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Research Article

The relationship between interleukin 8 and ki67 in cutaneous malignant melanoma

[1]Amalia-Elena Anghel, [2]Corina-Daniela Ene, [3]Monica Neagu, [4]Ilinca Nicolae

[1] Department of Dermatology, Skinmed Clinic, Bucharest, Romania; [2] Department of Nephrology, "Carol Davila" Nephrology Clinical Hospital, Bucharest, Romania; [3] Department of Immunology "Victor Babeş" National Institute of Pathology, Bucharest, Romania; [4] Research Departament in Dermatology, "Dr. Victor Babeş" Clinical Hospital, Bucharest, Romania.

Abstract. Background and objectives: Malignant melanoma still represents one of the most aggressive types of cutaneous cancers with a growing incidence all over the world. Interleukin 8 (IL-8) is an extremely valuable molecular marker, involved in oncogenesis and tumor progression. Ki 67 is a well-known tumor proliferation marker. Immunostaining cells percentage for Ki 67 correlated with IL-8 tumoral concentration could represent a way to appreciate the stage of development in cutaneous malignant melanoma. Our study analyses two markers of proliferation, assessing the relationships between tissue levels of IL-8 and Ki67 immunoexpression depending on the Clark level of the tumors. Material and methods: In the current study we included 88 patients with primar malignant melanoma, histopathological confirmed, without associated pathologies. We determined IL-8 tissue level using ELISA method and we assessed the immunohistochemical staining for Ki67. Both techniques were performed on tissue fragments with malignant melanoma and on peritumoral healthy tissue obtained from oncological surgical excision (88 cases). Results: Ki67 antigen was positively identified in 75% of the studied melanomas: weak positive immunostaining for Ki67 (5-25% positive cells) was found in 34% of tumors (41.2% of Clark II, 24.2% of Clark III, 40.9% of Clark IV and 75% of Clark V), immunostaining moderately positive was identified in 17.0% of melanomas (5.8% for Clark II, 27.3% for Clark III, 22.7% for Clark IV and 6.3% for Clark V), immunostaining strongly positive for Ki67 was identified in 17% of melanomas (27.3% for Clark III, 22.7% for Clark IV and 6.3% for Clark V). The obtained values for IL-8 in melanoma was estimated to be 33.6±6.9 pg g^{-1} protein and in peritumoral tissue, 2.9±0.6 pg g^{-1} protein. IL-8 concentration was significantly increased in malignant melanoma compared to healthy skin tissue. Compared to Clark level II melanomas (24.5 ±7.4 pg g^{-1} protein) we observed a significant increase of IL-8 in Clark level III melanomas (34.4±8.3 pg g^{-1} protein, $p<0.05$), Clark level IV melanomas (37.5±16.3 pg g^{-1} protein, $p<0.05$) and Clark level V (32.2±7.8 pg g^{-1} protein, $p<0.05$). Pearson correlation coefficient showed a positive association, statistically significant, between the amount of IL-8 and the intensity of immunostaining for Ki67 antigen in Clark III level melanomas ($r=0.672$, $p<0.05$) and Clark level IV melanomas ($r=0.590$, $p<0.05$). Conclusion: Tissue concentrations of IL-8 were correlated with the Clark level and the Ki67 immunoexpression in cutaneous malignant melanoma. Both, immunohistochemical detection of Ki67 and the tumoral quantification of IL-8, in conjunction with morphological and biological parameters, could be of real value in the management of cutaneous malignant melanoma.

Key Words: melanoma, interleukin 8, Ki 67, melanocytic proliferation.

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Corresponding Authors: A.-E. Anghel, e-mail: amalia.anghel@yahoo.com.

Introduction

Malignant melanoma (MM) represents a tumor derived from the genetically modified epidermal melanocytes that develops as a result of complex interactions between genetics and environmental factors. Malignant melanoma still represents one of the most agressive types of cutaneous cancer, with a high risk of metastasis and a low response rate to the oncologic treatments (Hahn et al 2002). The extraordinary tumor resistance to conventional oncological therapies requires immediate new therapy strategies based on molecular pathogenic aspects. It was demonstrated that the genetic pattern implicated in mitogenic transduction, which involves tumor suppressor genes inactivation, as PTEN/MMAC1 (PTEN) gene and ras oncogenes activation, has a dominant role in melanocytic oncogenesis (Tsao et al 2000). The result of this inactivation-activation process is the tumoral cells resistance to apoptosis, as an effect of inadequate activation of some survival pathways, especially those mediated RAF/MEK/ERK and PI3K/Akt (Tsao et al 2000).

