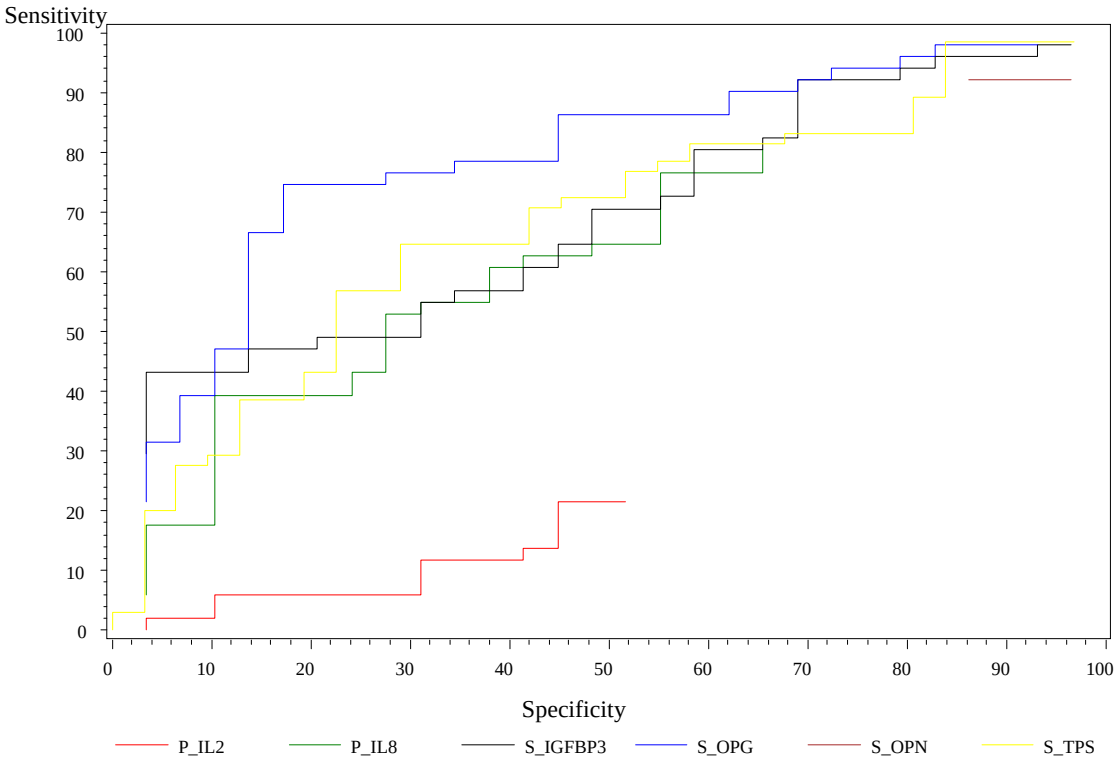


Figure 9 - **ROC - S_TPS S_100A S_OPG S_OPN S_IGFBP3 P_IL2 P_IL8**



15. Discussion

Malignant melanoma is one the most aggressive cancers and is potentially lethal if not detected at an early stage and treated properly. As the incidence rate is increasing worldwide, efforts are made to better understand the behavior of this heterogeneous cancer. Understanding the correlations between the prognostic factors and the biology of the disease is a major objective in melanoma research (20). The Breslow thickness of a tumor and the status of the sentinel lymph node are still the most important prognostic factors for recurrence and survival. Tumor infiltrating lymphocytes, or the mitotic index, is increasingly playing a more important prognostic role. These prognostic factors do not give us accurate information to predict melanoma behavior in an individual patient, the aggressiveness of the disease or the way of tumor disseminates. Melanoma does not behave or progress in the same manner and equally quickly in all patients and tumor can in addition stay in a state of tumor dormancy.

We do not have any clinical, histological, immunohistochemical, or molecular marker that would allow us to precisely identify the tumor characteristics concerning its behavior and patient's prognosis.

Other than morphological and histopathological biomarkers, an increasing number of biomarkers have been identified to provide us with more detailed prognostic information. Efforts are made in gene expression profiling, genomic hybridization, etc. to better understand the biological activity of melanoma and to use this information in new therapy development.

Tumor markers play an important role in all aspects of cancer care. Modern personalized medicine tends to use individual biomarkers to subdivide traditional tumor stages to subunits that behave in a different way. In melanoma, prognostic markers are needed to refine a risk of progression and predict an outcome. As melanoma is supposed to be a heterogeneous group of disorders, there is a need for individualization of melanoma diagnosis, prognosis and treatment. Melanoma biomarker research is an open field for understanding of molecular events in melanoma progression and should provide new molecular targets for therapeutic intervention (89) (90).

Unfortunately, there is no reliable biomarker in melanoma that would be used in clinical practice. Some European countries recommend determination of S100B or lactate

dehydrogenase in serum of patients with malignant melanoma, others do not support this process because of controversial results in different studies.
The search for new biomarkers that could potentially be used in clinical practice continues. As we can offer our patients new therapeutic modalities, there is a need for careful follow-up and patient monitoring and to predict the possible benefit from a therapy.

Our study has been performed in direct continuation to other tumor markers studies in our Immunoanalytical laboratory. These studies have been mainly related to breast cancer, colorectal cancer, prostate and ovarian cancer.

Our study represents one of few studies that present the broad multi-marker screening of serum/plasma different biomarkers using a novel xMAP technology. As biomarkers we used different proinflammatory, proliferative or proangiogenic factors that reflect the host response of patients with melanoma. We present the analysis of 14 tumor markers in well-defined groups of patients, who were participants in a prospective study. We selected these substances according to literature data in other cancers, most of these have not been examined in such a broad screening in precisely defined group of patients yet.

