Research Article

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Fluorescence biomarkers of malignant melanoma detectable in urine

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Abstract: Malignant melanoma (MM) is a cancerous transformation of melanocytes. It is a disease with the worst response to therapy and, compared to other malignancies, presents much earlier with metastases. MM still belongs to relatively late-detected malignant diseases. Even so, the MM mortality rate is up to 96% for a relatively small incidence (5%). The gold standard for MM diagnosis is a histopathological examination that requires invasive surgery. An invasive sampling method of a biological material can be a stressful factor for the patient, which is often the reason why patients do not seek medical assistance as soon as possible. Our goal was to find a link between metabolites in urine and the stage of MM. Two excitation peaks at 360–370 nm and 450 nm were characterised in spectra of urine samples. The emission spectra have shown one significant peak at 410–460 nm. After addition of glutathione reductase to the samples, fluorescence dropped down only in patient samples and hidden fluorophores appeared. Malignant diseases are associated with the presence of specific metabolites that can be detected fluorescently in biological material such as urine, which can be a suitable alternative for an early detection of cancer or for tracking changes during and after treatment.

Keywords: NAD(P)H, lipofuscin, HIF-1 α , MITF-M, IGF1

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1 Introduction

Urine contains a number of endogenous fluorophores that can be used to monitor the course of a disease, the response to treatment or identify the potential threat of disease development. Known compounds with altered fluorescence intensity observed in case of some cancers (bladder, breast, etc.) include free (unbound) NAD(P)H molecules, flavins, porphyrins, proteins and pigments [1–4].

NADH and NADPH (collectively referred to as NADH hereafter) are increasingly produced by cancer cells as a consequence of their preference for cytosolic metabolism, the so-called Warburg effect [5]. Another feature of neoplastic tissue is the accumulation of porphyrins, the so-called porphyria [6,7]. Water-soluble porphyrins (5-aminolevulinic acid [ALA], porphobilinogen [PBG], uroporphyrin) are partially excreted into the urine and their amount increases with disease progression.

The tumour microenvironment is also defined by an extracellular matrix rearrangement. The physiological presence of protein structures (collagens, elastin, laminins) is reduced by their degradation. Elastin fibres surround the nevus root [8]. Melanomas have significantly fewer elastic fibres in the melanocyte roots. Melanoma cells exert pressure on the elastin layer in the papillary dermis, forming a compressed layer that distinguishes melanoma from the nevus [8]. The elastin layer is still present, however less evident. Elastin fragments affect the presence of matrix metalloproteinases (MMP-2, -4) and activate melanoma cells [9]. Higher levels of elastin remnants in tumours are associated with a higher stage of disease and metastasis [10]. The breakdown of elastin and its loss increases with Breslow tumour categorisation, mitotic degree as well as tumorigenic transcriptional and growth factors aberration, which are negative factors for malignant melanoma (MM) progression.

Another endogenous fluorophore, the concentration of which increases as a result of the presence of melanocyte cancer transformation, is lipofuscin (water-soluble pigment) [11]. Accumulation of aberrant lipofuscin results in a marked pigmentation of the affected

tissue [12], which usually appears as an abnormally brown to black colouration. Lipofuscin accumulation has been reported in retinal pigment epithelium cells and macrophages in several types of benign tumours, lesions simulating tumours and MM and choroid tumours [13]. Choroid tumours, unlike MM, do not exhibit typical tumour pigmentation; they are almost invisible ophthalmoscopically, but the presence of lipofuscin can be detected by fluorescence. Lipofuscin is produced as a waste material of intracellular structures accumulated in the lysosomes of affected cells [14]. The formation of the lipid pigment is the result of decreased cysteine protease activity in the lysosomes, which diminishes by age, and it is therefore also referred as the old-age pigment [15,16]. Its accumulation under conditions of oxidative stress due to reactive oxygen species (ROS) attack on lysosomes and mitochondria (mitochondria undergo autophagocytosis) leads to cell damage by destabilising membrane structures through the release of lipofuscin [17,18]. Non-physiological pigment accumulation (not associated to high age) is a sign of the tumour transformation of melanocytes. Implication of degenerative changes due to the development of MM, lipofuscin may occur in the urine [19].