Interleukin 8 is produced by the stimulated monocytes, macrophages, fibroblasts, endothelial cells, keratinocytes, melanocytes, hepatocytes, chondrocytes and a large number of tumoral cellular lines. It is an important pro inflammatory cytokine, with chemoattractant and neutrophil-activating properties (Baggiolini et al 1994). From a chemical and ultrastructural point of view, IL8 is an 8kDa un-glycosylated protein (72 aminoacids) derived from an processed protein with 77 aminoacids. A lot of studies demonstrate that the tumoral cells determine IL-8 overexpression, which is induced by the tumoral microenvironment in the context of the hypoxia associated with malignant tumors. Increased synthesis of IL-8 determines increased expression of the receptors CXCR1 and CXCR2 in the tumoral cells, tumor-associated endothelial cells, neutrophils and macrophages (David et al 2008; Schraufstatter et al 2001).

Changes of serum level of Il-8 reflects the importance of the pathogenic events in the pathology of melanocytic neoplasia (Anghel et al 2011). The interaction between IL-8 and receptors

induces kinases serine/threonine activation, which is one on the main effectors of the neutrophil chemotaxis, and it triggers the phosphorylation of serine/kinase substrate, PKB/Akt (Cheng et al 2008). Increasing the expression of the AKT activity was identified in numerous types of neoplasia, together with survival modulation, tumor angiogenesis and cellular migration, all off these representing a therapeutic targets. The induced signal IL-8 adjust MAPK activity (Mitogen activated protein kinase), a signal cascade with a large number of kinases serine/threonine that mediates the interaction between proteins and surface receptors, determining the activation of a large number of signaling pathways, the most important being Raf-1/MAP/ERKkinase 1/Erk cascade (Figure 1) (Venkatakrishnan et al 2000). It was demonstrated that IL-8 signaling induces the classic MAPK cascade, with downstream phosphorylation by Erk1/2 detected in neutrophils and tumoral cells (Alonso et al 2004).

One of the most studied etiopathogenic pathways in melanocytic oncogenesis is the MAP kinase (MAPK). The ligand binding to the tyrosine kinase receptor induces a conformational change with Ras (H, N, and K) and Raf kinases (A, B, and C) with phosphorylation and activation (Morrison et al 2002). Activated Raf dimers induces MEK phosphorylation, that activates ERK, which goes into the nucleus and acts as a transcription factor over some genes and leads to cellular proliferation and survival. Two of the most frequent studied genes, with an important role in tumoral melanocytic cells proliferation, are represented by CDKN2A and p21/WAF-1. CDKN2 encodes a 16 kd product that inhibits CDK 4, a key enzyme which regulates the cellular cycle in competition to cyclin D. The complex CDK4 cyclin D is responsible for the sequential phosphorylation and inactivation of the retinoblastoma tumor suppressor gene family (p107 [RBL1] and p130 [RBL2]) (Hanahan et al 2000). The R point is a moment in cellular cycle that represents the transition from the G0/G1 phase to DNA synthesis (S phase) and it is regulated by transcription factors from E2F family. Through E2F pathway, the RB gene family regulates the cellular cycle in the S phase. E2F induced replication imply the activation of the genes responsible for the cellular cycle progression (A, D, E cyclins, DNA polymerase, transcription factors c-myc, c-myb). In melanoma, there are large studies on the genetic changes that induce activation of cell proliferation, Koenig et al., notes, in order of frequency, dramatic loss of the p16 and PTEN expression, followed by RB genes and p53 exclusion from the nuclear compartment. These findings suggest that inactivation of p16/RB pathway is an important step in the pathogenesis of melanoma (Koenig et al 2002; Volgelstein et al 2000).

For melanomas with BRAF mutations (approximately 50% of total melanomas, the most frequent is BRAF600 with activating valine/glutamic acid at position 600), MAPK pathway is constitutively activated due to the presence of BRAF kinase mutations and high levels of Raf.