Tissue polypeptide specific antigen is a circulating complex of polypeptide fragments of cytokeratins that have been showed to correlate well with cell growth rate and tumor burden. This was confirmed in our study; TPS correlated with tumor size, there were statistically significant difference in serum levels when comparing the control and melanoma group and also increasing levels in the serum of patients with advanced melanoma in comparison to preoperative levels in melanoma patients. This observation can be explained by increasing serum levels of circulating cytokeratins fragment following tumor growth and extension. TPS have not been studied in melanoma patients , excluding the study of Barak et al. concerning the dynamics of serum tumor markers in predicting metastatic uveal melanoma, where TPS were not statistically significant (91). Some authors have demonstrated that TPS is a marker for proliferation of cells. Chen at al. have shown that higher preoperative expression of serum TPS is closely related to clinicopathological characteristics of breast cancer and overall survival. TPS was correlated with tumor size and lymph node metastases (92), similarly tour study. According to study from Ahn at al., preoperative TPS is a valuable biomarker for clinical use in predicting outcomes in breast cancer patients (93) . Concerning the results of studies

performed in our faculty hospital, TPS appears to be a suitable marker for NSCLC follow-up (94), cytokeratins are also elevated in patients with colorectal carcinoma and show association with response to primary therapy and prognosis (95), TPS can be also recommended as a good tool for differential diagnosis between liver metastases of breast cancer and benign liver lesions (96). Finally, TPS is an important predictive marker for OS an DFI after liver resections and radiofrequency ablations for colorectal liver metastases (97).

Thymidine kinase is an enzyme involved in DNA synthesis and its level and activity are dependent on the growth state and cell cycle phase. We have found no correlation in TK serum levels and studied parameters. TK have not been studied in malignant melanoma yet, excluding a study from Wu et al., who have found an increased TK serum level correlating with metastatic site in patients with melanoma. According to this study, TK might be involved in the deep lymphatic dissemination and progression of melanoma metastases. Patients involved in this study received both chemotherapy and immunotherapy for metastatic melanoma (98). In our study we have had only 2 patients with distant metastases so our results could not have been significant. Our results are also in discrepancy to the results in other studies in various carcinomas. A logical correlation between this marker and growth stage of the cell and tumor growth has been proven. The insufficient amount of patients with advanced melanoma involved in our study made these results impossible to explain. TK has been extensively studied in hematological malignancies where TK seemed to be a powerful discriminator of disease stage and to provide prognostic information. Some data is dedicated to problems in lung cancer, where TK was not confirmed as a tool for diagnosis or therapy monitoring, but it had a promising prognostic relevance (99). In breast or colorectal cancer research, TK has been found to play a potential role in cancer disease monitoring as was found in our faculty hospital as well (100) (101).

The S100 protein family consists of twenty members. They are multifunctional proteins expressed in a diverse spectrum of tissues. The protein S100B is the most studied member in malignant melanoma from this group and is considered to be the traditional biomarker in this cancer. Several studies have demonstrated that S100B concentrations are significantly related to clinical stage, are useful in treatment monitoring and increasing serum S100B level is an independent prognostic marker for overall survival and disease-free interval. The sensitivity of serum S100B in patients with stage I and II has been

reported to be 15% compared to 60-85% sensitivity for stage IV (102) (61) (62) (63). But further clinical trials have to be done to use S100B protein as tumor marker in routine clinical practice. In our research, we have studied S100A that has not been studied in melanoma yet, to our knowledge. Serum levels of S100A have correlated with lymphatic involvement, with Breslow thickness in advanced melanoma, with tumor ulceration, with localization of the tumor, and there were significantly higher serum levels in melanoma group compared to healthy controls. According to literature data, our results are identical to those presented in breast, colorectal, ovarian, lung or prostate cancer (60).

Osteopontin is an adhesive glycoprotein involved in tumor angiogenesis and bone turnover. High levels of osteopontin in variety of cancers are associated with poor prognosis, overall and disease-free interval are inversely related to osteopontin levels, there is a correlation with stage for early progression in lung, breast, prostate or liver cancer (88) (103) (104) (105) (106). Consistent with these observations that serum levels of osteopontin are useful tumor markers in a variety of cancers, we have found significant correlation in OPN serum levels and lymph node involvement as well as with positivity/negativity of sentinel lymph node. Serum levels were significantly elevated in patients with malignant melanoma compared to healthy donors. Increasing levels in serum of patients with advanced melanoma in comparison to preoperative levels in primary melanoma patients have been observed. Kadkol et al. and Barak et al. performed a study concerning metastatic uveal melanoma where serum levels of OPN were significantly higher in patients with metastatic melanoma compared with patients who were DF for 10 years and levels of metastatic patients were also significantly higher than those of the controls in conformity to our results (107) (87). Rangel et al. (108) has proven an association of high osteopontin expression and increased tumor thickness, OPN expression was also significantly predictive of sentinel lymph node metastases, confirming our results. We have not proven any association with Breslow thickness, this could probably be explained by the more accurate T groups distribution into “a” and “b” subgroups.

Osteoprotegerin is a potent proangiogenic factor, it regulates bone turnover and has additional roles in immune and vascular system. In our study, elevated OPG serum levels were found in melanoma group compared to healthy controls. No other important association was observed. In literature there is no study concerning OPG as potential biomarker in melanoma and few studies concerning OPG as biomarker in other cancers with controversial results. Martinetti et al. have not found any significant changes in OPG

serum levels during follow-up patients with advanced breast cancer treated with anastrozole, but there were short periods of follow-up and a small amount of patients included in this study (106). Tsukamoto et al. have found that overexpression of OPG was associated with significantly worse overall survival and relapse-free survival after curative resection in colorectal cancer (85).

We have found no important associations with the dynamics of epidermal growth factor serum levels and studied characteristics. There is limited data concerning EGF as a tumor marker in literature and only one study by Bracher et al. has evaluated EGF in melanoma, considering EGF an important factor in mediating melanoma lymph node metastasis (109).