The accumulation of metabolites, proteins or pigments is narrowly linked to the metabolic state of the cells which is genetically regulated. Malignant tumours are usually characterised by an insufficient supply of nutrients and oxygen to all areas of the tumour, resulting in so-called hypoxic or pseudohypoxic regions [20]. Cells in these malignant structures increasingly express hypoxia-inducible factor 1 alpha (HIF-1 α) which induces the expression of such regulatory genes that promote the adaptation of the cell to reduced oxygen and nutrients [21]. Activation of HIF-1 α in an effort to minimise the negative effects of low oxygen reduces cell demand for oxygen by responding to oxidative phosphorylation (OXPHOS) which as one of the main processes requires the most oxygen [22]. This adaptation is primarily mediated by favouring glycolysis as the main energy metabolic process [23]. Disconnection of OXPHOS is the cornerstone for the formation of ROS (ROS backstabilises HIF-1 α in hypoxia as well as normoxia) as a result of reduced respiratory complex activity [24]. Detachment of OXPHOS is a reversible process that when returned to the physiological state of oxygen leads to reduced oxygen consumption in response to activated HIF-1 α [25]. This dynamic equilibrium depends on the ability of oxygen to cross the membrane, which is affected by cholesterol, and lipid metabolism (closely linked to the viability of mitochondria and OXPHOS) [21].

Dysregulation of OXPHOS by HIF-1 α hyperactivation leads to limitation of pyruvate entry into the citrate cycle to mitigate the damage caused by non-physiological ROS generation [26]. Therefore, non-glucose metabolites especially from glutamine metabolism are favoured in the citrate cycle [27,28]. Originated by NADH, metabolic processes in mitochondrial matrix and cytosol do not regenerate on respiratory complexes but are transferred by the malate-aspartate shuttle system to the cytosol, where they accumulate and participate in the Warburg effect [29]. Abnormal stabilisation of HIF-1 α and NADH leads to malignant transformation and poor prognosis [30].

The mitochondrial protector is an insulin-like growth factor polypeptide [31,32] which is involved in the regulation of mitochondrial function, oxidative stress, and normal cell growth. Aberrant expression of IGF-1 increases expression of genes and proteins as BCL-2 and BCL-X(L) [33,34] which leads cells to immortality by resistance to apoptotic protective stimuli. Microphthalmia-associated transcription factor-melanocyte specific isoform (MITF-M) is involved in the maintenance of active mitochondrial metabolism [35], whose dysregulation leads to inhibition of OXPHOS. HIF-1 α decreases the expression of MITF-M [36] and increases the invasivity of the MM phenotype [37]. The main role of the MITF pathway is to regulate the cellular response to ROS stress by inducing tyrosinase synthesis which is a rate-limiting enzyme for melanin synthesis [38]. MITF-M depletion attenuates melanocyte response to autophagy induced by insufficient nutrient and oxygen intake (starvation) [39]. Downregulation of MITF leads to accumulation of lipofuscin [40,41] due to degradation of melanin.

Since NADH, FAD and lipofuscin belong to the endogenous fluorescent parameters which significantly change upon the transformation of melanocytes into malignant tumour, we explored their diagnostic potential in monitoring of MM progression.

2 Material and methods

2.1 Biological material

We studied the fluorescence intensity of endogenous fluorophores present in the urine and gene expression of genes related to starvation under hypoxia of MM patients and healthy probands. The demographic description of experimental groups is shown in Table 1.