Ki-67 protein (MKI67) is a well-known marker associated with cell proliferation. It is present in all active phases of the cell cycle (G1, S, G2 and M), but is absent in resting cells (G0). Ki-67 antigen can be exclusively detected in the cellular nucleus, while, in the most part of mitosis, the protein is transferred to the surface of the cell (Scolzen et al 2000; Straume et al 2000). Ki-67 has been the most used proliferation marker in melanoma and other tumor types. In a recent analysis, Ki-67 was considered a useful tool in distinguishing benign from malignant

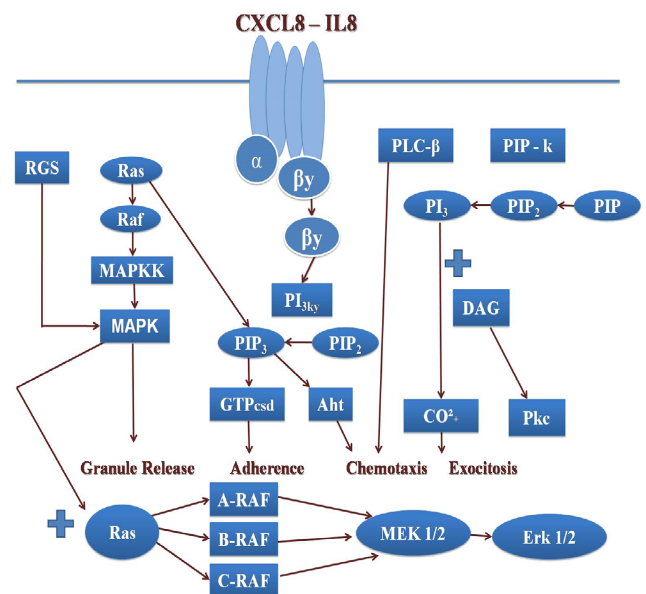

Figure 1: IL8-CXCL8 signal pathway: PLC-phospholipase C; PIP-K-phosphatidylinositol 4-phosphate kinase; IP3-inositol 1,4,5-trisphosphate; DAG-diacylglycerol; PIP2-phosphatidylinositol 4,5-bisphosphate; PIP3-phosphatidylinositol 3,4,5-trisphosphate; RGS-regulator of G protein signaling; PI3K- phosphatidylinositol 3-kinase; MAPK-mitogen-activated protein kinase; MAPKK-mitogen-activated protein kinase kinase; PH- plekitin homology; PKC-protein kinase C; Akt-protein kinase B; [Ca2+]i- intracellular calcium concentration; MEK- mitogen-activated protein kinase; Erk-Extracellular signal-regulated kinases

melanocytic lesions, as marker for malignancy (Henrique et al 2000). The proliferation rate of tumoral cells is an indicator of prognosis, correlated with the metastatic dissemination and long-term survival in a variety of human malignancies (Rothberg et al 2009). There are data in the literature, regarding melanoma, sustaining the association between increased expression of mitotic number and Ki-67 with unfavorable tumor characteristics, such as: tumor thickness, Clark level of invasion, presence of ulceration/tumoral necrosis and vascular invasion. Correlations between the Ki-67 expression and melanoma thickness are well documented, and an important link with survival was determined for thicker tumors (Gimotty et al 2005).

Significant proliferation of tumor cells, identified on histopathological/ immunohistochemical samples, measured with Ki6, correlated with a valuable biological marker, interleukin 8, can be important prognostic markers for cutaneous malignant melanoma. This is the aim of our study and the scientific literature does not have conclusive dates about this.

Our objective was to determine the possible involvement of IL-8 in cutaneous malignant melanoma proliferation by: evaluation of the immunohistochemical expression of Ki-67 antigen, quantitative determination of IL-8 in tumor tissue versus healthy tissue, assessing the statistical relationships between tissue levels of IL-8 and Ki67 immunnoexpression depending on the Clark level of the tumor.

Material and method

In the current study, approved by the Ethics Committee of the Hospital, were included patients with malignant melanoma without associated pathologies. The diagnosis of melanoma

was based on criteria established by the AJCC (American Joint Committee on Cancer). IL-8 quantitative determination and assessment of the Ki67 antigen immunohistochemical reaction were performed on tissue fragments with malignant melanoma single primary tumor, without detectable metastases, and on healthy tissue obtained from surgical excision (88 cases). All the patients gave their consent to the use of biological samples for diagnosis and research studies.