Tumor progression involves malignant transformation in which increased production of growth factors and cytokines enable autonomous melanoma growth. Melanoma cell lines produce different factors e.g. bFGF, VEGF, IL6 or IL8 (110) (111) . These factors promote cell growth, migration, angiogenesis, and enable tumor to survive and metastasis. Some studies have shown significantly increased serum IL6, IL8 and IL10 in melanoma patients (112) (113). Elevated serum levels of IL6 has been associated as negative prognostic factor in patients with stage IV melanoma and is a predictive factor of overall survival (114) (115). In the study of Lugowska et al., the serum levels of IL8 have been found significantly higher in melanoma patients compared to the healthy group (116). Elevated serum levels of IL10 have been associated with metastatic melanoma (117). According to the first broad multi-marker study from Yurkovetsky et al., concentrations of IL6 and IL8 were significantly higher in melanoma patients compared to healthy controls and pretreatment levels of IL6 positively correlated with disease-free interval (80). According to Brennecke et al., low IL8 serum levels after chemotherapy indicate response to chemotherapy in stage IV melanoma (118). In our study we have found an association with pretreatment serum levels of IL2 and sentinel lymph node involvement. We have found elevated serum levels of IL2 and IL8 in the melanoma group compared to the healthy controls. Preoperative serum levels of IL6 positively correlated with Breslow thickness and tumor localization. We have found no important correlation of IL10 serum levels and studied variables.

Vascular endothelial growth factor is a potent angiogenic factor and some studies have established its critical role in carcinogenesis. VEGF is overexpressed in almost all solid cancers (119). Thy dynamics of VEGF serum levels have been studied in the vast majority of solid cancers and its prognostic value and correlation with tumor status have been

confirmed by several studies. In our study we have found no important correlation with measured variables and VEGF serum levels. This is supported by literature data where we have found quite controversial results in published studies concerning malignant melanoma. Boon et al. found no correlation with VEGF serum levels and Breslow thickness, Clark invasion level or ulceration but there was a correlation with sentinel lymph node involvement; these results were confirmed by Vihinen et al. or Lugowska et al.; according to Tas et al. circulating levels of VEGF were significantly influenced by Breslow thickness and were elevated in patients with melanoma compared to healthy controls; according to broad multi-marker study from Yurkovetsky et al., a statistically significant increase in concentration of VEGF was found in sera of melanoma patients compared to healthy donors, high dose immunotherapy decreased levels of VEGF and no predictive value of VEGF serum levels were found (80) (120) (121) (122) (116). Regarding prognostic value of VEGF, Ugurel et al. have found elevated serum levels of VEGF strongly correlated with poor overall survival and disease-free interval (123) (124) (125).

Insulin-like growth factors binding proteins are substances that regulate mutagenic and anti-apoptotic effects of insulin-like growth factors. IGFBP3 has been shown to inhibit cell proliferation in breast, lung and prostate cancer cells; it may act as potential tumor suppressor (126). The mechanism regarding the involvement of IGFBPs and IGF axis remain uncovered. High circulating IGF-1 levels or low IGFBP-3 levels are associated with increased risk of several cancers (127). Little and contrasting data has been published regarding the relationship between these molecules and melanoma.

16. Conclusion

We have followed up selected biomarkers before surgery and during follow-up in patients with malignant melanoma and in patients with advanced disease for three years.

1. Our study represents one of a few studies that present the broad multi-marker screening of serum/plasma different biomarkers using novel xMAP technology.

2. We used different proinflammatory, proliferative or proangiogenic factors as biomarkers; these reflect the host response of patients with melanoma.

3. The correlation of protein S100A serum concentration with the tumor load, lymph node status and clinical prognostic information such as Breslow thickness, ulceration or tumor localization, makes it a useful tumor marker for follow-up patients after radical surgery and during subsequent treatment.

4. Serum levels of tissue polypeptide specific antigen have also correlated with tumor load and were increased in advanced melanoma compared to preoperative levels in primary melanoma. These results determine its use as a tumor marker in follow-up patients. Differences in protein S100A and tissue polypeptide specific antigen profiles between melanoma patients and healthy subjects allowed for discrimination between these two groups. No other proliferative markers in our study reflected any association with studied variables.

5. As for angiogenic factors reflected in the presented study - we found no association between serum levels of vascular endothelial factor, or basic fibroblast factor, and the studied parameters. Increasing osteopontin expression has been identified as a powerful predictor of sentinel lymph node involvement. Serum levels were correlated with lymph node status and higher serum levels were observed in advanced melanoma compared to preoperative levels in primary melanoma. This makes it a useful tumor marker for follow-up patients after radical surgery and during subsequent treatment. Differences in osteopontin and osteoprotegerin

profiles between melanoma patients and healthy subjects allowed us to differentiate these two groups.

6. Dynamic study of serum levels of interleukins have shown that serum levels of interleukin-2 correlated with sentinel lymph node positivity/negativity in preoperative levels and preoperative serum levels of interleukin-6 correlated with Breslow thickness or tumor localization. These results determine their use as prognostic markers. Interleukin-8 have been found to be elevated in melanoma group compared to the healthy controls.

7. Insulin-like growth factor reflected tumor load and was elevated in melanoma patients compared to healthy controls in our study.

8. As for sensitivity and specificity of studied markers - the ROC curves did not highlight any acceptable concentration.

9. We have proven the use of multiplex technology as a powerful tool in cancer monitoring and for research purposes.

10. We can recommend the use of protein S100A, tissue polypeptide specific antigen, osteopontin, osteoprotegerin, interleukin-2,6,8 or insulin-like growth factors as potentially useful biomarkers. Protein S100A and osteopontin were the substances that accurately reflected the biological activity of malignant melanoma. Their elevated serum/plasma levels reflected tumor load, angiogenic potential or tumor aggressiveness.

11. The search for an ideal circulating marker for malignant melanoma continues. The research in the field of tumor markers do not allow only a detailed prognostic information that is necessary for stratified patient's care and therapy, but also allow better understanding of the nature of malignancy.

