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Gangliosides and Antigangliosides in Malignant Melanoma

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1. Introduction

Cutaneous malignant melanoma is the most aggressive skin cancer with rising incidence in the last years [1]. Nowadays, the only treatment to cure melanoma is the early diagnosis and surgical removal of the primer tumor. New research directions developed in order to discover markers for early detection and therapeutic response of melanoma, treatments that could improve the survival rate. Recent studies showed that melanoma is a heterogeneous group of complex molecular disorders [2-16]. The diversity of these alterations sustain the importance of, on one hand, an individualized diagnosis, prognosis and treatment of melanoma patients, and, on the other hand, the detection of new biomarkers and therapeutic approaches in these patients.

The development of new research and investigation techniques in the last years, offered information regarding pathogenesis of melanoma. The skin is considered a hypoxic organ and the low level of oxygen induces the transcription of some hypoxic markers. The cells respond to hypoxia by stimulating the synthesis of some heterodimeric factors, composed by alpha inducible subunit (hypoxia inducible factor alpha-HIF alpha) and beta subunit (aryl hydrocarbon receptor nuclear transducer ARNT). Cutaneous melanocytes, from the dermic-epidermic junction, lay in a low oxygen medium [16, 17]. Tissue hypoxia can modify cellular behavior by direct influence on: cell cycle, cellular metabolism, differentiation, proliferation and survival, degradation and remodeling of extracellular matrix, tumor migration, invasion and metastasis, angiogenesis, apoptosis, cells sensitivity to antitumor therapy (Table 1) [16-29]. Cells ability to adapt to hypoxia is mediated by several transcription factors. A major role in cells response to hypoxia is played by HIF 1 alpha. Its expression and tissue distribution are influenced by many factors [3, 30-38]: modulators of cellular degradation (EPF, UCP, VDU2, Sumoylation, DeSUMOylation, Prolyl hydroxylases, PVLH, OS-9, SSAT 1, SSAT 2, GSK3 beta,

FOXO 4, Calcineurin A), modulators of translation (RNA-binding protein, PTB and HuR, PtdIns3k and MAPK pathways, IRES-mediated translation, calcium signaling bng, miRNA).

Function	Gene
Cell metabolism	Iron - Erythropoietin (EPO); Transferrin; Transferrin receptor (TfR); Ceruloplasmin pH - Carbonic anhydrase-9, 12 Nucleotide - Adenylate kinase; Ecto-5-nucleotidase Aminoacids - Transglutaminase 2; Glucose - Adenylate kinase; Aldolase A,C (ALDA,C); Carbonic anhydrase-9,-12; Enolase- 1 (ENO1); Glucose transporter-,1,3 (GLU1,3); Glyceraldehyde phosphate dehydrogenase (GAPDH); Hexokinase1,2 (HK1,2); Lactate dehydrogenase-A (LDHA); Pyruvate kinase M (PKM); Phosphofructokinase L (PFKL); Phosphoglycerate kinase 1(PGK1); 6-phosphofructo-2-kinase/fructose-2,6-bisphosphonate-3 (PFKFB3). Lipids -Sphingosine kinase 1 (Sphk 1); Lpin 1.
Matrix metabolism	Matrix metalloproteinases (MMPs) ; Plasminogen activator receptors and inhibitors (PAIs); Collagen prolyl hidroxylyase .
Cytoskeletal structure	Keratin 14,18,19 (KRT 14,18,19); Vimentin
Mitochondrial respiration	Pyruvate dehydrogenase kinase1 (PDK1) ; Monocarboxylate transporter (MCT4) ; Cytochrome c oxidase (COX1-4).
Transcriptional regulation	Differentiated embryo-chondrocyte expressed gene 1,2 (DEC1,20; Nuclear receptor 77 (NUR77); v-ets erythroblastosis virus E26 oncogene homolog1 (ETS1).
Vascular tone	Nitric oxide synthase2 (NOS2); Heme oxigenase ; Endothelin 1 (ET1) ; Adrenomedullin (ADM); Alpha-,beta-adrenergic receptor
Stem cells	KLF4; Nanog; Oct-3/4; Oct-4A,SOX2; Wnt/beta-catenin.
Cell proliferation/ survival	Insulin-like growth factor-2 (IGF2) ; IGF-factor binding protein 1,2,3 (IGF-BP1,2,3); Adrenomedulin (ADM); Coiled-coil-DIX1 (CCD1); Transforming growth factor alpha,beta (TGF alpha,beta); Cyclin G2; Survivin ; Notch-1; Nitric oxide synthase 2 (NOS2); P21; Erythropoietin.
Cell differentiation	Notch signal activation; Tertiary complex p300/HIF1/STAT3/ROR gamma; Multistructural complex Foxp3/pVHLE3/HIF1/nUb
Cell migration/invasion	Collagen V; Autocrine motility factor/ glucose-6-phosphate isomerase (AMF/GPI); Cathepsin D(CATHD); Integrin-linked kinase; Integrins; Lysyloxidase (LOX); Plasminogen activator receptor and inhibitor1 (PAI1); LDL receptor-related protein1 (LRP1); Microneme protein2/ CD99 (MIC2/CD99); Fibronectin; Urokinase plasminogen activator receptor (UPAR); Proto-oncogenes c-MET; Chemokine receptor type4 (CXCR4) ; MMP2
Angiogenesis	Proangiogenic - Vascular endothelial growth factor/receptors (VEGF, Flt-1 /VEGfR-1, Kdr/VEGF-R2); Endocrine-gland-derived VEGF (EG-VEGF); Leptin (LEP); Transforming growth factor –beta 3 (TGF-beta3); Angiopoietin 1,2 (Ang-1,2); TEK- tyrosine kinase endothelial (Tie-2); Adrenomedullin (ADM) ; Fibroblast growth factor (FGFs) ; Placenta

Function	Gene
	<p>growth factor (PLGF); Platelet-derived growth factor- beta (PDGF); Stem cell factor (SCF); Osteopontin; Plasminogen activator receptor and inhibitor -1 (PAI-1); Matrix metalloproteinases (MMPs); Tissue inhibitor of metalloproteinases (TIMPs); Nitric oxide synthase (NOS2); Cyclo-oxygenase-2 (COX-2); Endoglin; Alpha, beta-Adrenergic receptor; Endothelin-1; Semaphorin-4D; Integrins; Leptin; Endosialin; Adenosine A2A receptor ; Oxygen regulated protein-150; Stromal derived growth factor-1 (SDF-1); Interleukins (IL 1, 2, 4, 6, 8, 10).</p> <p>Antiangiogenic - Delta-like ligand 1-4 (DLL1-4); Carbonic anhydrase-9; Vasohibin-1; Thrombospondin-1; Regulator of G-protein signaling 5; Angiostatin; Endostatin; Canstatin; Interferons (alpha, beta, gamma).</p> <p>Neovessel formation - FGFs/FGFRs; PDGF-B/PDGFR; PLGF/VEGFR1(Flt-1); Thrombospondin-1/CD36,CD47,integrins; Integrins/Extracellular matrix; SDF-1/CXCR4L SCF/c-kit; DLL1-4/Notch; Interleukins/Interleukin receptors; Wnt/beta-catenin.</p> <p>Cell maturation - Vasohibin-1/vasohibin receptors; Endothelin receptors(Et-A,Et-B); Angiopoietin1/Tie-2; PDGF-B/PDGFR</p>
Cell apoptosis	<p>Bcl2/adenovirus E1B19KD-interacting protein3 (BNip3); Nip3-like protein X(NIX); Regulated in development and DNA damage responses1 (RTP801/REDD1); Redox factor-1 (Ref-1); Nuclear factor kappa B (NFkB); Heat shock protein 70 (HSP70); P53; Bax; Puma/ Bax; Bcl-2-Interacting Domain (BID).</p>
Metastasis	<p>Twist upregulation; CXCL chemokine CCL2/ JE/MCP-1; HGF-R/c-MET; CTGF/CCN2; ZEB1; TCF-3/E2A; Snail; MKP-1; MMPs upregulation; E-cadherin downregulation.</p>
Drug metabolism	<p>Multidrug resistance gene/P-glycoprotein (MDR1/P-GP); Multidrug resistance – associated protein1 (MRP1).</p>

Table 1. Representative target genes of Hypoxia-Inducible Factor1 (HIF1) and their functions [17-29].

Post-translational changes (polyubiquitination by PVHL-signaling for degradation, lysine acetylation by ARD1-facilitating PVHL binding) influence HIF activity. Biological disponibility of HIF 1 alpha might be altered by enviromental regulators (nickel, cobalt, arsenite, chromium, cadmium, desferroxamine, cigarette smoking, UVB, cytokines, hormones, oncogenes). Alterations in HIF-signaling pathways have major role in initiation and progression of malignant tumors (Table 1) [17-38]. Recent studies confirmed that HIF 1 alpha controls the expression of some genes involved in melanoma biology [39, 40]. Changes in lipids biochemistry associated with HIF overexpression were described in different types of neoplastic cells. Hypoxia promotes synthesis of free fatty acids, cholesterol, phospholipids, hormones, prostaglandins, leucotriens, sphingolipids. Cellular lipid uptake is increased in hypoxic conditions due to the interactions between lipids and hypoxia signaling pathways [41, 42]. These imbalances induce alteration of cell cycle, cellular proliferation, apoptosis, signal transduction, or alteration of antigenic structure of cellular membranes. Some studies reported the expression of an extensive adipose gene in pVHL cancer, in which HIF is constitutively activated [43]. Hypoxia inducible genes influence lipid droplet formation (hypoxia-inducible

protein 2), prostaglandin biosynthesis (cyclooxygenase 2), lipid signaling systems (lipoxigenase 12- lox , sphingosine kinase, SphK1) and synthetic processes (stearoyl-CoA desaturase-1, a rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids) [44-47]. Some of these genes are direct targets of HIF, while others are up-regulated by transcriptional factors HIF-dependent. Hypoxia interacts with transcriptional networks involved in lipid metabolism, including sterol response element binding proteins (SREBPs), DEC1/2 and GATA2/3 [9-11, 48-52].