Table 1: The demographic description of experimental groups

	Probands	Average age
MM patients	46	51
Controls	10	44
SSM	11	48
ALM	2	48
NM	8	50
LM	2	59
MMIS	2	50
Undefined	8	54
pT1ab	8	50
pT2ab	5	52
pT3ab	8	49
pT4ab	12	51
Undefined	13	54

2.2 Sampling and analysis

The urine samples were centrifuged for 5 min under 2,000 rpm at room temperature. Samples were diluted with deionised water in a 1:25 ratio. Excitation-emission matrices (EEMs), excitation (EX), absorption (AB) and specific emission fluorescence spectra (EM) were measured using a Horiba Dual FL dual spectrophotometer in the range 300–510 nm for the absorption spectra, in the range of 350–730 nm for EM ($\lambda_{EX} = 340$ nm), and in the excitation range of 240–800 nm ($\lambda_{EM} = 536$ nm). Addition of 2 μ L of glutathione reductase (GR) to 2 mL of urine samples and further analysis of emission spectra and EEM spectra were performed. GR is a homodimer containing FAD per monomer that restores intracellular glutathione (GSH) by reducing glutathione disulphide (GSSG) in the presence of NADH or NADPH (as hydrogen donor) [42].

The expression levels of the HIF-1 α , IGF-1 and MITF-M genes were analysed from blood (mRNA isolation by QIAamp RNA Blood Mini Kit (50), Cat. no.: 52304, Qiagen), reverse RT-PCR was performed by ProtoScript First Strand cDNA Synthesis Kit (E6300S, New England BioLabs) and quantification of the gene product was done by qRT-PCR (Maxima Hot Start PCR Master Mix (2x), K1059, Fermentas Life Sciences) and by Rotor-Gene Q (Ser. No.: R1114130, Qiagen).

2.3 Statistics

The obtained data were analysed with OriginPro 8 SR2 v8.0891 (OriginLab Corporation) and GraphPad Prism 5.04 (GraphPad Software). The presented data are mean $\pm$ standard error of the mean in three independent

experimental repeats. The unpaired *t*-test, *F*-test of variances and multiple *t*-test were used to compare the data obtained in the two groups. When comparing values among multiple groups, the one-way and two-way ANOVA statistical methods were used to evaluate statistical significance. Statistically significant results were found to have a *p* value (level of significance) below 0.05.

Informed consent: Informed consent has been obtained from all individuals included in this study.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance with the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee. Permission from the ethics committee was given by Ethics commission of Pavol Jozef Šafárik University in Košice, Faculty of Medicine is 20N/2016. This work was a part of doctoral thesis.

3 Results

Using EEM, excitation spectra and emission spectra, we analysed urine samples from patients with MM and healthy probands. We focused on the excitation spectra in the range of 300–500 nm, where two maxima were found; first found within the range 340–360 nm can be attributed mainly to NADH, collagens and elastin and second at 450 nm associated with porphyrins (Figure 1a). Typically, the maximum at 450 nm was more pronounced in MM patients. Based on the measured data, we have produced graphical representations of mean intensities for a maximum of 365 nm and at 450 nm in patients and in the controls (Figure 1b, c and Table 2). The calculated $R_{EX} = 450/365$ ratio for patient samples and for the controls is shown in Figure 1d and Table 2.

Absorption spectra were characterised by 1.42 times decrease in maxima at 280 nm which represent total value of proteins and amino acids (average of absorbance patients in Table 2). Next, the spectra showed increase at $\lambda_{AB} = 340$ nm, $\lambda_{AB} = 375$ nm and $\lambda_{AB} = 450$ nm in patient samples (Figure 1e, f and Table 2). Using two-way ANOVA, the Sidak's multiple comparison test showed strong significant decrease at $\lambda_{AB} = 280$ nm at $p < 0.0001$ and non-significant increase at $\lambda_{AB} = 340$, 375 and 450 nm.