12. Further research of the biomarkers may identify a population of melanoma patients who would be in high risk of cancer progression and would benefit the most of new therapeutic approaches.

17. Selected pictures of patients involved in our study

In figure 10 to 25 there are some examples of malignant melanoma in patients involved in our study and having surgery at the Department of plastic surgery.

Figure 10 – Malignant melanoma of the Face, pT1aNxMx

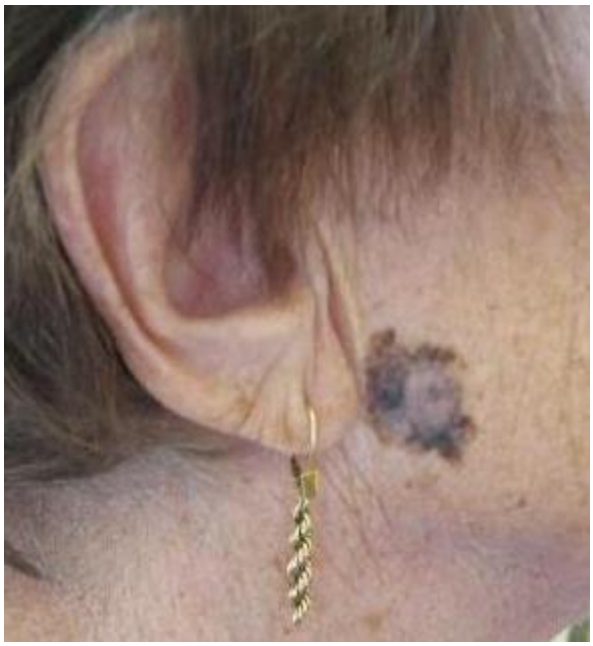


Figure 11 – Malignant melanoma of the trunk, pT1aNxMx

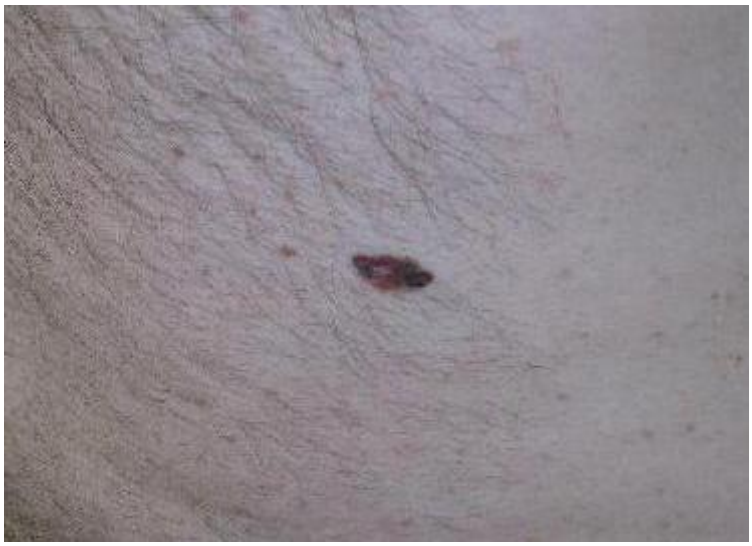


Figure 12 – Malignant melanoma of the back, pT1aN0Mx

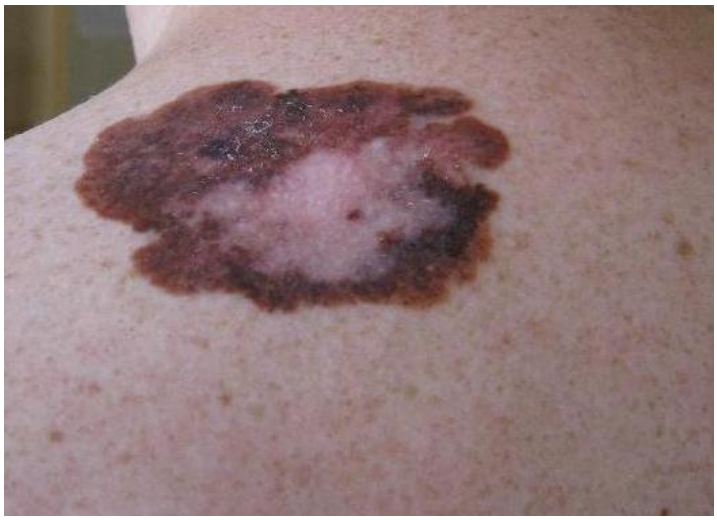


Figure 13 – Malignant melanoma of the lower limb, pT1aN0Mx

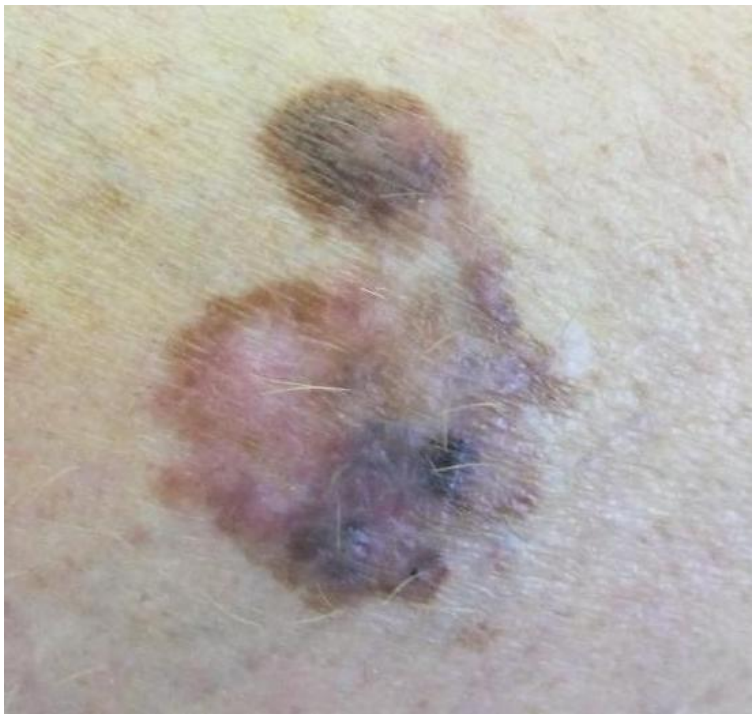


Figure 14 – Malignant melanoma of the trunk, pT1bN0Mx

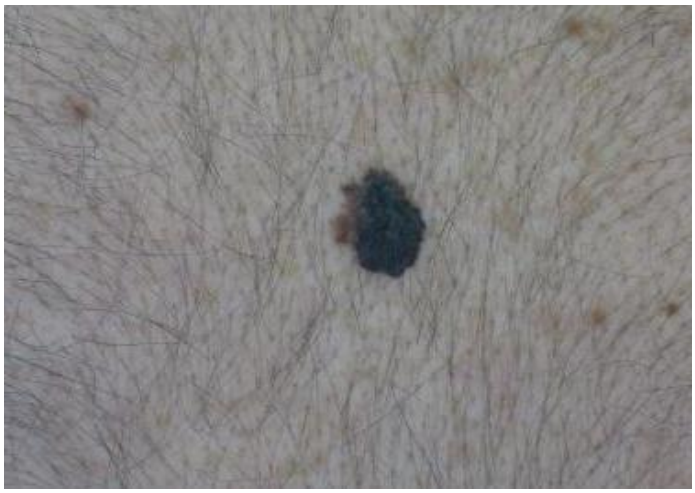


Figure 15 – Malignant melanoma of the lower limb, pT2pN2M0