Ki 67 antigen immunohistochemical evaluation

Histological samples were obtained from fragments of tumoral tissue and healthy tissue from operatory protocols. Immunohistochemical staining indicates the nuclear and nucleolar localization of Ki67 in the cells from sections, based on the specificity of antibody binding to tissue antigens. The method for assessing the intensity of immunohistochemical staining for Ki67 was: <5% (inconclusive/negative), 5-25% (weak positive), 25-50% (moderately positive),> 50% (strongly positive).

IL-8 and proteins determination in tissue homogenates

The tissues can be processed immediately or can be stored at -80 degrees Celsius. In this study we chose the freezing of the surgically excised tissues and we performed all determinations together due to technical reasons. At the time of processing, the tissues were weighed, homogenized, and subjected to extraction with phosphate buffer saline, pH 7.4. After separating the supernatant by centrifugation at 4000 rpm for 10 minutes, we made the quantitative determinations for proteins in tissue homogenates (spectrophotometric method) and IL-8 (ELISA). The results for interleukin 8 were expressed pg g -1protein.

Statistical analysis

We use for data processing the SPSS program. For comparing the quantitative variables of the two groups we performed t test. It was chosen as a test of statistical significance 0.05 (5%), 95% confidence level showing that the decision is fair. The correlations between variables were determined by linear regression, and for the presentation of the link between two variables we use Pearson correlation coefficient.

Results

Immunohistochemical expression of Ki67 staining reaction

Ki67 antigen was positively identified in 75% of the studied melanomas (> 5% of cell nuclei were stained) and inconclusive (<5% of stained nuclei) in 25% of melanomas and normal skin tissue (Table 1). In melanoma tumors, correlated with Clark level, Ki 67 was found positive in 47% of Clark level II, 78.7 % of Clark III tumors, 81.8% of Clark IV and 87.5% Clark level V (Tabel 2).

Table 1. Biological parameters studied in melanoma and control tissues

Parameter	Melanoma	Control	p
Ki67 (+) / Ki67(-) (n/n)	66/22	0	-
IL-8 (pg g^{-1} protein)	33.6±6.9	2.9±0.6	<0.001

Ki67 staining was unconclusive (under 5% of stained cells) in 25% of analyzed tumors, distributed as follows: 53.0% of Clark level II melanomas, 21.3% of tumors with Clark level III, 18.2% of Clark level IV tumors and 12.5% of Clark level V tumors. This weak positive immunoreactivity for Ki67 (5-25% positive cells) was found in 34% of tumors: 41.2% of Clark level II tumors, 24.2% of Clark level III tumors, 40.9% of Clark level IV tumors and 75% of Clark level V tumors.

Table 2. Ki67 antigen immunostaining in melanoma according to Clark level

Clark level	II	III	IV	V
Samples (n)	17	33	22	16
Ki67(+) (n,%)	8 (47.0%)	26 (78.7%)	18 (81.8%)	14 (87.5%)
Ki67 (-) (n,%)	9 (53%)	7 (21.3%)	4 (18.2%)	2 (12.5%)

Immunostaining moderately positive for Ki67 (25-50% positive cells) was identified in 17.0% of melanomas: 5.8% for Clark II, 27.3% for Clark III, 22.7% for Clark IV and 6.3% for Clark V (Figure 2).

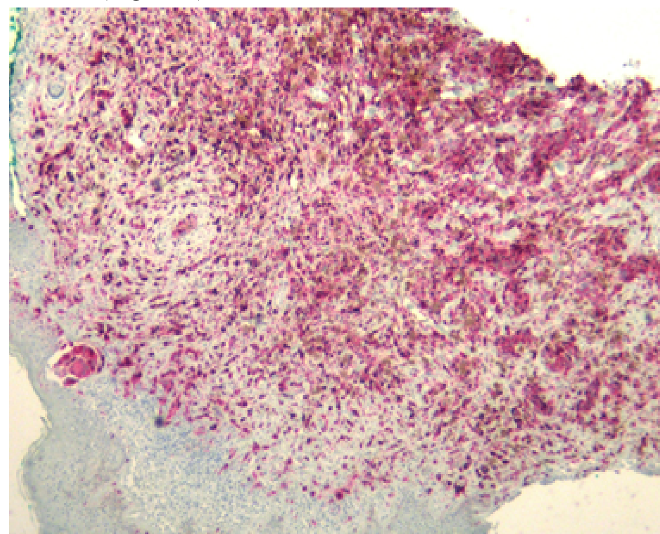

Figure 2. Ki 67 Immunohistochemical image Ki 67 brown chromogen; HE 10X.: homogeous nuclear staining (40%) in a cutaneous malignant melanoma

Immunostaining strongly positive for Ki67 (over 50% of stained cells) was identified in 17% of melanomas: 27.3% for Clark III, 22.7% for Clark IV and 6.3% for Clark V.