Gangliosides form lipoproteic domains in cells membrane and affect series of fundamental biological processes like cell signal transduction, cell growth and differentiation, immune responses, cell transformation and degradation. Reference researches about gangliosides system in malignant tumors offer new data, useful for a better understanding of melanoma biology. Melanoma development needs high levels of oxygen and substances over the offer of the existent blood vessels. Angiogenesis is promoted under these conditions [10, 51]. The main stimuli of the angiogenic switch are: HIF 1 α , low pH, hypoglycemia, presence of reactive oxygen/nitrogen species, inflammation. In hypoxic conditions, melanoma cells release proangiogenesis (VEGFs, FGFs, PDGF, HGF, TGF, TNF, PGF, Ang-1, IL8) and antiangiogenesis (trombospondin, angiostatin, endostatin, Ang2, IFNs, IL12, fibronectin, TIMPs, PAI-1, dopamine, retinoic acid, vitamin D) factors [56-58]. These molecules interact with RTKs signaling pathway from melanoma cells, endothelial cells, pericytes, immune cells. Gangliosides and their degradation products could influence angiogenesis in malignant melanoma by multiple mechanisms [59-61]. Monosialogangliosides and gangliosides in b series counteract the effects of proangiogenic growth factors, while complex gangliosides control endothelial cell response to the action of proangiogenic factors. Lisogangliosides and sphingosine inhibit the activity of protein-kinase associated growth factors, GM1 and GM3 inactivate PDGFR, GD1a and GD1b inactivate PKC, GM3 inhibits EGFR, GD1a and GQ1b intensify the activity of calcium/calmoduline dependent kinase [54, 61].

Gangliosides control cell differentiation and proliferation [62-64]. Monosialogangliosides suppress cell growth (GM1-MMP9, GM1-PDGF, GM1-NGF, GM3-EGF, GM3-IGFs, GM2-pFAK), while polysialogangliosides stimulate cell growth (GD3 binding integrin, p-FAK, pp130Cas, p-paxilin, p-Yes or GD2 binding p-FAK, p-p38, c-Met) [65]. The transduction mechanism is complex including complex signaling pathways (p38-MAPK, Erk1/2, p13k/AKT, JNK1/2/3) [65-70]. Gangliosides act as inhibitors or promoters of cell apoptosis in melanoma [71, 72]. GD3 induces apoptosis by activating caspases and stimulates production of reactive oxygen species (ROS). GD3 acetylation cancels apoptotic effect of GD3, conferring resistance to antitumor therapy [73, 74]. GM3 induces apoptosis by intracellular accumulation of ceramide; GM1 stimulates apoptosis by disrupting the flow of intramitochondrial calcium [73-75].

GD1b, GT1b, GQ1b have inhibitor effects on adenylate cyclase in peripheral T cells and suppress the production of cytokines, controlling humoral immune response (IL4, IL5) [76]. GM1 and GD1a inhibit the activity of protein-kinases involved in the immune response. GQ1b promotes production of immunoglobulins [74, 77, 78]. Anti-GD2 antibodies modify integrin conformation through signaling pathways FAK, ERK, p38/MAPK [74, 77, 78].

Gangliosides influence anticancerous therapy through several mechanisms [51, 79-85] i) overexpression of proangiogenic factors; ii) functional mutations in tumor suppression genes; iii) overexpression of some oncogenes conferring resistance to hypoxia, and though instability of genes and inhibition of apoptosis; iv) impaired detoxification and DNA repair mechanisms; v) mutations of endothelial cells; vi) selective activation of genes associated with melanoma resistance to apoptosis; vii) cells inability to accumulate sphingosine, sphinganine and ceramide; viii) ability of melanoma cells to acetylate gangliosides.

In a recent study the authors showed that malignant phenotype was associated with overexpression of ganglioside sialic acid on the membranes of melanoma cells. The tissue level of ganglioside sialic acid was correlated with histological markers of melanoma (Breslow index, Clark level, presence/absence of ulceration) [53]. The gangliosides identified in melanoma were complex (Fig. 1) and they could be used as a differentiation marker between normal and malignant tissue [55]. The model of membranous gangliosides was characteristic for a cell type and for a tumor stage. The observed gangliosides in malignant melanoma were: $GD3>GM3>GD2>GM2>O-Ac$ $GD3>GD1a>GT1b>GD1b>GQ1b>GM1$; in dysplastic nevi: $GD3>GM3>GD2>GM2>GM1$; in healthy tissue around the tumor: $GM3>GM2>GD3>GD2>GM1$. The transition from radial to vertical growth of melanoma was followed by high synthesis of polysialogangliosides [53].

The compositional analysis of gangliosides in melanocytic tumors showed that aberrant glycosylation of sphingolipids could stimulate or inhibit invasion and metastasis of malignant tumor cells. High levels of monosialogangliosides could be found in benign tumors with slight evolution and in healthy tissue. Polysialogangliosides had high levels in malignant proliferations with quick and irregular evolution. A special attention was given to gangliosides acetylation in malignant melanoma. Acetylated glycosphingolipids were determined only in melanoma cells with vertical development. O-acetylation was selectively on disialogangliosides and was associated with metastasis of malignant melanoma [53].

Another research subject was the immunogenic potential of melanoma-associated gangliosides. Several tumor gangliosides induced the synthesis of antiganglioside antibodies in melanoma patients [9, 85-92]. The role of these antibodies is a subject of great current interest in medical research [9, 10, 78, 90, 93]. The data regarding endogenous immune response against gangliosides in melanoma patients and its pathophysiological relevance in management of melanoma were the main interests of the authors.

The **objective** of the study was to evaluate the role of gangliosides and antiganglioside antibodies in detection, staging and progression of cutaneous malignant melanoma. We aimed to determine the status of serum gangliosides and antiganglioside antibodies in patients with untreated malignant melanoma compared with patients with dysplastic nevi and control, their variation with surgical removal of the tumor and the relation between these parameters and some histological/biochemical factors used for melanoma staging (accepted by American Joint Committee of Cancer).

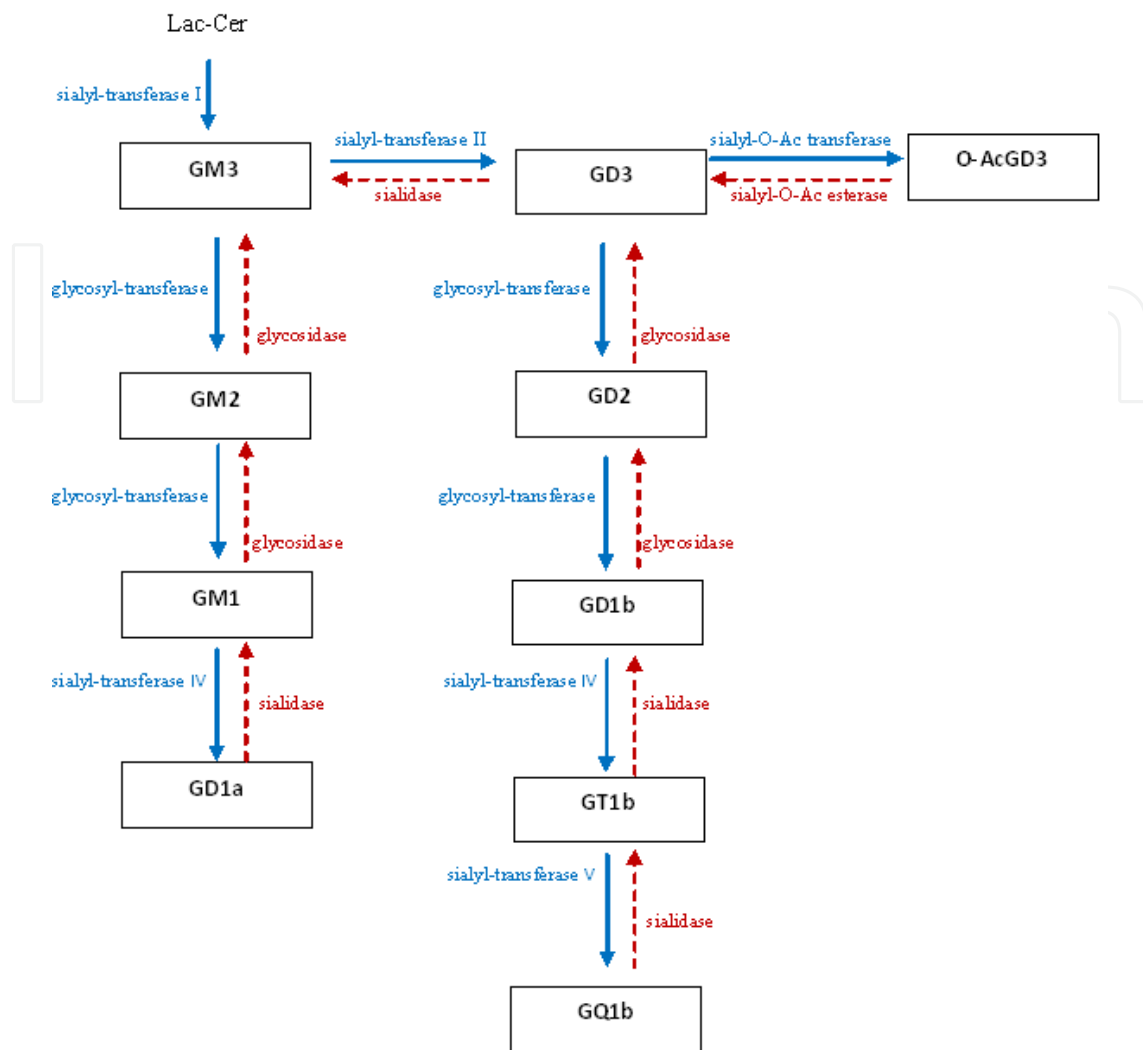


Figure 1. Metabolism of gangliosides in malignant melanoma. **GM1**=Gal-3GalNAc-4(Neu5Ac-3)Gal-4GlcCer; **GM2**=Gal-NAC-4(Neu5Ac-3)Gal-4GlcCer; **GM3**=Neu5Ac-3Gal-4GlcCer; **GD1a**=Neu5Ac-3Gal-3GalNAc-4(Neu5Ac-3)Gal-4GlcCer; **GD1b**=Gal-3GalNAc-4(Neu5Ac-8Neu5Ac-3)Gal-4GlcCer; **GD2**=GalNAC-4(Neu5Ac-8Neu5Ac-3)Gal-4GlcCer; **GD3**=Neu5Ac-8Neu5Ac-3Gal-4GlcCer; **GT1b**=Neu5Ac-3Gal-3GalNAc-4(Neu5Ac-8Neu5Ac-3)Gal-4GlcCer; **GQ1b**=Neu5Ac-8Neu5Ac-3Gal-3GalNAc-4(Neu5Ac-8Neu5Ac-3)Gal-4GlcCer; **O-acetyl-GD3**=Acetyl-O-Neu5Ac-8Neu5Ac-3Gal-4Glc1Cer; Glc=glucose; Gal=galactose; GalNAC=N-acetyl-galactosamine; NeuAc=neuraminic acid; Cer=ceramide; NANA=N-acetyl-neuraminic-acid.