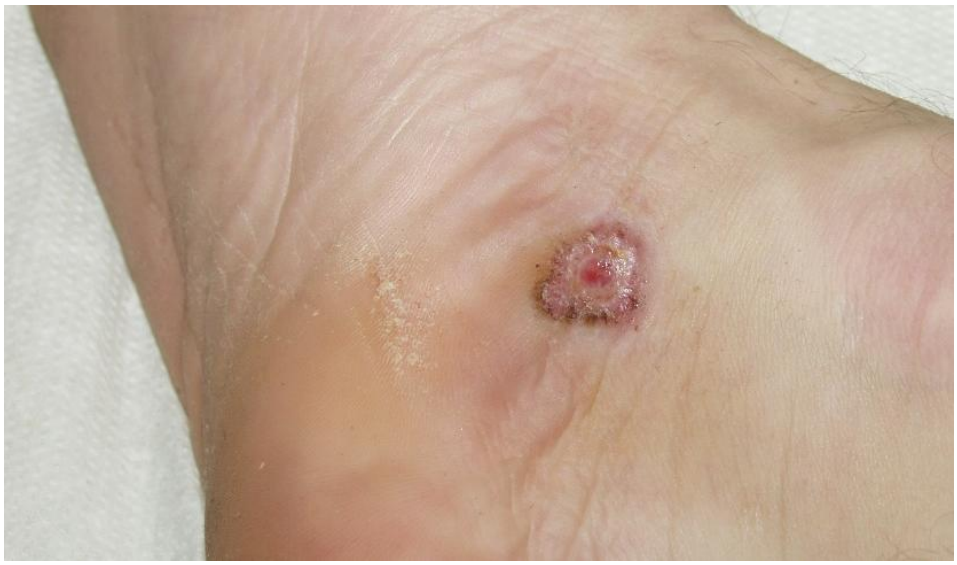


Figure 16 – Malignant melanoma of the back, pT3bN0Mx

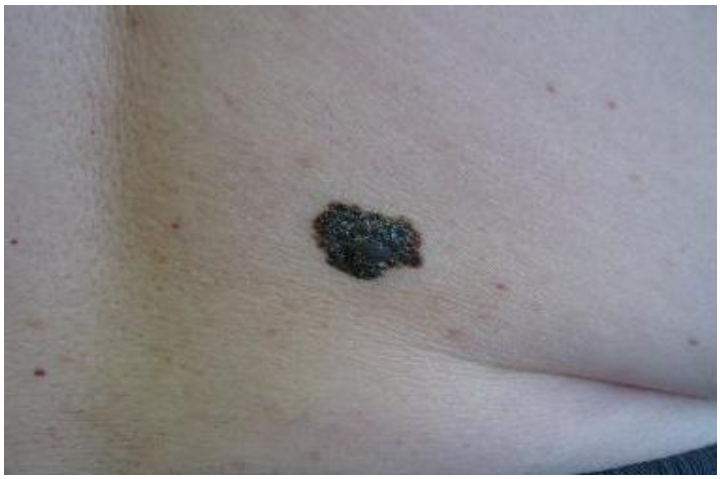


Figure 17 – Malignant melanoma of the back, pT3bN2Mx

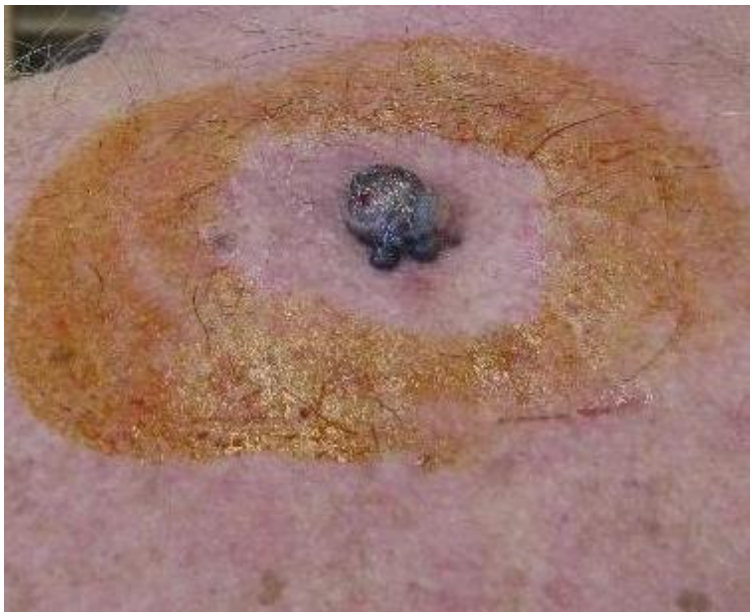


Figure 18 – Acrolentiginous malignant melanoma, pT3aN0Mx

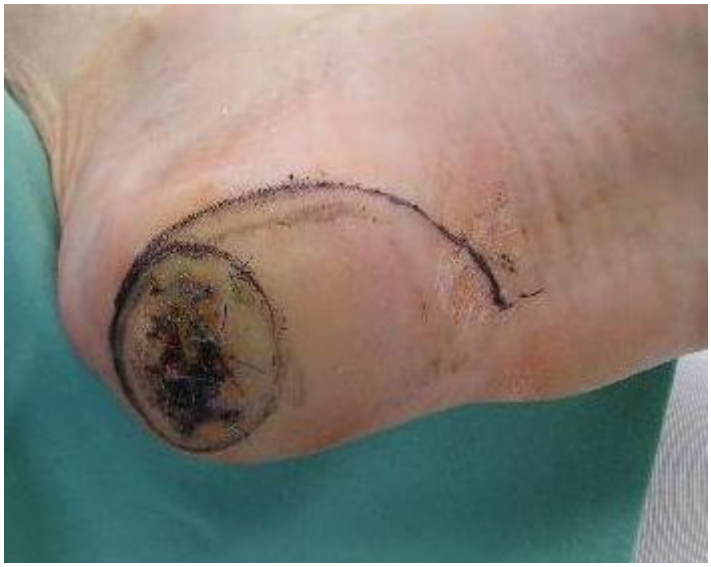


Figure 19 – Malignant melanoma of the back, pT4aN1Mx

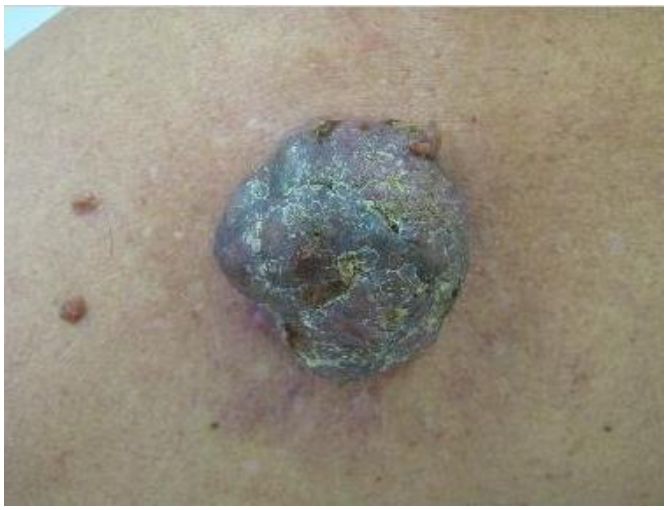


Figure 20 – Malignant melanoma of the back, pT4bN1Mx