Quantitative determination of IL-8 (pg g^{-1} protein) in tumoral tissue versus healthy tissue.

The amount of IL-8 was evaluated in melanoma tumors and in peritumoral healthy tissue. The IL-8 level in melanoma was estimated to be 33.6±6.9 pg g^{-1} protein. In peritumoral tissue we obtained values of 2.9±0.6 pg g^{-1} protein (Table 1). IL-8 concentration was significantly increased in malignant melanoma tumors compared to normal skin tissue (Table 1). The amount of IL-8 was analyzed according to the Clark level (Table 3). Compared to Clark level II melanomas (24.5±7.4 pg g-1 protein), we noticed a significant increase in IL-8 for Clark level III melanomas (34.4±8.3 pg g^{-1} protein, $p<0.05$), Clark level IV

melanomas (37.5±16.3 pg g^{-1} protein, $p<0.05$) and Clark level V (32.2 ±7.8 pg g^{-1} protein, $p<0.05$).

Table 3. IL-8 concentration in melanoma cells according to Clark level

Clark level	II	III	IV	V
Samples (n)	17	33	22	16
IL-8 (pg g^{-1} protein)	24.5±7.4	34.4±8.3	37.5±16.3	32.2±7.8

The relationship between IL-8 and the intensity of the immunostaining for Ki67 antigen in melanoma cells.

Pearson correlation coefficient showed a positive association without statistical significance between tumoral production of IL-8 in melanomas and the intensity of immunohistochemical staining for Ki67 antigen in Clark level II tumors ($r=0.119$, $p>0.05$) and Clark level V tumors ($r=0.171$, $p>0.05$). A positive association, statistically significant, was obtained between the amount of IL-8 and the intensity staining for Ki67 antigen in Clark III level melanomas ($r=0.672$, $p<0.05$) and Clark level IV melanomas ($r=0.590$, $p<0.05$) (Table 4).

Table 4. Statistical relationship between IL-8 and Ki67antigen in melanoma according to Clark level

Clark level	Ki67 versus IL-8	
	r	p
II	0.119	0.214
III	0.672	0.003
IV	0.59	0.012
V	0.171	0.088

Discussions

The results of this study prove that malignant melanoma malignant phenotype has been associated with the excessive production of IL-8 in the tumoral environment. This statement is supported by the following results:

- in malignant melanoma we determined an amount of IL-8 to 12 times higher than in the adjacent skin tissue;
- the increased amount of IL-8 detected in the malignant melanoma was associated with increased Clark level;
- the transition from horizontal growth phase to vertical growth phase of melanoma, corresponding to the progression from Clark level II to Clark level III, was correlated with quantitative increase of IL-8.

Based on these experimental data it can be estimated that stimulation of the IL-8 synthesis in malignant melanoma could be associated with tumor development. The possible mechanisms that accompany the rapid and uneven growth of melanoma and IL-8 over synthesis might be explained by the ability of malignant cells and recruited inflammatory cells in the tumor microenvironment to develop large amounts of cytokines. In the literature there are reported some data that supports constitutive expression of IL-8 in various types of cancer: melanoma, breast cancer, ovarian cancer, prostate cancer (Jenkins et al 2015). Positive association between circulating IL-8 and the tumor environment has been documented in human solid tumors (Chow et al 2015). The increase in IL-8 in the tumor microenvironment has been associated with the proliferation, migration, angiogenesis, chemo-resistance in vivo and in vitro for various types of cancers (Anghel 2011; Ene et al 2015; Ning et al 2011).