2. Materials and method

The study lasted five years and was based on the prospective-observational analysis of patients with melanocytic lesions. All the patients in the study signed the informed consent accordind with the Declaration from Helsinki in 1964. Our study included three groups: malignant melanoma group (128 adult patients diagnosed with malignant melanoma, with adequate nutritional status, without associated diseases, with no treatment for melanoma before the inclusion in the study), dysplastic nevi group (48 adult patients with dysplastic nevi without associated diseases) and control group (48 healthy subjects). The groups were similar for age, sex and nutritional characteristics.

No patients with age under 18 years, pregnancy, alcoholism or drug dependence, with any hormonal, antidepressive, antioxidant, with MAO inhibitors or blockers of dopaminergic receptors treatment or with associated diseases (neurological, psychiatric, digestive, endocrine, cardiovascular, hepatic, renal, pulmonary, metabolic, autoimmune disorders, chronic infections/inflammation, others neoplastic diseases) were included in the study.

The *diagnosis protocol* was based on clinical examination, common haematological and biochemical determinations for all the patients in the study. The histological and immunohistochemical analysis were made for the patients with malignant melanoma and dysplastic nevi. After the diagnosis, in malignant melanoma and nevi groups the tumors were surgically removed. In all the patients included in the study we determined molecular markers that could indicate melanoma progression (lactate dehydrogenase, interleukin 8, C reactive protein); gangliosides and antigangliosides.

In nevi and melanoma groups, the variations of these factors were evaluated in six moments: T0-when included in the study; T1 – 8 weeks after surgical removal of the tumour, at 3(T2), 6 (T3), 12 (T4), 18 (T5) and 36 (T6) months after surgical removal of the tumour. In melanoma group, the variations of gangliosides and antigangliosides were analysed in relation to serum factors – lactate dehydrogenase, interleukin 8, C reactive proteins and histological factors – Breslow index, Clark level, presence/absence of ulceration.

2.1. Clinical and biological characteristics of melanoma patients

The clinical examination showed the following localization of cutaneous malignant melanoma: 64% on trunk, 21% on limbs, less than 10% on head and neck (Fig. 2). By histological characteristics, we found nodular melanoma in 60 patients (47%), superficial melanoma in 22 patients (17%), acral lenticular melanoma in 9 patients (7%) and unclassified melanoma in 22 patients (17%) (Fig. 3).

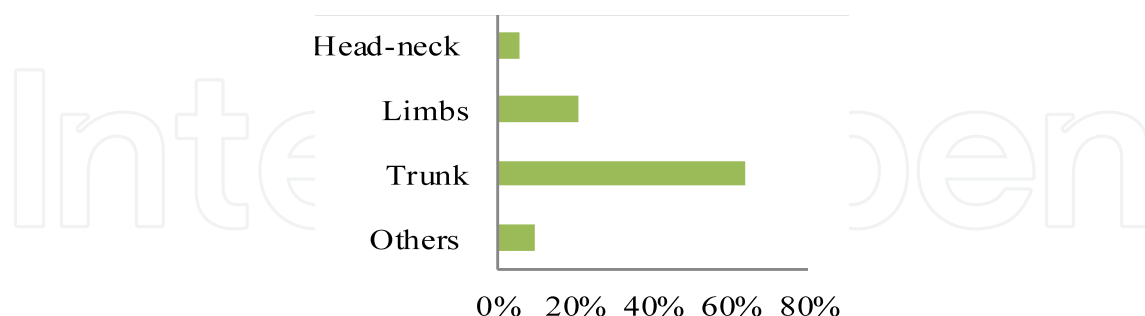


Figure 2. Anatomical site of melanomas

From patients with melanoma, 28 cases (22%) had Clark II, 47 cases (37%) had Clark III, 36 cases (28%) had Clark IV, 17 cases (13%) had Clark V. None of the patients included in the study had Clark I melanoma. From the patients with melanoma, 20.68% had Breslow under 1.00mm, 24.82% Breslow 1.01-2.0mm, 19.71% Breslow 2.01-3.0mm, 18.49% Breslow over 3.01mm. The ulceration was present in 12.5% patients with melanoma. After staging melano-

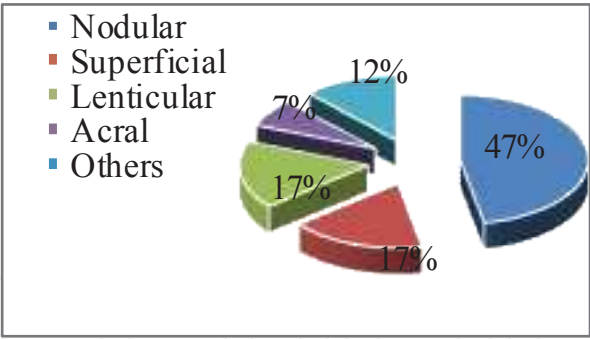


Figure 3. Histological features of melanomas

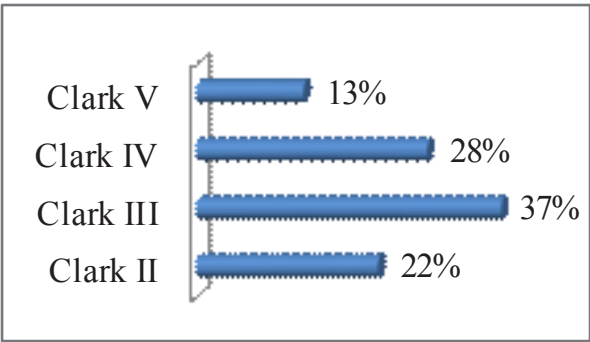


Figure 4. Melanomas Clark levels

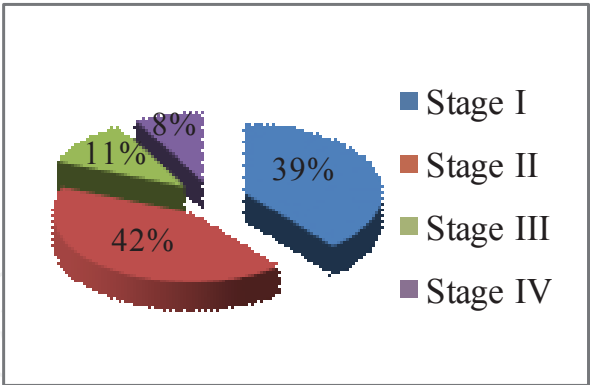


Figure 5. Clinical stages of melanomas

ma, we identified melanoma stage I – 51 cases, stage II – 54 cases, stage III – 54 cases, stage IV – 9 cases (Fig. 5).

The researchers in melanoma field considered lactatdehydeogenase (LDH), C reactive protein (CRP), interleukin 8 (IL8) useful for melanoma follow-up. High serum levels of those markers indicate the progression of melanoma. We presented their levels in the studied groups in Table 2.

Groups	LDH (U/L)	CRP (mg/dl)	IL8 (pg/ml)
Melanoma	389±217	1.07±0.88	68.9±17.2
Dysplastic nevi	241±88	0.26±0.23	28.7±13.3
Control	207±82	0.12±0.12	10.9±4.6

Table 2. Serum level of LDH, CRP and IL8 in melanoma, dysplastic nevi and control groups

In melanoma group, LDH had the following levels at T0: in 0.5% cases smaller than 120U/L, in 73% between 120 and 450 U/L and in 26.5% over 450U/L. In dysplastic nevi patients and in control group, LDH level varied between 120 and 450 U/L. We considered the interval 120-450U/L as normal values for LDH. CRP was in 19% patients between 0 and 0.30mg/dl, in 47%patients between 0.30-0.60mg/dl and in 33% over 0.60mg/dl. We considered the interval 0.30-0.60mg/dl as normal level for CRP. IL8 is a marker of angiogenesis. We considered the normal level for IL8 5-42pg/ml. In melanoma group, IL8 had the following levels: in 16% patients under 15pg/ml, in 22% patients between 15 and 42pg/ml, and in 62% over 42pg/ml.

2.2. Statistical analysis

All the results were analysed using SPSS, a soft for statistic determinations. The results were presented as mean±standard deviation. The variations between groups were determined using t test or ANOVA test. The correlations between groups were calculated using linear regression and Pearson coefficient. $p < 0.05$ was considered with statistical significance. We evaluated the relapse-free survival using Kaplan-Meier curves.

2.3. Laboratory methods

Serum determination of gangliosides. The assessment of gangliosidic acid (LASA) had the following steps: 50 microliters serum were diluted with 150 microliters of cold distilled water and the solution was shaken. There were added 3 ml of chloroform:methanol (v/v) 2:1, at 4-5 celsius degrees. The extraction and partition was finished after adding 0.5ml of cold distilled water. After separating the phases by centrifugation, sialic acid was titred with resorcinol-chlorhidric acid [94].

Sialic acid assessment by resorcinol method. Sialic acid, after the cleavage from glycoconjugates through acid hydrolysis, reacted with resorcinol in acid solution, in the presence of divalent cooper ions. Thus, it formed a blue-violet complex. The complex was evaluated by photometric determination at 580nm, after extraction in a mixture of butanol-acetate and butyl-acetate [95, 96].

