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Metallothionein Electrochemically Determined using Brdicka Reaction as a Promising Blood Marker of Head and Neck Malignant Tumours

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There are more than half of million patients who fall ill with head and neck carcinoma per year, which 6 % of annually newly diagnosed malignant diseases in the world. Group of head and neck tumours includes tumours occurring in the upper respiratory tract and upper digestive tract. They include tumours occurring in the area of oral cavity, tumours of hard and soft palate, gingivae and tongue. However, suitable markers in the case of head and neck carcinoma have not been satisfactory identified. Metallothioneins as a group of proteins with unknown but important role in development of a tumour can be assumed as a potential marker. Therefore, electrochemical monitoring of metallothionein levels in patients suffering from primary malignant tumour in head and neck area and examination of a suitability of electrochemical detection as a technique to be used in clinical practise are the most important aims of this study. Differential pulse voltammetry Brdicka reaction was optimized (time of accumulation 240 s, dilution of a sample 100 times and sample injection 10 μl) and used for analysis off blood obtained from 145 patients with newly diagnosed malignant tumour disease

in the head and neck area. As a control, fifty eight blood samples were used. The obtained data enabled us to suggest reference MT level in blood of healthy human within the interval from 0.2 to 0.8 μM. In the tumour blood samples, the most extend group was represented by patients suffering from oropharyngeal cancer (n = 69, 47.5 %), laryngeal cancer (n = 36, 24.8 %), hypopharyngeal cancer (n = 14, 9.6 %), oral cavity cancer (n = 18, 12.4 %) and rarely occurring nasal cavity and paranasal sinus cancer (n = 4, 2.7 %) and parotid carcinoma (n = 4, 2.7%). Age median of the studied group was 60 years. Determined MT levels in blood of patients varied from 1.08 to 6.39 μM, whereas average values differed in the accordance with tumour localization. Differences between individual localizations are discussed. In conclusion, MT levels are closely associated with the rate of tumour differentiation, stage of tumour disease and tumour cell characteristics. Our study demonstrates not only changes in blood MT levels in patients suffering from malignant tumour disease in head and neck area, but also suitability of electrochemical techniques for blood analysis.

Keywords: electrochemical detection; head and neck cancer; voltammetry; catalytic signal; metallothionein; marker

1. INTRODUCTION

There are approximately 650 000 of patients who fall ill with head and neck carcinoma per year, which 6 % of annually newly diagnosed malignant diseases in the world. This type of malignant disease has increasing tendency in the Central and East Europe [1]. Almost 98 % of all diseased is older than 40 years in Europe, however, this age limit still declines [2]. Men represent the most endangered group [3,4]. Mutual rate between men and women is almost 10:1 in some counties [5]. Percentage of five-year survival varies from 91 % (patients in the I. stage) through 77 % (patients in the II. stage), 61 % (III. stage), 32 % (IV. stage), 25 % (IVb. stage) to only 4 % (patients in the final IVc. stage). Group of head and neck tumours includes tumours occurring in the upper respiratory tract and upper digestive tract. They include tumours occurring in the area of oral cavity, tumours of hard and soft palate, gingivae and tongue [6]. Other types occurring in this area without connection with upper respiratory or digestive tract are not included in this category, i.e. brain tumours, malignant melanoma, etc. More than 90 % of the tumours of the mucosal lining are classified as squamous cell carcinoma developed from premalignant lesions such as leukoplakia and erythroleukoplakia [7]. Head and neck tumours mostly occur in the oral cavity (40 %) followed by larynx (25 %) and pharynx with app. 15 %. The rest of tumours is of more rare localization, such as thyroid gland and salivary glands. Causations of genesis of head/neck cancer are not fully known. Nevertheless, factors contributing to its induction as smoking and drinking of alcohol have been identified [7]. In addition, combination of both factors seems to be the most hazardous (separately they can increase risk of disease incidence five times, in combination more than fifteen times) [8]. In addition, the effect of food and food habits has been also investigated, however , specific connection between food and food habits and occurrence and development of this type of tumour disease has not been found. On the other hand, consumption of fruit and vegetable, especially of that rich on carotenoids, has chemoprotective effect and reduce risk of a tumour disease including head and neck carcinoma [5]. Poor oral hygiene can represent next risk factor with the effect on cancerogenesis in this area. Mechanism of this process is probably based on