In this study, Ki67 was investigated as a possible marker of cell proliferation in cutaneous malignant melanoma. It is known that the Ki67 antigen is expressed in the active stages of cell division (S, G1, G2, M), but not in G0. Until today, there is no compelling analytical evidence to recommend Ki67 as a prognostic factor of clinical utility in cancer (Alco et al 2015; Zheng et al. 2015). In addition, there is not rigorous data regarding the interpretation methodology of nuclear immunostaining for Ki67 antigen. To assess the results of this study, we choose a value of 5% for Ki67 immunostaining to distinguish between a negative and a positive result in a section examined by immunohistochemistry. By analysis of the immunohistochemical expression distribution of Ki67 in cutaneous malignant melanoma reported to the Clark level, it was revealed the following data:

- immunoexpression <5% was dominant in Clark level II melanomas;
- expression 5-25% for Ki67 was maximum in Clark level II and V melanomas;
- expression 25-50% for Ki67 was dominant in Clark level III and IV tumors;
- incidence >50% for Ki67 was maximum in Clark III level tumors. It is estimated that tumors with low Ki67 have a low proliferative activity. Moderate and strongly positive immunostaining is associated with high inflammatory infiltrates, increased angiogenesis, invasive tumor, enhanced proliferation (Alco et al 2015; Zheng et al 2015).

Although it couldn't be identified a direct relationship between IL-8 and Ki 67 antigen, in the present study we demonstrated that:

- tissue concentrations of IL-8 and the intensity of immunohistochemical expression of Ki67 antigen increases with the Clark level;
- a strong positive association has been established between IL-8 and Ki67 in Clark level III and IV melanomas.

As a result, IL-8 and Ki67 could give valuable information regarding the tumor mass, tumor volume and clinical staging of malignant melanoma (Ene et al 2015; Anghel et al 2011; Ning et al 2011; Zheng et al 2015). After surgical removal of the melanoma, the authors noticed a favorable evolution of the patients with low levels of IL-8 and Ki67. Kaplan-Meier curves regarding the determination of recurrence-free survival in patients with cutaneous malignant melanoma, depending on the tumoral level of IL-8 and the intensity of the immunohistochemical staining for Ki67, will be a part of an ongoing study. This analysis involves collecting data on the postoperative evolution of cutaneous malignant melanoma patients (unpublished results). In the scientific literature we do not found studies regarding the analysis of recurrence-free survival and overall survival in patients with cutaneous malignant melanoma, depending on IL-8 tumoral concentration and immuno-expression of Ki67. The tissue determination of IL-8 and Ki-67 status may serve as molecular markers for the post-surgery monitoring of the patients with malignant melanoma. The molecular mechanisms by which a series of factors amplify the tumor proliferation are not completely identified. It is known that the tumor microenvironment influences the tumor progression. Malignant melanocytes, and

also fibroblasts, endothelial cells, immune cells, inflammatory cells, keratinocytes produce extracellular matrix, sialo conjugates, which affect antigenicity, chemokines, adjacent hyperplasia (Kodet et al 2015; Nicolae et al 2012; Nicolae et al 2011). Type b gangliosides, namely GD1b, GT1b, GQ1b, might suppress tumor growth and angiogenesis through the negative effect exerted on the synthesis and secretion of IL-8 (Ene et al 2015). Other studies have shown that IL-1alpha and TNF-alfa stimulates IL-8 and GRO alpha growth, thus inducing epidermal hyper proliferation, keratinocytes migration, angiogenesis (Steunde et al 2002). These results strengthen previous observations according to which blocking IL-8 production would represent an alternative therapy in malignant melanoma.

Conclusions

The study of IL-8 and Ki67 could give useful information regarding the tumor growth. Malignant phenotype has been associated with excessive synthesis of IL-8 in melanoma . Tissue concentrations of IL-8 were correlated with the Clark level and the Ki67 immunoexpression in cutaneous malignant melanoma. The reduction of IL-8 and blocking the production of Ki67 could have an important role in the management of patients with malignant melanoma.

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Authors

- Amalia-Elena Anghel, Department of Dermatology, Skinmed Clinic, 8 Splaiul Unirii, 40032 Bucharest, Romania, EU, email: amalia.anghel@yahoo.com
- Corina-Daniela Ene, Department of Nephrology, "Carol Davila" Clinical Hospital, 010701, Bucharest, Romania, EU, email: koranik85@yahoo.com
- Monica Neagu, Department of Immunology, "Victor Babeş" National Institute of Pathology, 99-101 Splaiul Independenţei, 050096, Bucharest, Romania, EU, neagu.monica@gmail.com
- Ilinca Nicolae, Research Department in Dermatology, "Dr. Victor Babes" Infectious and Tropical Diseases Clinical Hospital, 281 Mihai Bravu Street, 030303, Bucharest, Romania, EU, email: drnicolaei@yahoo.ro

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