Serum assessment of antiganglioside antibodies. Antiganglioside antibodies were assessed using Euroline kits, by Immunoblot, a technique for in vivo determination (serum or plasma) of human antiganglioside antibodies Ig G and IgM type. We evaluated the immune response against seven types of ganglioside: GM1, GM2, GM3, GD1a, GD1b, GT1b and GQ1b. The test kit contained strips coated with parallel lines of purified antigens [97].

3. Results

3.1. Serum profile of gangliosides

Serum gangliosides had increased levels in melanoma and nevi patients compared with control, before the surgical removal of the tumours. In metastatic melanoma, gangliosides levels were statistically significant higher than in melanoma without metastasis (Table 3). Serum gangliosides had a statistically significant decrease after the surgical removal of the tumour in patients with primary melanoma (39.82 ± 21.13 vs 57.29 ± 17.61 mg/dl, $p < 0.05$). In patients with metastatic melanoma, respectively, dysplastic nevi, no statistically significant variations were determined with surgical removal of the tumour.

Groups	Gangliosides (mg/dL)	P ₁	P ₂
Control	18.02±2.78	-	1
Dysplastic nevi	18.86±3.27	1	0.88
Malignant melanoma	57.29±17.61	0.04	0.03
Metastatic melanoma	80.14±19.26	0.00	0.00

p₁-melanoma, metastasis vs dysplastic nevi, p₂-melanoma, metastasis, dysplastic nevi vs control group

Table 3. Serum levels of gangliosides in melanoma, dysplastic nevi and control groups

Serum gangliosides were analyzed in relation to age, sex, histological characteristics of melanoma (Breslow index, Clark level, histological type of the tumor, presence/absence of ulceration) (Table 4). Serum gangliosides did not vary significantly with age and sex, with tumor localization. Serum gangliosides had statistically significant increased values in patients with high Breslow index, respectively high Clark level. We analyzed serum level of gangliosides for the same Clark level in relation to Breslow index and histological type of melanoma (Fig 6).

Parameters	Serum gangliosides (mg/dl)	P
Women	19.06±2.66	p ₁ =0.18
Men	18.17±2.85	
Tumor localization		
Head-neck	49.20±6.23	p ₂ =0.30
Trunk	77.31±19.42	
Limbs	66.81±12.51	
Histological type		
Nodular melanoma	81.61±27.43	p ₃ =0.03
Extensive in surface melanoma	36.42±18.55	
Lenticular malignant melanoma	72.53±27.51	

Parameters	Serum gangliosides (mg/dl)	P
Acral lenticular malignant melanoma	44.17±16.51	
Breslow index		
0-1.0 mm	32.67±9.23	p ₄ =0.02
1.01-2.0 mm	50.88±9.74	
2.01 – 3.0mm	73.12±23.19	
3.01-4.0mm	89.15±2.31	
Clark level		
II	25.92±8.93	p ₅ =0.01
III	33.42±16.75	
IV	62.37±20.91	
V	78.01±20.66	
Ulceration		
Melanoma with ulceration	55.87±13.11	p ₆ =0.04
Melanoma without ulceration	39.72±12.33	

p<0,05 was considered with statistically significance for IC=95%. p₁-women vs men, p₂-trunk vs limbs, p₃-nodular melanoma vs extensive in surface melanoma, p₄-Breslow<1.0mm vs Breslow>3.01mm, p₅-Clark II vs Clark V, p₆-ulcerated melanoma vs melanoma without ulceration

Table 4. Serum gangliosides in relation to clinical and histological features of melanoma

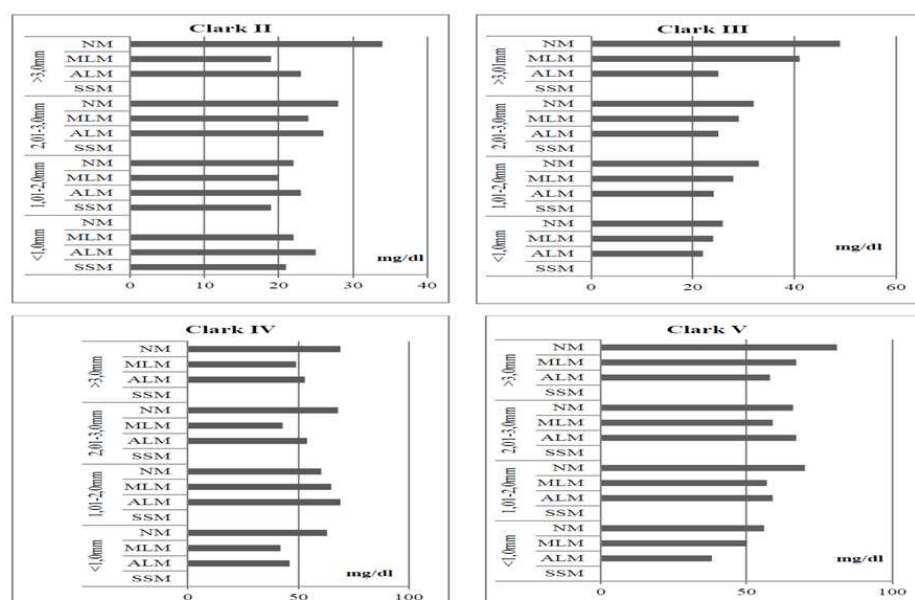


Figure 6. Graphical representation of serum levels of gangliosides in melanoma for the same Clark level in relation to Breslow index (p<0.05) and histological type of tumour (p>0.05) NM-nodular melanoma, MLM-lenticular melanoma, ALM-acral lenticular melanoma, SSM-extensive in surface melanoma

The correlation coefficient showed a positive relation between ganglioside levels and Clark level ($r=0.60$, $CI=0.30-0.90$, $p<0.05$), respectively, Breslow index ($r=0.31$, $CI=0.19-0.58$, $p<0.05$). For the same Clark level and Breslow index, the production of gangliosides was higher in nodular melanoma and in lentiginous acral malignant melanoma compared with lenticular acral melanoma and extensive in surface melanoma (Figure 6).

3.2. Serum profile of antiganglioside antibodies

The immune response against gangliosides GM1, GM2, GM3, GD1a, GD1b, GT1b, GQ1b was evaluated by antiganglioside antibodies IgG and IgM type, before the surgical removal of the tumor. The types of antiganglioside antibodies in melanoma, dysplastic nevi and control group will be presented in this section only for moment 0. We considered antiganglioside antibodies negative when signal intensity was undetectable (0-5) or low (5-10) and positive when signal intensity was medium (11-50) or high (>50).

3.2.1. Anti-GM1 prevalence in studied groups

In patients with malignant melanoma, antiganglioside antibodies anti-GM1 IgG type were undetectable in 90.62% patients, with low intensity of the signal in 4.69% patients, with medium intensity of the signal in 4.68% patients. In dysplastic nevi group, 91.64% patients had undetectable IgG antibodies, in 8.33% the signal was low. In the control group we did not detect any positive anti-GM1 of IgG type. 72.65% patients with melanoma had signal of anti-GM1 IgM class negative, 11.71% low signal, 13.29% medium signal and 2.34% high signal. 88.33% patients with dysplastic nevi had negative IgM anti-GM1 signal, in 10.42% the signal intensity was low, in 6.25% medium. In 93.75% healthy patients IgM antibodies had negative signal, in 4.17% the signal was low, in 2.08% medium. In melanoma patients anti-GM1 IgG type were positive in 4.68% cases and IgM in 15.63% cases. No statistical differences were observed in anti-GM1 IgG class status between melanoma, dysplastic nevi and control group. Anti-GM1 IgM class varied significantly between the studied groups (Table 5).

Groups	Antibodies type	Positive	Negative	P
Melanoma	IgG	6	122	NS
	IgM	20	108	0.00
Dysplastic nevi	IgG	0	48	NS
	IgM	3	45	NS
Control	IgG	0	48	1
	IgM	1	47	1

p-melanoma vs control, dysplastic vs control, NS=no statistical significance

Table 5. Anti-GM1 IgG and IgM class in patients with malignant melanoma, dysplastic nevi and control

Due to its statistically significant variation, we analysed IgM status in relation to clinical and histological features of melanoma. Anti-GM1 status was analyzed in relation to age, sex, histological characteristics of the tumor (Breslow index, Clark level, histological type of the tumor, presence/absence of ulceration) (Table 6). The intensity of the signal did not vary with sex and age. Anti-GM1 varied with histological type: in patients with nodular melanoma the intensity signal was significantly increased compared with acral lenticular melanoma ($p<0.05$), extensive in surface melanoma ($p<0.05$) or lenticular melanoma ($p<0.05$).

Parameters	IgM intensity signal	P
Sex		
Women	8.96±18.28	p ₁ =0.14
Men	5.14±11.09	
Tumor site		
Head-neck	13.28±9.86	p ₂ =0.02
Trunk	8.87±17.30	
Limbs	0.88±1.24	
Histological type		
Nodular melanoma	13.26±19.28	p ₃ =0.00
Extensive in surface melanoma	0.82±1.11	
Lenticular melanoma	0.77±0.97	
Acral lenticular melanoma	1.31±0.65	
Breslow index		
0-1.0 mm	0.82±1.11	p ₄ =0.00
1.01-2.0 mm	2.53±1.15	
2.01 – 3.0mm	9.18±6.82	
>3.01 mm	13.65±15.59	
Clark level		
II	1.07±1.30	p ₅ =0.00
III	1.07±0.97	
IV	12.61±14.85	
V	18.38±27.59	
Ulceration		
Melanoma with ulceration	36.43±23.86	p ₆ =0.00
Melanoma without ulceration	2.46±4.39	

$p<0.05$ was considered with statistically significance for IC=95%. p_1 -women vs men, p_2 -trunk vs limbs, p_3 -nodular melanoma vs extensive in surface melanoma, p_4 -Breslow<1.0mm vs Breslow>3.01mm, p_5 -Clark II vs Clark V, p_6 -ulcerated melanoma vs melanoma without ulceration

Table 6. Anti-GM1 antibodies IgM class in relation to clinical and histological features of melanoma

Anti-GM1 IgM intensity signal was significantly increased in melanoma on head and neck, compared with melanoma on trunk ($p<0.05$), respectively on limbs ($p<0.05$) (Table 6). Anti-GM1 signal varied statistically significant with Breslow index and Clark level. Compared with Breslow $<1.0\text{mm}$, signal intensity was significantly higher in patients with Breslow $2.01\text{--}3.0\text{mm}$ ($p<0.05$), respectively Breslow $>3.01\text{mm}$ ($p<0.05$). Compared with Clark II, signal intensity was significantly higher in patients with Clark IV ($p<0.05$), respectively, Clark V ($p<0.05$). Signal intensity of anti-GM1 IgM type antibodies was statistically significant increased in melanoma with ulceration compared with melanoma without ulceration ($p<0.05$).

