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PROGNOSTICKÉ FAKTORY U MALIGNÍHO MELANOMU

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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.

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4. Introduction

Malignant melanoma is as old as humanity itself. 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).

5. List of current knowledge

Malignant melanoma currently represents a serious medical problem worldwide (especially in the Caucasian population) (1). With the rapid increase of melanoma over the last decades, melanoma has come to be considered an epidemic cancer in these areas (4). Melanoma incidences have continuously increased over the last 30 years. In contrast, melanoma mortality rates have not increased as dramatically as the rate of diagnosed incidence (5) (6). The incidence rate of melanoma has been increasing by about 5 percent per year (7). Over the past 40 years, the incidence of CM in the Czech Republic has risen by more than 600% (8).

There are many factors influence melanoma development. Genotype, phenotype and environmental factors play their roles in this process (1). Sun exposure is the only factor that significantly influences the development of melanoma (9)(10). No specific gene has so far been discovered as being responsible for melanoma (11).

Cancer is characterized by unregulated cell growth of autonomous nature with impaired regulatory mechanisms of cell proliferation, altered cell differentiation and inhibition of apoptosis (12).

Melanoma has two growth phases, radial and vertical (4). We distinguish lentigo maligna melanoma Dubreuilh (13), superficial spreading melanoma (14), nodular melanoma (15), acral lentiginous melanoma, desmoplastic and mucosal melanoma (16).

Skin disorders are easily recognized by simple inspection. The precise diagnosis and early detection of melanoma significantly improves 5-year survival rates (17) (18). The exact diagnosis is made by lesion biopsy (19).

Understanding the correlations between the prognostic factors and biology of the disease is a major objective of melanoma research (20). The main prognostic factors are: age, sex, anatomical site, Breslow thickness, ulceration, regression, mitotic rate, microsatellites, lymph node involvement and tumor infiltrating lymphocytes (21)(22)(23)(24). The status of the sentinel lymph node is the most important prognostic factor for recurrence and survival (14).

Formal staging of cancer is fundamental in providing clinicians with prognostic information, developing treatment strategies, and directing and analyzing clinical trials (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)(26).

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. Sentinel lymph node biopsy identify patients with occult nodal metastasis and expectedly poorer outcome that could benefit from a completion of nodal dissection and evaluation for systemic adjuvant therapies. 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 (27) (28) (29) (30) (31).

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 (33)(34).

Surgery is also the treatment of choice for single or few local or regional metastases (35) (36). Surgery can also be used as a palliative option for carefully selected patients with symptomatic metastases (37).

Locally invasive melanomas bring risks of local and distant relapse (38). The role of radiation therapy as primary or adjuvant treatment for melanoma is controversial (39) (40).

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). Interferon alpha is the major drug that has been considered for adjuvant therapy. 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) (43).

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 interaction of the immune system with the tumor shows a promising pathway for intervention (45).

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 (46).

A number of immunotherapy trials were conducted in recent years (47). IFN-α is discussed above, IL-2 is a potent immune modulator that stimulates activation and proliferation of T-lymphocytes (48).

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).

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

Trials on novel investigational therapies are currently ongoing. 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).

6. Tumor markers

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 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, tumor-associated antigens, enzymes, hormones, special serum proteins, miscellaneous markers (54). The diagnostic efficiency of tumor marker examination depends on variety of factors such as sensitivity, specificity, positive predictive value, and negative predictive value. (55) (56). 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 (57) (58). At the present moment, no ideal biomarker exists in the field of melanoma (59).

7. Tumor markers in our study

a) **Protein S100A**

S100 proteins have been implicated in many intracellular and extracellular functions such as cell growth and differentiation, cell cycle progression, transcription, inflammatory response, etc. (60). 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. TK1 is a useful marker for cell proliferation and hence for malignancy (64) (65). The serum levels of TK 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)

Tissue polypeptide antigen is a circulating complex of polypeptide fragments of cytokeratins 8,18 and 19. 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 (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 (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. 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. 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 (81). Osteoprotegerin / osteoprotegerin ligand pathway is a key regulator of bone metabolism through its effect on development and activation of osteoclasts (82). Several studies have demonstrated the involvement of OPG in vascular complications. It increases endothelial cell survival, proliferation and migration, as well as endothelial cell formation in angiogenesis (82) (83). A number of studies have been performed assessing the role of OPG in tumorigenesis (84). OPG has an important role in tumor angiogenesis, a key process in cancer development and metastasis. 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. OPN is an important factor in bone remodeling (86). OPN actively promotes the tumorigenic phenotype and contributes to metastasis. 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).

8. 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.

9. The patients and methods

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.

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 melanoma tissue was assessed by a histopathologist at the Department of Pathology.

b) **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. 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. 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. A statistical analysis was carried out.

10. 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 1).

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 1).

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 1).

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 2).

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 2).

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 2).

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 3).

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 4).

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 5).

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 6).

According to our results, only protein S100A positively correlated with presented tumor ulceration, $p<0.01$. No other interesting associations have been found (Table 7).

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 8).

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 9).

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.

11. Tables associated with results

Table 1 - 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 2 - 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 3 - 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 4 - 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 5 - 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 6 - 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 7 - 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 8 - 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 9 - 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

12. 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.

13. 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.

14. References

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15. 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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