Furthermore, we focused on emission spectra in the studied group in the range of 350–730 nm after excitation at $\lambda_{EX} = 340$ nm. Emission spectra were characterised by one

Figure 1: Excitation spectra (a), levels of excitation maxima at 365 (b) and 450 nm (c), ratio of excitation maxima 450/365 nm (d), absorption spectra (e), absorbance maxima at 280/340/375/450 nm (f).

single peak in the range of 410–460 nm which consists of multiple fluorescence peaks for NADH, elastin/collagen, FAD, etc. Patient samples were generally characterised by a

higher fluorescence intensity of this maximum than the control samples (Figure 2a). The difference before and after addition of GR in the range 355–500 nm with the maxima at

Table 2: Intensity values for absorption, excitation and emission spectra (significance, $p > 0.05$ is non-significant (ns); $p < 0.05$ is *; $p < 0.01$ is **; $p < 0.001$ is ***)

	MM patients		Controls		Significance
	Average (μA)	$\pm\text{SD}$	Average (μA)	$\pm\text{SD}$	
$\lambda_{\text{EX}} = 365 \text{ nm}$	3010.94	635.43	2844.00	671.74	ns
$\lambda_{\text{EX}} = 450 \text{ nm}$	2750.26	656.24	1990.60	10.10	ns
$R_{\text{EX}} = 450/365$	0.89	0.23	0.74	0.18	ns
$\lambda_{\text{AB}} = 280 \text{ nm}$	1.04	0.47	1.49	0.11	***
$\lambda_{\text{AB}} = 340 \text{ nm}$	0.45	0.15	0.24	0.07	ns
$\lambda_{\text{AB}} = 375 \text{ nm}$	0.42	0.11	0.21	0.08	ns
$\lambda_{\text{AB}} = 450 \text{ nm}$	0.37	0.09	0.21	0.08	ns
$\lambda_{\text{EM}} = 410 \text{ nm (GR)}$	3528.45	2938.04	11711.64	1512.40	
$\lambda_{\text{EM}} = 440 \text{ nm}$	8287.58	2356.42	11140.03	863.98	ns
$\lambda_{\text{EM}} = 440 \text{ nm (GR)}$	3462.85	1028.90	1073.78	1048.09	***
$\lambda_{\text{EM}} = 460 \text{ nm}$	6423.26	1656.42	9905.10	1032.54	**
$\lambda_{\text{EM}} = 460 \text{ nm (GR)}$	3112.79	775.31	8505.65	1394.84	***
$\lambda_{\text{EM}} = 525 \text{ nm}$	2356.13	851.20	1180.24	227.54	***
$\lambda_{\text{EM}} = 525 \text{ nm (GR)}$	2702.53	256.08	2573.22	334.41	***
$\lambda_{\text{EM}} = 565 \text{ nm}$	1353.48	492.07	1376.66	103.66	ns
$\lambda_{\text{EM}} = 565 \text{ nm (GR)}$	480.86	43.00	801.50	33.49	***
$\lambda_{\text{EM}} = 620 \text{ nm}$	441.15	105.43	387.97	13.95	ns
$\lambda_{\text{EM}} = 620 \text{ nm (GR)}$	370.14	102.40	391.02	13.72	ns
$R_{\text{EM}} = 620/460$	0.07	0.04	0.05	0.01	*
$R_{\text{EM}} = 620/460 \text{ (GR)}$	0.18	0.13	0.04	0.01	***

440 nm or 460 nm (possibly NADH and NADPH emission after excitation by 340 nm) is caused by NADPH fluorescence intensity which is sensitive to dehydrogenation by GR [43]. Upon the addition of GR to the urine, the emission of free NADH dropped while peaks representative mainly for elastin/collagen (410 nm), NADH (440, 460 nm), flavins (FAD 525 nm), porphyrins (basic 585 nm, acidic/neutral 620–630 nm) and pigments (lipofuscin soluble in water 565 nm) showed up (Figure 2b). Samples with GR were characterised by a significant reduction in fluorescence within the 410–460 nm region in MM patients, while no significant effect was observed in the controls. After the removal of NADH emission using GR, some maxima at 410, 440, 460, 525 and 565 nm were discovered (Figure 2c–f). Other peaks that were visible after the addition of GR were expected for NADH at 440 nm, expected for NADPH at 460 nm, presupposed for FAD at 525 nm, assumed for lipofuscin-like pigments at 565 nm and presumed for acid/neutral porphyrins at 620 nm (values of intensities in Table 2). The $R_{\text{EM}} = 620/460$ ratio and the $R_{\text{EM}} = 620/460$ ratio after addition of GR for patient samples and for controls are in Table 2. Using comparison multiple t -test two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli between samples and controls gives significant result with $p = 0.031$ between patients and controls, and significance with $p < 0.001$