3.2.2. Anti-GM2 prevalence in studied groups

In patients with malignant melanoma, signal of antiganglioside antibodies anti-GM2 IgG type was undetectable in 96.87% patients, with low intensity in 1.57% patients, with medium intensity in 1.56% patients. In dysplastic nevi group, signal of antiganglioside antibodies anti-GM2 IgG type was undetectable in 97.92% cases, with low signal in 2.08% cases. In patients with malignant melanoma, signal of antiganglioside antibodies anti-GM2 IgM type was undetectable in 58.59% patients, with low intensity in 21.10% patients, with medium intensity in 4.68% patients and high intensity in 15.63% patients. In dysplastic nevi group, signal of antiganglioside antibodies anti-GM2 IgM type was undetectable in 70.83% cases, with low signal in 25.12% cases, and with medium intensity in 4.17% cases. In control group, anti-GM2 IgM type was undetectable in 91.67% volunteers, with weak signal in 6.25% and with medium signal in 2.08% volunteers. Only anti-GM2 IgM class varied significantly between the studied groups, therefore we analyzed IgM status by clinical and histological features of melanoma.

Groups	Antibodies type	Positive	Negative	P
Melanoma	IgG	2	126	NS
	IgM	26	102	<0.05
Dysplastic nevi	IgG	0	48	NS
	IgM	2	46	NS
Control	IgG	0	48	1
	IgM	1	47	1

p-melanoma vs control, dysplastic vs control, NS=no statistical significance

Table 7. Anti-GM2 IgG and IgM class in patients with malignant melanoma, dysplastic nevi and control

Anti-GM2 IgM status was analyzed in relation to age, sex, histological characteristics of the tumor (Breslow index, Clark level, histological type of the tumor, presence/absence of ulceration) (Table 8). The intensity level did not vary with sex and age, with anatomical site of tumor or histological type of melanoma, Breslow index, Clark level, respectively presence/absence of ulceration.

Parameters	IgM signal intensity	p
Sex		
Women	19.24±28.46	p ₁ =0.14
Men	12.51±23.70	
Tumor site		
Head-neck	13.00±24.96	p ₂ =0.35
Trunk	15.12±25.39	
Limbs	22.92±32.36	
Histological type		
Nodular melanoma	14.91±24.38	p ₃ =0.51
Extensive in surface melanoma	12.47±20.84	
Lenticular melanoma	14.51±26.60	
Acral lenticular melanoma	23.77±37.34	
Breslow index		
0-1.0 mm	12.13±20.41	p ₄ =0.30
1.01-2.0 mm	21.02±31.78	
2.01 – 3.0mm	11.93±22.99	
>3.01mm	18.80±28.25	
Clark level		
II	13.14±21.31	p ₅ =0.63
III	18.48±30.80	
IV	17.63±26.57	
V	17.23±26.56	
Ulceration		
Melanoma with ulceration	13.43±21.73	p ₆ =0.56
Melanoma without ulceration	16.91±27.43	

p<0.05 was considered with statistically significance for IC=95%. p₁-women vs men, p₂-trunk vs limbs, p₃-nodular melanoma vs extensive in surface melanoma, p₄-Breslow<1.0mm vs Breslow>3.01mm, p₅-Clark II vs Clark V, p₆-ulcerated melanoma vs melanoma without ulceration

Table 8. Anti-GM2 antibodies IgM class by clinical and histological features of melanoma

3.2.3. Anti-GM3 prevalence in studied groups

In patients with malignant melanoma, signal of antiganglioside antibodies anti-GM3 IgG type was undetectable in 96.10% patients, with low intensity in 3.12% patients, with medium intensity in 0.78% patients. In dysplastic nevi group, signal of antiganglioside antibodies anti-

GM3 IgG type was undetectable in 87.50% cases, with low signal in 12.25% cases. IgG did not vary significantly between groups. In patients with malignant melanoma, signal of antiganglioside antibodies anti-GM3 IgM type was undetectable in 74.22% patients, with low intensity in 8.59% patients, with medium intensity in 13.28% patients and high intensity in 3.91% patients. In dysplastic nevi group, signal of antiganglioside antibodies anti-GM3 IgM type was undetectable in 77.08% cases, with low signal in 18.75% cases, and with medium intensity in 4.17% cases. Anti-GM3 IgM class varied significantly between the studied groups (Table 9).

Groups	Antibodies type	Positive	Negative	P
Melanoma	IgG	1	127	NS
	IgM	6	122	<0.05
Dysplastic nevi	IgG	0	48	NS
	IgM	2	46	NS
Control	IgG	0	48	1
	IgM	0	48	1

p-melanoma vs control, dysplastic vs control, NS=no statistical significance

Table 9. Anti-GM3 IgG and IgM class in patients with malignant melanoma, dysplastic nevi and control

Anti-GM3 IgM status was analyzed in relation to age, sex, histological characteristics of the tumor (Table 10). The intensity level did not vary with sex and age, anatomical site or histological type of tumor, Breslow index or Clark level. Anti-GM3 antibodies had statistically significant higher intensity in ulcerated melanoma compared with melanoma without ulceration (Table 10).

Parameters	IgM signal intensity	p
Sex		
Women	10.42±19.74	p ₁ =0.08
Men	10.88±19.57	
Tumor site		
Head-neck	11.85±15.87	p ₂ =0.68
Trunk	9.43±18.08	
Limbs	15.01±25.07	
Histological type		
Nodular melanoma	7.73±12.84	p ₃ =0.57
Extensive in surface melanoma	14.81±24.57	

Lenticular melanoma	12.13±25.81	
Acral lenticular melanoma	10.75±16.50	
Breslow index		
0,0-1.0 mm	15.72±25.48	
1.01-2.0 mm	11.94±22.79	p ₄ =0.13
2.01 – 3.0mm	12.37±15.93	
>3.01mm	7.71±14.22	
Clark level		
II	13.11±23.58	
III	12.80±23.34	p ₅ =0.09
IV	9.13±13.89	
V	4.66±8.56	
Ulceration		
Melanoma with ulceration	2.81±2.31	p ₆ =0.00
Melanoma without ulceration	11.82±20.66	

p<0.05 was considered with statistically significance for IC=95%. p₁-women vs men, p₂-trunk vs limbs, p₃-nodular melanoma vs extensive in surface melanoma, p₄-Breslow<1.0mm vs Breslow>3.01mm, p₅-Clark II vs Clark V, p₆-ulcerated melanoma vs melanoma without ulceration

Table 10. Anti-GM3 antibodies IgM class by clinical and histological features

3.2.4. Anti-GD1a prevalence in studied groups

In patients with malignant melanoma, signal of antiganglioside antibodies anti-GD1a IgG type was undetectable in 89.06% patients, with low intensity in 10.16% patients, with medium intensity in 0.78% patients. In dysplastic nevi group, signal of antiganglioside antibodies anti-GD1a IgG type was undetectable in 95.83% cases, with low signal in 4.17% cases. No positive anti-GD1a IgG type were detected in nevi or control groups. In patients with malignant melanoma, signal of antiganglioside antibodies anti-GD1a IgM type was undetectable in 57.81% patients, with low intensity in 28.12% patients, with medium intensity in 3.12% patients and high intensity in 10.94% patients. In dysplastic nevi group, signal of antiganglioside antibodies anti-GD1a IgM type was undetectable in 77.08% cases, with low signal in 16.67% cases, and with medium intensity in 6.25% cases. No positive anti-GD1a IgM type were detected in nevi or control groups. No statistical differences were observed in anti-GD1a IgG class status between melanoma, dysplastic nevi and control group. Anti-GD1a IgM class varied significantly between the studied groups (Table 11). Due to this variation, we analyzed IgM status in relation to clinical and histological features of melanoma. In melanoma patients anti-GD1a IgG type were positive in 0,78% cases and IgM in 14,06% cases.

Groups	Antibodies type	Positive	Negative	P
Melanoma	IgG	1	127	NS
	IgM	18	110	<0.05
Dysplastic nevi	IgG	0	48	NS
	IgM	3	45	NS
Control	IgG	0	48	1
	IgM	0	48	1

p-melanoma vs control, dysplastic vs control, NS=no statistical significance

Table 11. Anti-GD1a IgG and IgM class in patients with malignant melanoma, dysplastic nevi and control

Anti-GD1a IgM status was analyzed in relation to age, sex, histological characteristics of the tumor (Breslow index, Clark level, histological type of the tumor, presence/absence of ulceration) (Table 12). The intensity level did not vary with sex and age or histological type of melanoma. The antibodies varied with tumor site: the highest intensity was determined in melanoma situated on trunk ($p<0.05$), followed by melanomas on head and neck ($p<0.05$) compared with melanomas on limbs. High intensity of anti-GD1a were observed in melanoma with Breslow 2.01-3.0mm ($p<0.05$), respectively Breslow>3.01mm ($p<0.05$) compared with Breslow 0.0-1.0mm. Increased intensity of anti-GD1a were determined in Clark IV ($p<0.05$), respectively Clark V ($p<0.05$) compared with Clark II melanomas. Anti-GD1a signal intensity varied significantly with ulceration, with higher levels in ulcerated melanomas ($p<0.05$) (Table 12).