the formation and presence of acetaldehyde in saliva (similar mechanism of induction as with alcohol) [9]. Human papiloma virus (HPV) is also ranged to the group of risk factors of head/neck cancer [10-13]. Generally, this localization is highly predisposed to the contact with potent cancerogens (air/food contaminants and pollutants).

Based on the above mentioned facts it is not surprising that the temporal trends in incidence of these tumours relate to environmental factors. There is an increased tendency in countries without prevention and a decrease in countries having an active policy of prevention of alcohol and tobacco consumption. In contrast, an increased incidence occurs in the world at tumour sites related to human papilloma virus infection in relation to changes in sexual habits [7]. Retrieval of suitable marker/markers of malignant tumour disease belongs to the most important factors of prevention. In addition, well-timed detection may significantly increase possibility of complete cure. However, suitable markers in the case of head and neck carcinoma have not been satisfactory identified [14].

Most of the examined predictors of head and neck cancer are in the phase of preclinical testing. Hanahan et al. describe six important steps that are considered as necessary for the development of cancer at the cellular level [15]: 1) the autonomous proliferative signals, 2) inhibition of growth inhibitory signals, 3) avoidance of programmed cell death (apoptosis), 4) immortalization, 5) obtaining blood supply to feed the tumour (angiogenesis), 6) tissue invasion and metastasis. Given the complexity of carcinogenesis, determination of one biological tumour marker is of little importance and is often appropriate to determine a battery of biomarkers. For cancers of the head and neck, higher expression of epidermal growth factor receptor (EGFR) growth factor is observed [16]. EGFR levels are increased during the transition from dysplasia to cancer in advanced stages of cancer and poorly differentiated tumours. It is assumed that external influences such as smoking and alcohol abuse, lead to increased production of EGF and increased expression of EGFR and may play a role in tumour development [17,18]. In addition, it was found that the better the prognosis is correlated to EGFR gene amplification [19-21]. Another important step necessary for the development of cancer is loss of normal anti-growing signals. Oncogenesis suppressor gene TP53 belongs to the most studied genes [22-24]. It is very interesting that mutations associated with HPV infection may damage of TP53 gene. Gene mutations can be also caused by the interaction of DNA with benzpyrene, which are part of cigarette smoke. It has been shown the increase p53 positivity in the progression of head and neck cancer - 19% were positive in normal epithelium, 29 % positivity in hyperplastic lesions, 45 % positivity for dysplastic lesions and 58 % positivity in invasive carcinoma [23,25]. p53 protein and its relation to Ki-67, and cyclin D1 belong to other proteins intensively studied in relation to cancer of the head and neck [21]. Experimental work has shown that telomerase is expressed in 90 % of human tumours, whereas is not detectable in normal somatic cells [15,26,27]. Available data indicate the potential importance of telomerase in the pathogenesis of head and neck cancer. Telomerase activity is already detectable in late stages of carcinogenesis and in the early stages of cancer [28]. However, immortalization have not been detected during the process of carcinogenesis in some head and neck cancers [29]. Inadequate blood supply of tumours has not only affects its growth, but also to treatment. The lack of blood supply tumours are hypoxic and the hypoxic cells are radioresistant [30]. Most tumours are capable of stimulating endothelial cell proliferation and formation of new blood vessels. This process is intricately regulated angiogenesis stimulatory and inhibitory factors. CD34 antigen of