between patients and controls after addition of GR (multiple t -test two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli is a procedure for controlling the false discovery rate of a family of hypothesis tests where the null hypothesis is true).

From the intensity difference before and after addition of GR at the range 355–500 nm, we calculated the capacity of area under the curve and percentage presume of NAD(P)H, which was 96% in average for the patients compared to average of the area for patients and 51% in average for the patients compared to average of the area for controls (Figure 3).

After distribution of measured excitation and emission maxima after addition of GR by type of MM (Superficial spreading melanoma [SSM], nodular melanoma [NM], acral lentiginous melanoma [ALM], lentigo melanoma [LM], malignant melanoma in situ [MMIS]), we can see the tendency that there is dependency on emission maxima at 440 and 460 nm which increases with increasing malignancy (increasing pT stage of MM). On the contrary, after addition of GR we can see the decrease of the fluorescence intensity at 440 and 460 nm which corresponds with incorporation of NADH and NADPH into enzyme (Figure 4a and b). For ulcerated and non-ulcerated tumours (Figure 4c), we determined opposite leaning that ulcerated tumours had slightly lower fluorescence than non-ulcerated tumours.

Figure 2: Emission spectra of urine samples $\pm$ GR (a), detail of emission region 350–750 nm (b), emission maxima ($\pm$ GR) at 440 nm (c), 460 nm (d), 565 nm (e), 620 nm (f).

Based on distribution of Melan A (Figure 4d), we did not determine any significant change between MM patients. The statistics of unpaired t -test, F -test of variances (no association among groups analysis) and multiple t -test of emission intensities are in supplementary data. The two-way ANOVA showed strong significant change with p value <0.0001 if compare differences between types of MM at $\lambda_{EM} = 440, 460$, and 525 nm by the Dunnett's multiple comparisons test (Dunnett's test is pairwise comparison of multiple treatment groups with a single control group). At $\lambda_{EM} = 565 \text{ nm}$, the significance difference was between LM vs controls at the p level 0.0075 and undefined type of MM vs controls at the p level 0.013 . The rest were non-significant

(see supplementary data). Next, the two-way ANOVA gave the strong significant change with the p level <0.0001 if compare differences between types of MM at $\lambda_{EM} = 440, 460, 525 \text{ nm}$ (except for pT4ab vs controls which was at the level 0.013) and 565 nm by the Dunnett's multiple comparisons test. At $\lambda_{EM} = 620 \text{ nm}$, there was no significance between groups (see supplementary data). Finally, the two-way ANOVA showed up the strong significant change at the p level <0.0001 if compare differences between presented and non-presented melan A parameters at $\lambda_{EM} = 440$ and 460 nm by the Dunnett's multiple comparisons test. At $\lambda_{EM} = 525 \text{ nm}$, the significance difference was between presented melan A vs controls with $p = 0.008$, non-presented melan A

Figure 3: Amount area under the plot probable of NADH in urine samples based on emission spectra.

Figure 4: Fluorescence intensities for excitation at 365 and 450 nm, and emission at 410/460/565 and 620 nm ($\pm$ GR) based on EEM by MM type (a and b), MM pT stage (c and d), MM ulceration (e and f), MM melan A (f and g).