Parameters	IgM signal intensity	p
Sex		
Women	12.96±24.08	p ₁ =0.93
Men	13.32±23.34	
Tumor site		
Head-neck	10.42±8.31	p ₂ =0.03
Trunk	16.26±6.65	
Limbs	2.38±83.22	
Histological type		
Nodular melanoma	25.25±30.38	p ₃ =0.70
Extensive in surface melanoma	2.37±1.76	
Lenticular melanoma	2.54±1.76	
Acral lenticular melanoma	2.33±1.38	
Breslow index		
0,0-1.0 mm	2.45±1.71	p ₄ =0.03
1.01-2.0 mm	2.41±1.74	
2.01 – 3.0mm	8.93±5.48	

Parameters	IgM signal intensity	p
> 3.01 mm	25.01±35.92	
Clark level		
II	2.40±1.71	
III	4.57±10.34	p ₅ =0.01
IV	19.55±26.08	
V	38.66±36.54	
Ulceration		
Melanoma with ulceration	46.65±38.07	p ₆ =0.00
Melanoma without ulceration	8.33±16.16	

p<0.05 was considered with statistically significance for IC=95%. p₁-women vs men, p₂-trunk vs limbs, p₃-nodular melanoma vs extensive in surface melanoma, p₄-Breslow<1.0mm vs Breslow>3.01mm, p₅-Clark II vs Clark V, p₆-ulcerated melanoma vs melanoma without ulceration

Table 12. Anti-GD1a antibodies IgM class in relation to clinical and histological features of melanoma

3.2.5. Anti-GD1b prevalence in studied groups

In patients with malignant melanoma, signal of antiganglioside antibodies anti-GD1b IgG type was undetectable in 61.71% patients, with low intensity in 34.37% patients, with medium intensity in 3.12% patients. In dysplastic nevi group, signal of antiganglioside antibodies anti-GD1b IgG type was undetectable in 81.25% cases, with low signal in 18.75% cases. In patients with malignant melanoma, signal of antiganglioside antibodies anti-GD1b IgM type was undetectable in 64.84% patients, with low intensity in 25.10% patients, with medium intensity in 4.68% patients and high intensity in 5.46% patients (Table 13).

Groups	Antibodies type	Positive	Negative	p
Melanoma	IgG	4	124	NS
	IgM	13	115	<0.05
Dysplastic nevi	IgG	0	48	NS
	IgM	2	46	NS
Control	IgG	0	48	1
	IgM	0	48	1

p-melanoma vs control, dysplastic vs control, NS=no statistical significance

Table 13. Anti-GD1b IgG and IgM class in patients with malignant melanoma, dysplastic nevi and control

In dysplastic nevi group, signal of anti-GD1b IgM type was undetectable in 81.25% cases, with low signal in 5.46% cases, and with medium intensity in 1.56% cases. No positive anti-GD1b IgM type were detected in nevi or control groups. In melanoma patients anti-GD1b IgG type were positive in 3.12% cases, while IgM in 10.14% cases. Anti-GD1b IgG class had no significant variations between groups, while, IgM class modified significantly (Table 13).

Anti-GD1b IgM status was analyzed in relation to age, sex, histological characteristics of the tumor (Breslow index, Clark level, histological type of the tumor, presence/absence of ulceration) (Table 14). We did not determine significant variations of anti-GD1b except with the histological type of melanoma. Compared with nodular melanoma, we detected statistically increased intensity in extensive in surface melanoma ($p<0.05$) and acral lentiginos melanoma ($p<0.05$).

Parameters	IgM signal intensity	P
Sex		
Women	7.67±12.57	p ₁ =0.52
Men	9.36±16.02	
Tumor site		
Head-neck	10.57±21.36	p ₂ =0.14
Trunk	6.74±12.44	
Limbs	14.30±20.88	
Histological type		
Nodular melanoma	6.00±11.40	p ₃ =0.00
Extensive in surface melanoma	13.72±18.44	
Lenticular melanoma	7.36±15.95	
Acral lenticular melanoma	16.55±19.00	
Breslow index		
0-1.0 mm	13.72±18.49	p ₄ =0.14
1.01-2.0 mm	11.52±18.17	
2.01 – 3.0mm	6.43±14.07	
>3.01 mm	5.65±8.33	
Clark level		
II	12.44±16.85	p ₅ =0.35
III	9.29±15.93	
IV	6.44±13.88	
V	5.88±7.22	
Ulceration		
Melanom with ulceration	4.56±6.19	p ₆ =0.23
Melanom without ulceration	3.92±5.36	

$p<0.05$ was considered with statistically significance for IC=95%. p_1 -women vs men, p_2 -trunk vs limbs, p_3 -nodular melanoma vs extensive in surface melanoma, p_4 -Breslow<1,0mm vs Breslow>3,01mm, p_5 -Clark II vs Clark V, p_6 -ulcerated melanoma vs melanoma without ulceration

Table 14. Anti-GD1b antibodies IgM class in relation to clinical and histological features of melanoma

3.2.6. Anti-GT1b prevalence in studied groups

In patients with malignant melanoma, signal of anti-GT1b IgG type was undetectable in 83.59% patients, with low intensity in 16.40% patients. In dysplastic nevi group, signal of anti-GT1b

IgG type was undetectable in 85.41% cases, with low signal in 14.58% cases. In patients with malignant melanoma, signal of anti-GT1b IgM type was undetectable in 53.24% patients, with low intensity in 33.59% patients, with medium intensity in 10.93% patients and high intensity in 3.12% patients. In dysplastic nevi group, signal of anti-GT1b IgM type was undetectable in 68.75% cases, with low signal in 27.08% cases, and with medium intensity in 4.16% cases. No positive anti-GT1b IgM or IgG type were detected in control group. The statistical analysis showed that IgM antibodies varied significantly between the studied groups (Table 15). We analyzed IgM status in relation to clinical and histological features of melanoma.

Groups	Antibodies type	Positive	Negative	p
Melanoma	IgG	0	128	NS
	IgM	18	110	<0.05
Dysplastic nevi	IgG	0	48	NS
	IgM	2	46	NS
Control	IgG	0	48	1
	IgM	0	48	1

p-melanoma vs control, dysplastic vs control, NS=no statistical significance

Table 15. Anti-GT1b IgG and IgM class in patients with malignant melanoma, dysplastic nevi and control

Anti-GT1b status was analyzed in relation to age, sex, histological characteristics of the tumor (Breslow index, Clark level, histological type of the tumor, presence/absence of ulceration) (Table 16). We did not determine significant variations of anti-GD1b except with the tumor site of melanoma. Compared with melanomas on head-neck, we detected statistically increased intensity in melanomas on trunk ($p<0.05$) or on limbs ($p<0.05$).

Parameters	IgM signal intensity	p
Sex		
Women	8.59±13.30	p ₁ =0.53
Men	9.23±14.97	
Tumor site		
Head-neck	2.85±2.79	p ₂ =0.02
Trunk	7.36±13.46	
Limbs	16.15±19.12	

Histological type		
Nodular melanoma	6.61±14.96	p ₃ =0.10
Extensive in surface melanoma	13.27±17.92	
Lenticular melanoma	6.31±4.84	
Acral lenticular melanoma	16.33±16.62	
Breslow index		
0-1.0 mm	13.27±17.92	p ₄ =0.07
1.01-2.0 mm	11.47±11.04	
2.01 – 3.0mm	2.75±2.04	
>3.01 mm	7.85±16.35	
Clark level		
II	12.18±16.28	p ₅ =0.28
III	9.74±10.76	
IV	5.33±13.25	
V	9.44±19.85	
Ulceration		
Melanom with ulceration	2.75±1.69	p ₆ =0.23
Melanom without ulceration	9.86±15.03	

p<0.05 was considered with statistically significance for IC=95%. p₁-women vs men, p₂-trunk vs limbs, p₃-nodular melanoma vs extensive in surface melanoma, p₄-Breslow<1.0mm vs Breslow>3.01mm, p₅-Clark II vs Clark V, p₆-ulcerated melanoma vs melanoma without ulceration

Table 16. Anti-GT1b antibodies IgM class in relation to clinical and histological features of melanoma

3.2.7. *Anti-GQ1b prevalence in studied groups*

In all patients with malignant melanoma, dysplastic nevi and control group, antiganglioside antibodies anti-GQ1b IgG type were negative. In patients with malignant melanoma, signal of antiganglioside antibodies anti-GQ1b IgM type was undetectable in 90.62% patients, with low intensity in 4.68% patients, with medium intensity in 3.90% patients and high intensity in 0.78% patients. No positive anti-GT1b IgM type were detected in dysplastic nevi, respectively, control groups. No statistical differences were observed in anti-GQ1b IgG class status between melanoma, dysplastic nevi and control group. Anti-GQ1b IgM class varied significantly between the studied groups (Table 17). We analyzed IgM status by clinical and histological features of melanoma.

Groups	Antibodies type	Positive	Negative	p
Melanoma	IgG	0	128	NS
	IgM	6	122	<0,05
Dysplastic nevi	IgG	0	48	NS
	IgM	0	48	NS
Control	IgG	0	48	1
	IgM	0	48	1

p-melanoma vs control, dysplastic vs control, NS=no statistical significance

Table 17. Anti-GQ1b IgG and IgM class in patients with malignant melanoma, dysplastic nevi and control

Anti-GQ1b IgM status was analyzed in relation to age, sex, histological characteristics of the tumor (Breslow index, Clark level, histological type of the tumor, presence/absence of ulceration) (Table 18) The intensity level did not vary with sex and age, tumor site or histological type of melanoma, Clark level, Breslow index, presence/absence of ulceration.