endothelial cells is a marker of angiogenesis. Determination of microvascular density using CD34 is considered an independent prognostic factor in breast, ovarian, prostate and stomach cancer. Results of studies in head and neck cancer are not sufficient. Some studies have found a link between increased microvascular density and mestating [31], in another study, the higher density was associated with better prognosis in contrary [32], other studies have not found a correlation between microvascular density and clinical outcome in general [33]. Vascular endothelial growth factor (VEGF) has an effect on mitosis of endothelial cells, their motility, organization, and also on endothelial permeability [34]. Invasion and metastasis of tumour cells is an important part of the progression of a tumour. At this stage of tumour growth, numerous proteins are involved. For invasion of tumour epithelial cells three steps are required: attachment to the basal cell membrane, intracellular matrix proteolysis and migration of tumour cells. Some integrins, E-cadherin and a group of cytoplasmic proteins (catenins) are involved in the attachment of tumour cells to basement membrane. Cadherin are transmembrane proteins providing a link between cells in the tissue. It was found that reduced levels of E-cadherin were associated with metastatic genotype in malignant head and neck cancer [35]. Critical phase of the invasion of tumour cells is extracellular matrix proteolysis. At this step, involving a group of proteinases known as matrix metalloproteinase (MMP), which activity requires the presence of metal ions, are required. Particular attention should be given to MMP-2 and MMP-9, also known as collagenase type IV with a molecular mass of 92 kDa. Both proteases were detected in invasive forms of head and neck cancer [36]. The presence of increased activity of MMP-9 was associated with more invasive cancer of the oral cavity [21]. It was also found that staining for MMP-9 correlated with increased microvascular density and VEGF, suggesting a possible cooperation between the two factors in tumour angiogenesis. It was also observed and verified the interaction between MMP-9 and the metal-bearing protein metallothionein [37].

Metallothioneins (MT) were discovered by Margoshes and Vallee in 1957 after isolation from horse kidneys [38]. They occur across all animals with the high rate of homology. In addition, similar proteins are expressed also by bacteria, fungi and plants. Mammalian MT are small proteins (6-10 kDa) with the high content of cysteine in its primary structure (up to 30 %) and with absence of aromatic amino acids. Main function of metallothioneins in organisms consists in transport of heavy metal ions, maintenance of oxidation-reduction status and regulation of gene expression. They are usually of intracellular localization (cytoplasm and organelles), which are closely connected with oxidative metabolism (mitochondria, lysozomes). Nevertheless, they have important function in nucleic acids protection [39]. Increased expression of metallothionein genes is closely connected with proliferating cells [40] and with the stage of cell cycle. The highest metallothionein values were detected in the late G1 phase and during the moving to S phase [41]. Attention is focused on the role of metallothionein in processes of cancerogenesis and its relation to the protection of proliferation malignant tumour cells [42-51]. Electrochemical techniques are very suitable for metallothionein detection. This fact has been confirmed and verified on various types of samples including human tissue and blood samples [37,52-79]. Especially electrochemical methods based on the detection of catalytic signals such as peak H and Brdicka reaction are the most suitable and promising techniques for monitoring of these proteins in body fluids and tissues. Electrochemical monitoring of metallothionein levels in patients suffering from primary malignant tumour in head and neck area and

examination of a suitability of electrochemical detection as a technique to be used in clinical practise are the most important aims of this study.

2. EXPERIMENTAL PART

2.1. Chemicals

Rabbit liver MT (MW 7143), containing 5.9 % Cd and 0.5 % Zn , were purchased from Sigma Aldrich (USA). Tris(2-carboxyethyl)phosphine (TCEP) was prepared by Molecular Probes (Eugene, USA). MT stock standard solutions were prepared with ACS grade water (Sigma-Aldrich, USA) and stored in the dark at –20 °C. Working standard solutions were prepared daily by dilution of the stock solutions. To pipette volumes down to micro and nanolitres, pipettes used were purchased from Eppendorf Research (Eppendorf, Germany) with the highest certified deviation (±12 %).