Figure 5: Emission fluorescence at 565 nm divided by MM type and MM pT stage.

vs controls with $p = 0.029$, undefined vs controls with $p = 0.008$, and at 620 nm there was no significance between groups (see supplementary data). When comparing emission fluorescence intensities at 565 nm (presumably lipofuscin), it shows the significant increase in the intensity with increasing the pT stage of MM after addition of GR (Figure 5, supplementary data).

EEM of urine and healthy controls were characterised by four endogenous fluorescent regions (Figure 6a). Fluorescence maximum marked as A ($\lambda_{EX} = 340$; $\lambda_{EM} = 410$ nm) after addition of GR could represent the fluorescence of elastin/collagens and elastin/collagen fragments, fluorescence maximum marked as B ($\lambda_{EX} = 340$ nm; $\lambda_{EM} = 440$ – 460 nm) could represent the endogenous fluorophore-free NADH and

maximum marked as C could either represent bilirubin and other lipid components (found at $\lambda_{EX} = 370\text{--}380\text{ nm}$; $\lambda_{EM} = 490\text{--}510\text{ nm}$) or it could be lipofuscin and lipofuscin-like lipopigments (ceroids) (found at $\lambda_{EX} = 400\text{--}500\text{ nm}$; $\lambda_{EM} = 480\text{--}700\text{ nm}$). The isolated maximum marked as D ($\lambda_{EX} = 450\text{ nm}$, $\lambda_{EM} = 520\text{ nm}$) can be attributed to the fluorescence of flavins (FAD) and their metabolites. Some urine specimens of patients also exhibited a further maximum marked as E, consisting of three further peaks (E1–E3) ($\lambda_{EX} = 390\text{--}400\text{ nm}$; $\lambda_{EM} = 565, 620, 680\text{ nm}$), which may have resulted from endogenous fluorescence of lipofuscin, and its derivative luminescent ferrous clusters (porphyrins), or bilirubin.

When comparing the EEM of the control group and MM patients, there was specific decrease of fluorescence intensity after the addition of GR to the urine sample prior to the analysis. These changes were mainly present in the excitation region of $350\text{--}460\text{ nm}$ and in the emission region of $500\text{--}650\text{ nm}$ (Figure 6b and d). In the control group, the decrease of fluorescence after GR addition was not as pronounced in comparison with MM patients at above-mentioned emission wavelengths (Figure 6c and e).

The level of measured endogenous fluorophores in urine reflects not only the metabolic but also the genetic state of MM cells. Genes which are directly or indirectly involved in energetic and pigment metabolism of MM cells were analysed. The gene expression of HIF-1 α , MTF-M and IGF-1 (Figure 7a–f) for single pT stages of MM and for

ulcerated and non-ulcerated MM corresponds with malignancy of the disease. The average values of relative gene expression and p values of significance of unpaired t -test and F -test of variances are in Table 3. The ordinary one-way ANOVA for HIF-1 α expression between groups of pT stages resulted in significance with the p level 0.038, for MTF-M the significance was with $p = 0.003$ and for IGF1 the significance was with $p < 0.0001$. The Brown–Forsytlett’s test for HIF-1 α was non-significant with the p value 0.4829, for MTF-M the test significance was with the p value 0.0120, and for IGF1 test was non-significant with $p = 0.102$. The two-way ANOVA Dunnett’s multiple comparisons test showed significance change for comparing MTF-M gene expression in the pT1ab stage with the p value 0.006 and the pT2ab stage with $p = 0.002$, and if comparing IGF1 for pT1ab with the $p = 0.021$, pT2ab with $p = 0.027$, pT3ab with $p < 0.0001$, pT4ab with $p < 0.001$ and undefined stage with $p = 0.003$. The rest of gene expression for HIF-1 α and MTF-M was non-significant (Table 3).

4 Discussion

MM is one of the most aggressive forms of cancer. Neoplastic changes in skin cells are predominantly due to physical damage, such as an inappropriate exposure to UV

Figure 6: (a) EEM with denoted maxima; (b) EEM MM urine; (c) EEM MM urine + GR; (d) EEM control urine; (e) EEM control urine + GR.