Parameters	IgM signal intensity	p
Sex		
Women	4.69±8.91	p ₁ =0.32
Men	4.84±10.27	
Tumor site		
Head-neck	2.00±1.52	p ₂ =0.30
Trunk	4.60±7.61	
Limbs	6.69±16.76	
Histological type		
Nodular melanoma	3.45±5.94	p ₃ =0.16
Extensive in surface melanoma	6.81±17.13	
Lenticular melanoma	5.90±9.64	
Acral lenticular melanoma	9.77±12.22	
Breslow index		
0-1.0 mm	6.81±17.13	p ₄ =0.70

1.01-2.0 mm	4.44±7.03	
2.01 – 3.0mm	2.81±1.72	
>3.01 mm	5.03±8.92	
Clark level		
II	5.66±15.60	
III	5.68±8.64	p ₅ =0.60
IV	2.97±1.62	
V	4.72±10.68	
Ulceration		
Melanoma with ulceration	2.87±1.78	p ₆ =0.82
Melanoma without ulceration	5.05±10.33	

p<0.05 was considered with statistically significance for IC=95%. p₁-women vs men, p₂-trunk vs limbs, p₃-nodular melanoma vs extensive in surface melanoma, p₄-Breslow<1.0mm vs Breslow>3.01mm, p₅-Clark II vs Clark V, p₆-ulcerated melanoma vs melanoma without ulceration

Table 18. Anti-GQ1b antibodies IgM class in relation to clinical and histological features of melanoma

3.3. The relationship between gangliosidic system and biochemical factors used for melanoma staging

In this section we will present the statistical correlations between LDH, CRP and IL8 in melanoma patients, before the surgical removal of the tumor, and also, the relation between the biochemical factors and serum gangliosides/antigangliosides in melanoma patients (Table 19).

	LDH	CRP	IL8
IL8	r=0.36 p=0.00	r=0.92 p=0.00	-
CRP	r=0.40 p=0.00	-	-
Serum gangliosides (LASA)	r=0.44 p=0.00	r=0.43 p=0.00	r=0.47 p=0.00

Table 19. The correlations between gangliosidic system and staging factors in malignant melanoma patients

In melanoma group positive statistical significant correlations were determined between LDH and IL8, LDH and CRP, respectively, CRP and IL8. A strong positive correlation was detected between serum gangliosides and LDH, CRP and IL8 in melanoma group.

	LDH	CRP	IL8
Anti-GM1	r=0.75 p=0.00	r=0.33 p=0.00	r=0.37 p=0.00
Anti-GM2	r=0.07 p=0.38	r= - 0.06 p=0.47	r= - 0.03 p=0.67
Anti-GM3	r= - 0.12 p=0.16	r= - 0.17 p=0.05	r= - 0.15 p=0.02
Anti-GD1a	r=0.140 p=0.00	r=0.45 p=0.00	r=0.53 p=0.00
Anti-GD1b	r= - 0.15 p=0.06	r= - 0.24 p=0.00	r= - 0.22 p=0.00
Anti-GT1b	r= - 0.16 p=0.05	r= - 0.29 p=0.00	r= - 0.25 p=0.00
Anti-GQ1b	r= - 0.02 p=0.81	r= - 0.13 p=0.11	r= - 0.15 p=0.09

Table 20. The correlations between antigangliosides and staging factors in malignant melanoma patients

Anti-GM1 correlated positive, statistically significant with LDH, CRP and IL8. Anti-GM2 IgM type correlated weakly, statistically insignificant with the markers used for melanoma staging. Anti-GM3 was negatively associated before surgical intervention, with the markers that indicate the progression of melanoma: statistically insignificant with LDH, statistically significant with CRP respectively, IL8. Anti-GD1a was strongly positive associated with the markers for melanoma staging, before surgical intervention. The b serie of antiganglioside antibodies correlated weakly, negatively, with LDH, CRP and IL8.

3.4. Variation of gangliosidic system in melanoma patients after surgical intervention

From the 128 patients with malignant melanoma, we selected 30 cases with the same clinical stage (I or II), that were monitored for 36 months using the same investigation protocol. The decision to continue monitoring without receiving any treatment was based on oncologist decision, no signs of melanoma evolution, and also on patients attitude/agreement. The moments of evaluation were: T0-when included in the study; T1 – 8 weeks after surgical removal of the tumor, at 3(T2), 6 (T3), 12 (T4), 18 (T5) and 36 (T6) months after surgical removal of the tumor (Table 21).

	T1	T2	T3	T4	T5	T6
LASA	40.73±28.40	41.86±28.49	42.30±30.55	44.60±32.22	45.53±34.54	53.11±41.26 ⁽¹⁾
Anti-GM1	8.14±12.16	7.80±11.40	6.22±7.12	8.15±9.30	9.33±7.24	13.10±10.25 ⁽¹⁾
Anti-GM2	14.35±7.85	10.26±8.20	12.15±5.12	9.37±8.75	10.6±6.25	5.22±6.10
Anti-GM3	10.66±12.37	6,15±10.27 ⁽¹⁾	4.06±3.86 ⁽¹⁾	6.04±9.05 ⁽¹⁾	4.60±8.15 ⁽¹⁾	3.90±5.10 ⁽¹⁾
Anti-GD1a	17.42±18.32	14,05±9.61	20.10±16.10	21.17±9.25	18.00±10.82	23.10±11.50 ⁽¹⁾
Anti-GD1b	11.15±9,10	17.10±6.33	15.16±5.15	11,10±8.32	9.11±8.17	8.30±6.25
Anti-GT1b	11.15±18.10	16.22±17.02	10.61±8.33	8.62±6.55	7.14±8.09 ⁽¹⁾	8.32±10.12
Anti-GQ1b	4.10±6,44	6.22±5.17	8.77±10.21 ⁽¹⁾	9.40±7.25 ⁽¹⁾	6.18±7.35	10.40±7.35

1)=statistically significant variation compared with moment 1

Table 21. Variation of serum gangliosides and antiganglioside antibodies after surgical removal of melanoma

LASA increased after surgical intervention, having the biggest level at T6, its variation being statistically significant ($p<0.05$). Anti-GM1 decreased in the first six months after surgical removal of melanoma, afterwards its level increased, at T6 being statistically significant higher compared with its level at T1 ($p<0.05$). Anti-GM2 decreased during the 36 months of evaluation, but its variation was not statistically significant ($p>0.05$). Anti-GM3 decreased significantly at T2, T3, T4, T5, and respectively, at T6 compared with T1. Anti-GD1a increased significantly during the 36 months of evaluation compared with its level at T1. Antiganglioside antibodies from b series had a sinuous variation during evaluation: anti-GD1b increased in the first 12 months after surgical removal of the tumor, afterwards, its intensity decreased compared with T1; anti-GT1b increased at T2, and then, its intensity decreased during evaluation; anti-GQ1b increased during evaluation.

After surgical removal of melanoma we detected anti-GM3 IgM type in 12 patients, data that could indicate the regression of melanoma. High pathological levels of serum gangliosides and detectable levels of anti-GD1a IgM were determined in 11 patients, data that could be associated with progression of melanoma and possible metastasis. The increase of serum gangliosides, during evaluation and the absence of antiganglioside antibodies in 7 patients suggest tumoral recurrence.

3.5. Determination of relapse-free survival in malignant melanoma patients

We determined the relapse free survival using Kaplan-Meier curves, in patients with operated malignant melanoma, monitored for 36 months. Survival rate was assessed in relation to serum levels of gangliosides, anti-GM3 IgM type and anti-GD1a IgM type. We choose a cut-off value suitable for estimating the prognosis.

Biological parameters		Free-relapse survival (months)	p
Serum gangliosides (mg/dl)	≤25	22.15±1.19	1
	>25	16.33±1.72	0.01
Anti-GM3/IgM	≤14.20	17.82±0.94	1
	>14.20	21.32±1.53	0.04
Anti-GD1a/IgM	≤15.35	21.67±1.04	1
	>15.35	13.85±1.37	0.00
p –statistical significance			

Table 22. Free-relapse survival in patients with malignant melanoma

The relapse-free survival curves in relation to serum gangliosides showed an increase in survival in patients with serum gangliosides lower than 25mg/dl compared with those that had gangliosides over 25mg/dl (22.15±1.19 versus 16.33±1.72 months, $p<0.05$) (Table 22). The relapse-free survival curves in relation to serum anti-GM3 IgM type showed an increase in survival in patients with signal intensity over 14,20 compared with patients with lower anti-GM3 intensity signal (21.31±1.53 versus 17.82±0.94 months, $p<0.05$) (Table 22). The relapse-free survival curves in relation to serum anti-GD1a IgM type showed an increase in survival in patients with signal intensity under 15.35 compared with patients with higher anti-GM3 intensity signal (13.85±1.37 versus 21.67±1.04 months, $p<0.05$) (Table 22).

4. Discussions

There is little information about quantitative variations of serum gangliosides, their origin and progression of malignant melanoma in medical literature. Serum gangliosides are derived on one hand, from tumor microenvironment, and, on the other hand, from membranous components turn-over. The ability of melanoma cells to synthesize and release gangliosides in extracellular space are sustained by the results of this study.

Though, in systemic circulation, we detected high levels of gangliosides in patients with primary melanoma and metastatic melanoma compared with dysplastic nevi and control groups. The patients with metastatic melanoma had significantly increased levels of gangliosides compared with patients with primary tumor. The serum levels of gangliosides were similar in nevi and control group.

Important data regarding the origin of serum gangliosides were obtained by analyzing their levels before and after surgical removal of melanocytic tumors. The levels of serum gangliosides varied statistically significant with surgical intervention in patients with localized melanoma, and without statistical significance in patients with metastatic melanoma, respectively, dysplastic nevi. Therefore, serum concentration of gangliosides could give important data about tumor mass, tumor volume or tumor progression. We consider that low levels of

gangliosides in multiple determinations after surgical removal of the tumor could indicate a correct surgical treatment of melanoma, while high levels in the same conditions could mean a progression of melanoma.

Our study showed a strong association between increased serum gangliosides and high Breslow index or high Clark level and presence of ulceration. Serum gangliosides correlated strong and positive with biological factors used for melanoma staging – LDH, CRP, IL8. High levels of LDH, CRP, IL8 are markers for melanoma progression. These correlations show that the principle elements involved in melanoma progression are vascularization and neoangiogenesis.