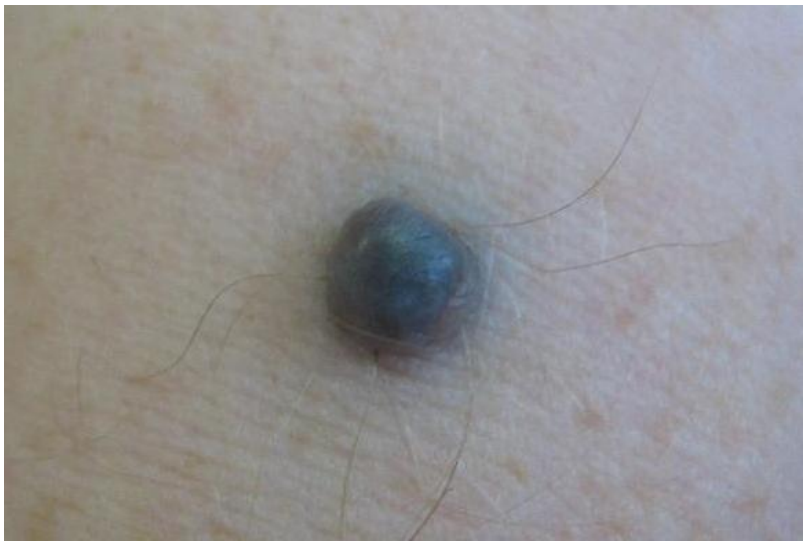


Figure 21 – Malignant melanoma of the back, pT4bNxMx

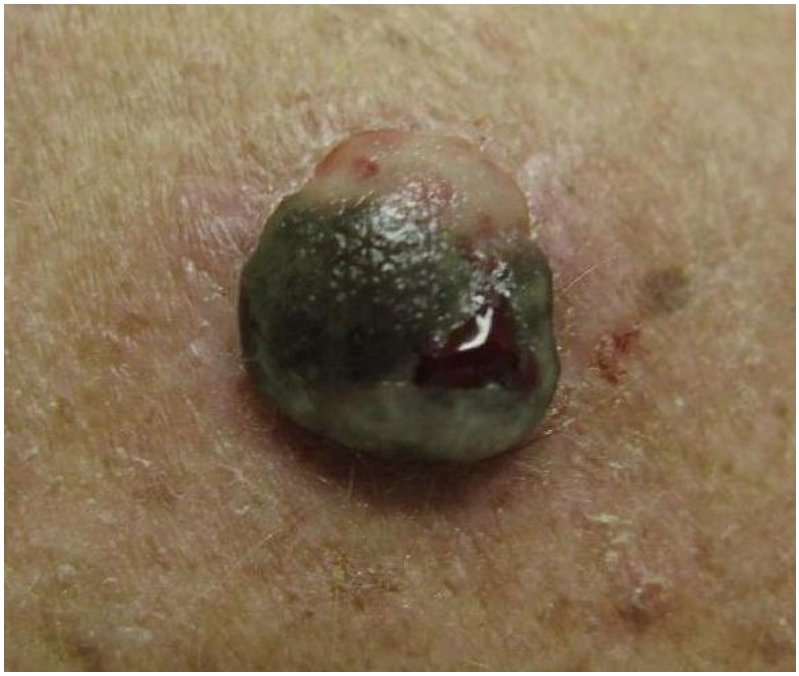


Figure 22 – Malignant melanoma of the back, pT4bN1Mx

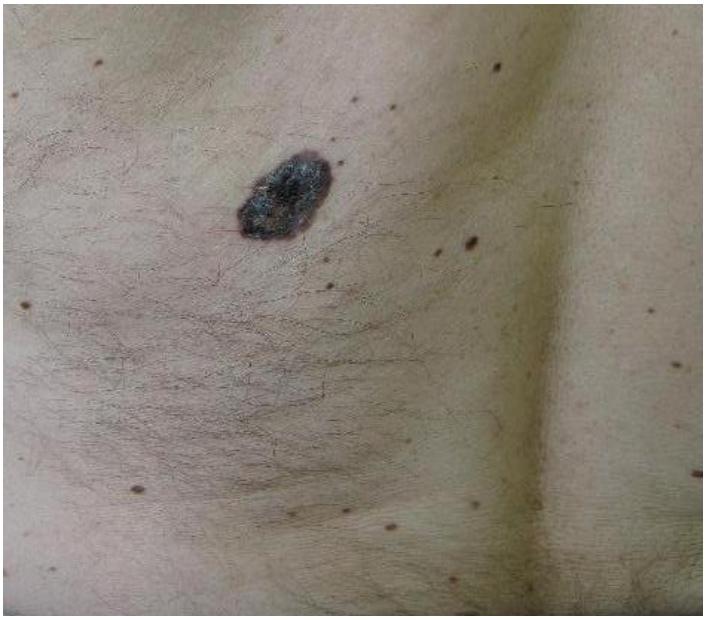


Figure 23 – Acrolentiginous malignant melanoma, pT4bN0Mx

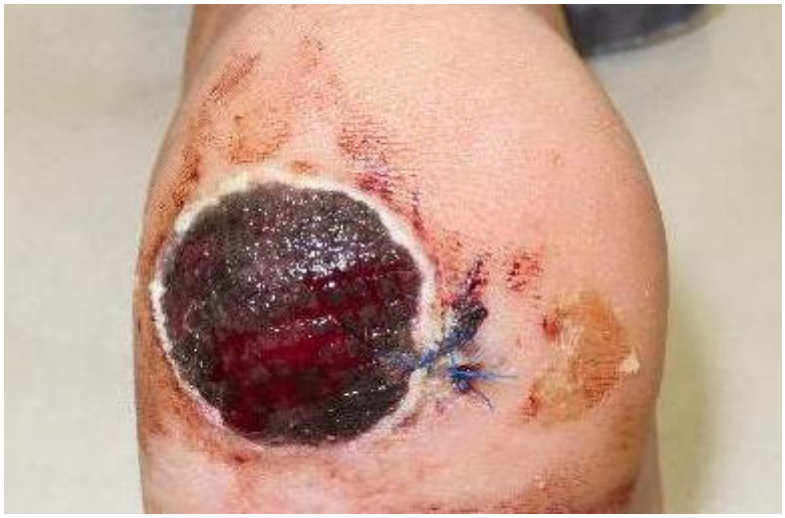


Figure 24 – Malignant melanoma of the back, pT4bNxMx

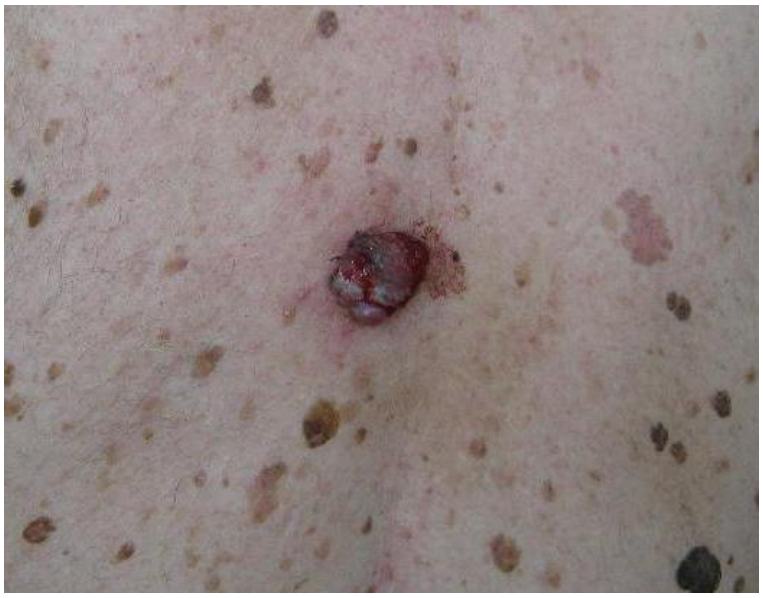
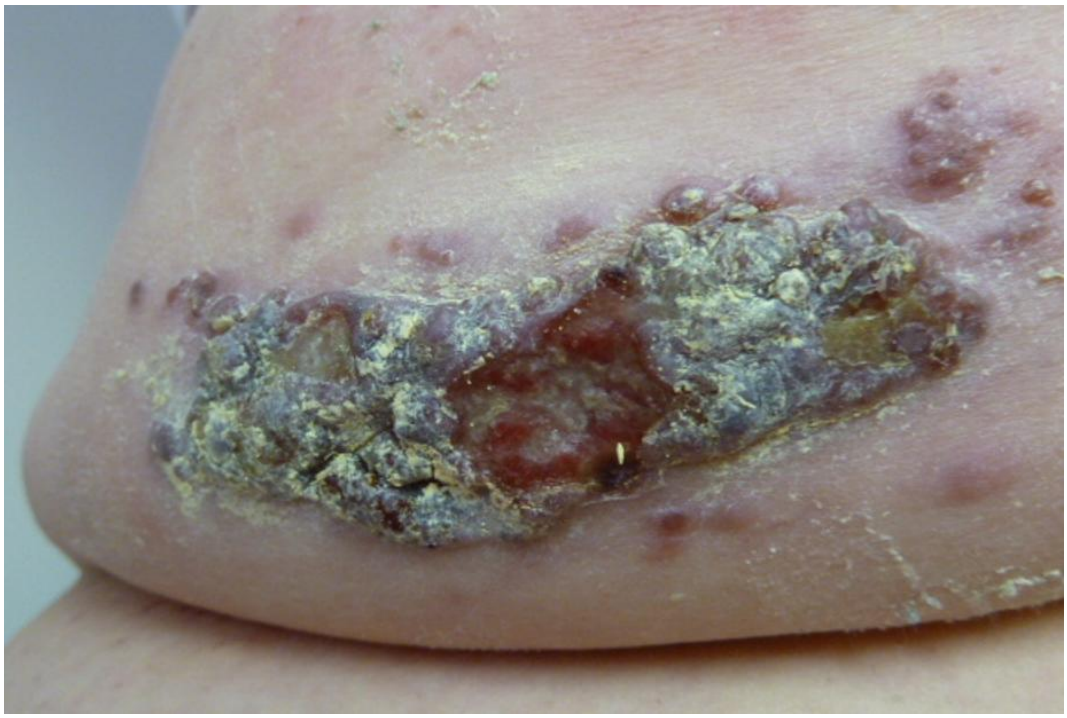