Figure 7: Gene expression by MM pT stage and MM ulceration of HIF-1α (a and b), MITF-M (c and d), IGF1 (e and f).

radiation (mainly UVC) [44], which damages the genetic information of healthy melanocytes and leads to tumour transformation (UVB radiation is required to activate provitamin D in the dermis). Repeated sunburn caused by inadequate exposure to sunlight, and therefore UV radiation, increases the risk of MM. Other risk factors include skin phototype (mainly I and II) [45], lifestyle (low frequency of exposure to sunlight during the year) or genetic predisposition (familial occurrence and numerous benign skin tumours). The Caucasian race, mainly people

living in the countryside, suffers predominantly from hypovitaminosis D and has light skin phototypes, and in developed industrial countries lifestyle (avoidance of direct sunlight) leads to more frequent skin malignancies [46–48]. Early diagnosis of the disease would certainly contribute to a reduction in MM mortality. Fluorescence methods utilising the luminescence of endogenous fluorophores characteristic for MM (e.g. lipofuscin) have still unexplored potential in detection of both presence and predisposition of MM.

Table 3: Gene expression and p values of gene expression compared to controls

Relative gene expression					
	pT1ab	pT2ab	pT3ab	pT4ab	Undefined
HIF-1 α	0.43	0.37	0.71	0.78	0.57
MITF-M	0.29	0.21	0.81	0.79	0.60
IGF1	3.10	3.04	5.43	5.07	3.56
Unpaired t -test two-tailed					
	pT1ab	pT2ab	pT3ab	pT4ab	Undefined
HIF-1 α	0.19	0.20	0.0003	0.004	0.07
MITF-M	0.01	0.004	0.25	0.40	0.08
IGF1	0.0002	0.003	<0.0001	<0.0001	0.0002
F -test of variances					
	pT1ab	pT2ab	pT3ab	pT4ab	Undefined
HIF-1 α	0.05	0.85	0.52	0.77	0.01
MITF-M	0.66	0.02	0.08	0.08	0.15
IGF1	<0.0001	<0.0001	0.0022	0.001	<0.0001
One-way ANOVA Dunnett's multiple comparisons test					
	pT1ab	pT2ab	pT3ab	pT4ab	Undefined
HIF-1 α	0.64	0.85	0.02	0.08	0.19
MITF-M	0.01	0.002	0.56	0.77	0.14
IGF1	0.02	0.03	<0.0001	0.0002	0.003

The absorption spectra were characterised by decrease maxima at 280 nm which represent total value of proteins and amino acids [49]. Next, the spectra showed increase at $\lambda_{AB} = 340$ nm (could correspond with NADH) [50], $\lambda_{AB} = 375$ nm and $\lambda_{AB} = 450$ nm (could present FAD) [51]. This change corresponds with presence of cancer disease [52].

As mentioned above, in the urine samples of MM patients and healthy controls, we detected various biomarkers of cancer diseases by fluorescence spectrophotometry. In excitation spectra, we mainly focused on area between 340 and 450 nm where could be detected, for example, NADH (340 nm) [53], elastin and collagens (340–360 nm) [54] and FAD (450 nm) [55], and their mutual ratio. The $R_{EX} = 450/365$ ratio was 0.8871 for the control group and 0.7422 for patients, thus 1.2-fold higher relative to patients. Change in the intensity at 365 nm in MM samples may reflect a lower relative protein concentration (elastin, collagens) as a result of malignant skin disease [56,57], and increased intensity at 340 nm reflects concentration of free NADH or NADPH, respectively [58]. NADH is used by GR to convert GSSG to reduced GSH [43]. This convert could be used for calculation of the NAD(P)H value in samples.

Values of the difference of emitted fluorescence signal at 440 and 460 nm (after excitation by $\lambda_{EX} = 340$ nm) before and after addition of GR reveal a difference between the patients (96% or 51%, respectively) and controls (0.15%).