Therefore, the levels of serum gangliosides could become a useful marker for clinical staging of melanoma, but its usage is limited by the lack of satisfactory criteria for interpretation. The problem becomes more complex, because there are multiple sources of serum ganglioside, being difficult to know if the exact source are the tumoral cells or the host organism. This statement is sustained by the variability in composition of serum gangliosides and altered immunologic reactivity of melanoma patients. In the model of serum gangliosides before and after surgical treatment, we observed a tendency of the body to adjust the biosynthesis of gangliosides, mainly by normalizing glycosyltransferase activity in melanoma patients. To demonstrate a possible link between ganglioside system and prognosis of patients with cutaneous malignant melanoma, we calculated relapse-free survival of the disease according to serum levels of gangliosides. Based on this analysis, the authors found that low levels of circulating gangliosides are positive prognosis factors, in terms of increasing the relapse free survival in patients with malignant melanoma. These results justify the role of serum gangliosides as potential biomarkers in the management of patients with melanoma, a finding that supports previous researches [10, 11, 53, 102, 103].

Data in literature about the involvement of gangliosides in tumor processes are controversial. Some studies claim that overexpression of gangliosides on cells membrane and their accumulation in intercellular space and in serum of patients with cancer may play a role in tumor growth, neovascularization, and lack of immune response. Gangliosides influence tumor metastasis and angiogenesis by modulating the autocrine production of growth factors and thereby, protect the tumor from the host's immune system [67, 98, 100, 101, 104, 105, 106].

In this sense, the relationship between ganglioside, tumor growth and progression have been the subject of several studies. A number of *in vivo* and *in vitro* studies have shown that gangliosides are involved in tumor suppression. Gangliosides metabolic products such as ceramides may be involved in apoptosis. Other studies argue that metastatic melanoma produces a variety of growth factors and interleukins that induce cell proliferation. Gangliosides can alter the growth of metastatic melanoma by modulation the activity of some growth factors, by regulating cAMP and some signaling pathways. Recently it has been demonstrated that gangliosides of human melanoma promote differentiation of dendritic cells from monocytes, maturation of Langerhans cells in the epidermis, and induce apoptosis of both cell types. Melanoma cells release active chemo attractants and other mediators to stimulate the migration and activation of macrophages, monocytes, granulocytes, keratinocytes, fibroblasts, platelets,

and other components of the native immunity. Metastatic melanoma release these molecules in the tumor microenvironment [52, 82, 99, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117].

Other studies claim that metastatic melanoma produces a number of growth factors such as interleukin-8, alpha protein that regulates the growth/the activity of melanocytes. It has been suggested that gangliosides could alter the growth of metastatic melanoma by modulating the production of an autocrine growth factor and by adjusting the 3'5'adenozin cyclic monophosphate (cAMP) and its corresponding signaling paths. Other studies have suggested that soluble gangliosides could be involved in tumor-induced immunosuppression [98, 111, 112, 115, 116, 117, 118, 119].

Melanoma cells, especially those of metastatic melanoma overexpress a variety of gangliosides. Aberrant gangliosides and their high level could be a marker of malignancy. Serum levels of compounds with sialic acid did not vary in patients with precancerous pigmentary lesions. We observed that in early diagnosed melanoma serum level of gangliosides was not significantly increased. Low levels of gangliosides in melanoma patients without ulceration or metastasis were associated with an increased relapse-free survival after surgical removal of the tumor. High levels of gangliosides in patients with metastatic melanoma were associated with progression of the disease and a decrease in relapse-free survival after surgical intervention [49, 53, 103].

The presence in the body of glycosphingolipids antigens, recognized by the immune system as nonself, determines the proliferation of some lymphocitary clones, that promote the synthesis of antibodies against these molecules. Glycosphigolipids associated with tumors, induce also, the synthesis of antibodies against gangliosides by a complex mechanism. It was accepted the idea of genetic similarity between some exogenous antigens and some components of nervous cells.

The umoral immune response in adult patients with untreated malignant melanoma, in dysplastic nevi and healthy volunteers was evaluated by the assessment of antigangliosides of IgG and IgM type against GM1, GM2, GM3, GD1a, GD1b, GT1b, GQ1b. In healthy individuals, antigangliosides antibodies IgG and IgM type were negative, in dysplastic nevi group, antibodies IgG type were absent, with the exception of anti-GM1 that were present in 4.17% cases. Antibodies IgM type had the following distribution: 6.25% anti-GM1; 4.17% anti-GM2; 4.17% anti-GM3; 6.25% anti-GD1a; 4.17% anti-GD1b; 4.17% anti-GT1b; 0% anti-GQ1b. In melanoma group, IgG antibodies had the following distribution: 4.68% anti-GM1; 1.56% anti-GM2; 0.78% anti-GM3; 0.78% anti-GD1a; 3.12% anti-GD1b, while the positive IgM were 15.63% anti-GM1; 20.31% anti-GM2; 17.19% anti-GM3; 14.06% anti-GD1a; 10,16% anti-GD1b; 14.06% anti-GT1b; 4.69% anti-GQ1b. No statistical differences were observed in IgG status between the studied groups. Significant variations of IgM antibodies were determined between melanoma and nevi, respectively control group.

Based on these results, we can appreciate that gangliosides expressed on melanoma cells induced the synthesis of antiganglioside antibodies. The presence of antiganglioside antibodies was associated with oncogenic transformation of melanocytes, but the moment of antibodies synthesis could not be determined. The antibodies identified in melanoma patients were mostly of IgM type.

To evaluate if the presence of IgM antibodies was associated with melanoma development, we determined their relation to clinical, histological and biological factors recommended by AJCC for melanoma staging. Anti-GM1 varied significantly with tumor site, histological site of melanoma, Breslow index, Clark level, presence of ulceration, anti-GM2 did not vary with histological characteristics of melanoma, anti-GM3 varied significantly with Clark level and presence of ulceration and anti-GD1a was influenced significantly by tumor site, Breslow index, Clark level and presence of ulceration. Anti-GD1b was influenced significantly by histological type of melanoma, anti-GT1b only by tumor site, while anti-GQ1b was not influenced by histological characteristics of melanoma.

Positive correlation with statistical significance were determined between anti-GM1 and LDH, CRP, respectively, IL8, between anti-GD1a and LDH, CRP, respectively, IL8. Negative significant correlations were observed between anti-GM3, anti-GT1b and LDH, CRP, respectively, IL8. The transition from radial to vertical growth of melanoma marked by high levels of LDH, CRP and IL8 was associated with an increase in anti-GM1, anti-GD1a and a decrease in anti-GM3, anti-GM2 antibodies of IgM type. Therefore, the IgM antibodies against GM1 and GD1a might be useful in malignant melanoma staging and diagnosis. Also, they could facilitate tumor growth by promoting neovascularization, inflammation and angiogenesis.

Other important findings of our study are the potential protective role of anti-GM2 and anti-GM3 antibodies of IgM type in melanoma patients. Anti-GM2 and anti-GM3 could affect gangliosides expression on melanoma cells, and though, might influence indirectly cell proliferation, transmembrane signaling and cells interaction. IgM antibodies against GD1b, GT1b, GQ1b offered no data about melanoma progression in relation to analyzed histological factors. Their negative correlation with IL8, LDH and CRP suggest that they could suppress tumor growth and angiogenesis indirectly.

Ganglioside and antiganglioside antibodies could be used for melanoma staging and though, they could increase the precision of the outcome. High levels of gangliosides and anti-GM1 and GD1a before the surgical removal of the tumor were associated with advanced melanoma and poor prognosis. The presence of anti-GM2,-GM3,-GD1b,-GT1b,-GQ1b in patients with dysplastic nevi could be suggestive of malignant transformation. Assessment of gangliosides and antigangliosides before the surgical intervention could be an important item for the post-surgical follow-up.

Other studies in patients with prostate cancer or soft tissue sarcoma showed that anti-GM1 antibodies had no value for diagnose and prognosis. In patients with thyroid cancer well differentiated, the level of anti-GM1 IgG and IgM type was associated with carcinogenesis, with no diagnose value in thyroid cancer [120, 121, 122]. In a big cohort of patients with lupus systemic erythematosus the presence of both anti-GM1 IgM and IgG type were associated with neuro-psychiatric disorders and depression. Anti-GM1 were identified in the serum of patients with chronic idiopathic hepatitis, in systemic infections, autoimmune disorders with neurological involvement (encephalopathy, HIV neuropathy) and after parenteral administration of gangliosides [123, 124, 125, 126, 127, 128, 129, 130].

Manipulation of cellular growth dependent of GD1b, GT1b, GQ1b gangliosides was demonstrated in several cellular systems. In vitro growth of human metastatic melanoma WM266-4

was inhibited by GD1b, GT1b, GQ1b, while other gangliosides (GM 1, GM2, GM3, GD1a, GD2 and GD3) had no effect. The action of gangliosides from b series was inhibited by IL8. This phenomena could be antagonized by exogenous anti-IL8. No other growth factor (regulating of oncogene alpha growth factor, platelet regulating growth factor, interleukin 6) influenced melanoma evolution. A possible mechanism through which GD1b, GT1b and GQ1b inhibit melanoma growth could be the suppression of IL8 secretion, of ARNm expression and activation. Other studies suggest that GD1b could determine melanoma progression in vivo by stimulating angiogenesis. GT1b also influenced growth and motility of endothelial cells, while GM3 had an angiostatic effect. Multiple results proved the role of anti-GD1b,-GT1b,-GQ1b IgM type in soft tissue sarcoma Erlich subcutaneous solid tumor, Erlich carcinoma with ascitis [76, 121, 131, 132, 133, 134].

The ability of the human organism to promote an anti-GM1,-GM2,-GM3,-GD1a,-GD1b-GT1b,-GQ1b immune response could influence the evolution of patients with malignant tumors. Taking into account the immunogenic capacity of gangliosides in malignant melanoma and their effect on tumor development, we consider that an action on gangliosides metabolism could be a strategy of reducing tumor angiogenesis. Low gangliosides and increased antibodies against gangliosides from melanoma cells confer an advantage in survival of melanoma patients compared with patients without antiganglioside antibodies. Based on our results regarding gangliosides and antigangliosides profile in patients with cutaneous malignant melanoma, the authors consider that pharmacological modulation of ganglioside-antiganglioside system could be a way to control melanoma development.

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