Figure 25 – Malignant melanoma of the back, pT4aNxM1



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19. Citations of author

a) Publications

Publications - author – article under revision

1. Česko-slovenská dermatologie – Prognostický význam osteoprotegerinu a osteopontinu u maligního melanomu
2. Rozhledy v chirurgii – Obtížná diferenciální diagnostika maligního melanomu - kazuistiky

Publications - coauthor

1. Complications of breast augmentation - a case report. Rozhl Chir. 2012 Aug;91(8):435-7. Czech. PubMed PMID: 23153428.
2. Spectrum of cutaneous and soft tissue lesions in two Carney complex patients-adnexal induction versus authentic adnexal neoplasms. Am J Dermatopathol. 2012 Oct;34(7):729-36. PubMed PMID: 22588545.
3. Non-colorectal liver metastases: surgical treatment options. Hepatogastroenterology. 2012 Jan-Feb;59(113):245-8. doi: 10.5754/hge10292. PubMed PMID: 22251545.
4. Growth factors and breast tumors, comparison of selected growth factors with traditional tumor markers. Anticancer Res. 2011 Dec;31(12):4653-6. PubMed PMID: 22199345.
5. Vitamin D in colorectal, breast, prostate and lung cancer: a pilot study. Anticancer Res. 2011 Oct;31(10):3619-21. PubMed PMID: 21965787.
6. Plasmatic levels of proinflammatory cytokines in abdominal aortic aneurysms. Rozhl Chir. 2011 Jan;90(1):37-41. Czech. PubMed PMID: 21634132.
7. Predictive value of serum biomarkers in patients after portal vein embolization (PVE): a pilot study. Anticancer Res. 2011 Jan;31(1):339-44. PubMed PMID: 21273621.
8. Abdominal aortic aneurysms--long-term treatment results. Rozhl Chir. 2010 May;89(5):300-5. Czech. PubMed PMID: 20666333.
9. Liver metastases of other than colorectal origin. Rozhl Chir. 2010 Mar;89(3):202-7. Czech. PubMed PMID: 20514918.

10. Ischemia-reperfusion injury in kidney transplantation from non-heart beating donor--do antioxidants or antiinflammatory drugs play any role?. Rozhl Chir. 2009 Feb;88(2):65-8. Czech. PubMed PMID: 19413262.

b) **Oral presentations - author**

1. CLAS annual meeting Boston, USA 10/2009
   The significance of perioperative tumor markers serum levels for the prognosis of patients with liver metastases
2. Postgraduální lékařské dny Plzeň 9.-11.2.2010
   Využití nádorových markerů u melanomu
3. Kongres české společnosti plastické chirurgie Harrachov 23.-24.4.2010
   Nádorové markery u maligního melanomu
4. XXXI.Imunoanalytické dny 2010, X.Cechtuma 2010 Mikulov 16.-18.5.2010
   Malignant melanoma and tumor markers
5. Perspectives in melanoma XIV 17.-18.9.2010 Amsterdam, Nizozemsko
   The changes of thymidine kinase levels in malignant melanoma - poster
6. International conference on biomarkers and clinical research Santa Clara, USA 22.-23.11.2010
   Monototal-prognosis and therapy control in patients with non small cell lung cancer
7. Diagnostika, léčba a prevence závažných civilizačních onemocnění Plzeň 25.11.2010
   Využití nádorových markerů u maligního melanomu - poster
8. XXXII.Imunoanalytické dny 2011 Karlovy Vary 8.-10.4.2011
   Stanovení biologické aktivity maligního melanomu pomocí X-map technologie
9. 1st Chinese european congress of plastic, reconstructive and aesthetic surgery 27.-29.10.2011 Peking Čína
   Prognostic factors of malignant melanoma – pilot study
10. Mezinárodní sympozium české společnosti plastické chirurgie Plzeň 17.-19.5.2012
    Prognostické faktory u maligního melanomu
11. 10th IQUAM congress and consensus conference Athens, Řecko 1.-4.11.2012
    Prognostic factors in malignant melanoma

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