Fluorescence spectra after excitation at $\lambda_{EX} = 340$ nm exhibit one large peak (410–460 nm) [53]. Several emission maxima such as NADH, elastin and collagens may be present in the described region. Moreover, the intensity of one fluorophore can mask other fluorophores in neighbouring region; therefore, a reduction of the NADH fluorescence signal by GR can be used to highlight other endogenous fluorophores emitting in the given region (350–730 nm). In this way, we could describe the presence probably of FAD, lipofuscin and porphyrins, the relative concentration of which was higher in patients than in healthy controls.

The metabolites in urine reflect the metabolic and genetic state of cells. We focused on genes which are involved in mitochondria metabolism. The genes such as HIF-1 α , MITF-M and IGF1 are directly involved in energy metabolism of MM. The pathological changes by malignant transformation of melanocytes give specific alternations in trash metabolites excluded by urine. The increased expression of HIF-1 α leads to decreased OXPHOS, preferred glycolysis and increased NADH values [59,60]. We determined increasing expression of HIF-1 α with increasing malignancy (relative gene expression for pT1ab was 2.18 times higher than for the controls, pT2ab was 1.89 times higher, pT3ab was 3.60 times higher, pT4ab was 3.96 times higher, undefined pT stage was 2.91 times higher). The same tendency was observed by other groups [61–64].

The MITF-M role is to regulate the cellular response to ROS stress. The low level of MITF-M leads to the worst fight with a damage caused by ROS [65,66]. It is well known that increased ROS production directs cells to oncogenic transformation [67]. In our experiments, we detected decreased level of MITF-M in all pT stages of MM. Downregulation of MITF-M gene tends to accumulation of lipofuscin [68] as the side effect of melanin degradation in the melanocytes caused by low activity of tyrosinase due to high level of ROS and HIF-1 α . Taken together, the low MITF-M expression correlates with increased presentation of lipofuscin in body fluids as well as in urine [69,70].

The last of studied genes was IGF1, known as mitochondrial protector, which protects cells from oxidative stress and regulates normal cell growth [71]. Its pathologically increased expression activates anti-apoptotic genes which are members of oncogenes (BCL-2,

BCL-X(L)) [33]. We determined increased expression of IGF1 gene in all pT stages of MM.

As we mentioned above, the differences in fluorescence intensities at $\lambda_{EM} = 440, 460, 525, 565$ nm between groups of type of MM, pT stages, melan A presentation or ulceration respectively is possible to use as marker of prediction of MM. This trend of increased NADH level occurs in early pT stages; therefore, the early pT stages still “work” on glucose TCA which is uncoupled and leads to accumulation of NADH. The pT4ab stage was described by lower level of NADH caused by glutamine TCA metabolism which compensates ROS production in mitochondria and regenerates NAD^+ [27,28]. “Lipofuscin” emission intensities divided by pT stages showed their increased values with increasing malignancy of MM.

In the end, in routine operation, technicians encounter enormous amounts of the biological material. Therefore, the measurement of concentration matrices is time-consuming. We managed to eliminate the effect of NADH using GR and thus visualise characteristic peaks (mostly suited for lipid pigments, porphyrins, elastin and others) regardless of their initial concentration in 50–96% of all analysed urine samples of MM patients. This trend was not observed in the urine samples of healthy subjects. All determined parameters based on excitation and emission spectra of urine discussed above, λ_{AB} (280, 340, 375, 450 nm), R_{EX} (365, 450 nm), λ_{EM} (440–460, 565 nm), show a potential in laboratory diagnostics for monitoring of MM progression. Since our findings correlate with gene expression of HIF-1 α , MITF-M and IGF1, potential changes in metabolic pathways associated with MM seem to be directly reflected in both excitation and emission spectra of urine samples. Therefore, spectral measurements of urine samples appear to be a rapid, inexpensive and non-invasive method suitable for monitoring of MM progression.

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