2.2. Differential pulse voltammetry Brdicka reaction for metallothionein determination

Differential pulse voltammetric measurements were performed with 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder (4 °C). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 was used as the working electrode. An Ag/AgCl/3M KCl electrode was the reference and glassy carbon electrode was auxiliary. For data processing GPES 4.9 supplied by EcoChemie was employed. Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer (NH_3(aq) + NH_4Cl, pH = 9.6) was used. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as follows: initial potential of –0.7 V, end potential of –1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude -250 mV, E_{ads} = 0 V, time of accumulation 240 s, volume of injected sample: 10 μl (100 × diluted sample with 0.1 M phosphate buffer pH 7.0). All experiments were carried out at temperature 4 °C employing thermostat Julabo F25 (Labortechnik GmbH, Germany) [54].

2.3. Preparation of deionised water and pH measurement

The deionised water was prepared using reverse osmosis equipment Aqual 25 (Czech Republic). The deionised water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

2.4. Blood samples and their preparation

Blood samples were obtained from Department of Otorhinolaryngology and Head and Neck Surgery, St. Anne´s University Hospital, between years 2006-2009. All samples originated from

patients suffering from malignant tumour disease in head/neck localization – oropharynx, oral cavity, hypopharynx, paranasal sinuses, larynx, and parotid gland (*glandula parotis*). Blood samples were collected before any type of treatment. Control blood samples were obtained from Department of Sports Medicine, Krizkovskeho 22, Brno, numbering of n = 58. There were no differences in technique of sample storage and preparation. Sample (blood) taking and subsequent processing was approved by Ethic Committee of Masaryk University.The samples were kept at 99 °C in a thermomixer (Eppendorf 5430, Germany) for 15 min with shaking in order to remove ballast proteins and peptides, which could influence the electrochemical response. The denatured homogenates were centrifuged at 4 °C, 15 000 × g for 30 min. (Eppendorf 5402, Germany) [80].

2.5. Mathematical treatment of data and estimation of detection limits

Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). Statistical significances of the differences between MT levels were determined using STATISTICA.CZ. Differences with $p < 0.05$ were considered significant and were determined by using of one way ANOVA test (particularly Scheffe test), which was applied for means comparison. The detection limits (3 signal/noise, S/N) were calculated according to Long and Winefordner [81], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

3. RESULTS AND DISCUSSION

Determination of biologically active compounds rich in cysteine moieties is not an easy task for analytical techniques due to the presence of free sulfhydryl (-SH) groups [45,53]. In our previous studies, electrophoretic [60,82,83] and especially electrochemical detection [54,55,61] of MT as a representative of such type of compounds have been suggested, optimized and used for analysis of sample obtained from patients with a tumour disease.

3.1. Electroanalytical detection MT by Brdicka reaction

Trends in electrochemical detection of metallothionein were described previously by Adam et al. [52] and, moreover, this topic was thoroughly overviewed by Ryvolova et al. [45]. Electrochemical detection of MT is relatively time and economically undemanding and brings highly reproducible results of MT concentration [61]. In addition, electrochemical methods seem to be a suitable tool for real blood sample analysis that can find use also in clinical practice [49,66]. Typical differential pulse voltammograms of the increasing concentrations of MT are shown in Fig. 1A. There are well separated and distinguishable signals called RS2Co at app. -1.05V, which represents current response of MT complex with components of Brdicka supporting electrolyte, Cat1 (-1.25 V) and Cat2 (-1.45 V) are catalytic signals of hydrogen evolution from the supporting electrolyte catalysed by the presence of

MT. More detailed explanation of these electrochemical processes can be found in the following papers, but the precise electrochemical process is not clear [77,80,84,85]. Based on the current knowledge on the nature of the mentioned signals Cat2 peak is the most proportional to concentration of MT. Dependence of Cat2 height on MT concentration is shown in Fig. 1B. This dependence was strictly linear within the studied range from 1 to 50 µM as follows y = 1.9126x - 0.0369, R^2 = 0.9991. The suggested technique of detection was highly repeatable with relative standard deviation (R.S.D) = 2.8%. The detection limit for MT estimated as 3 S/N was 1 nM.

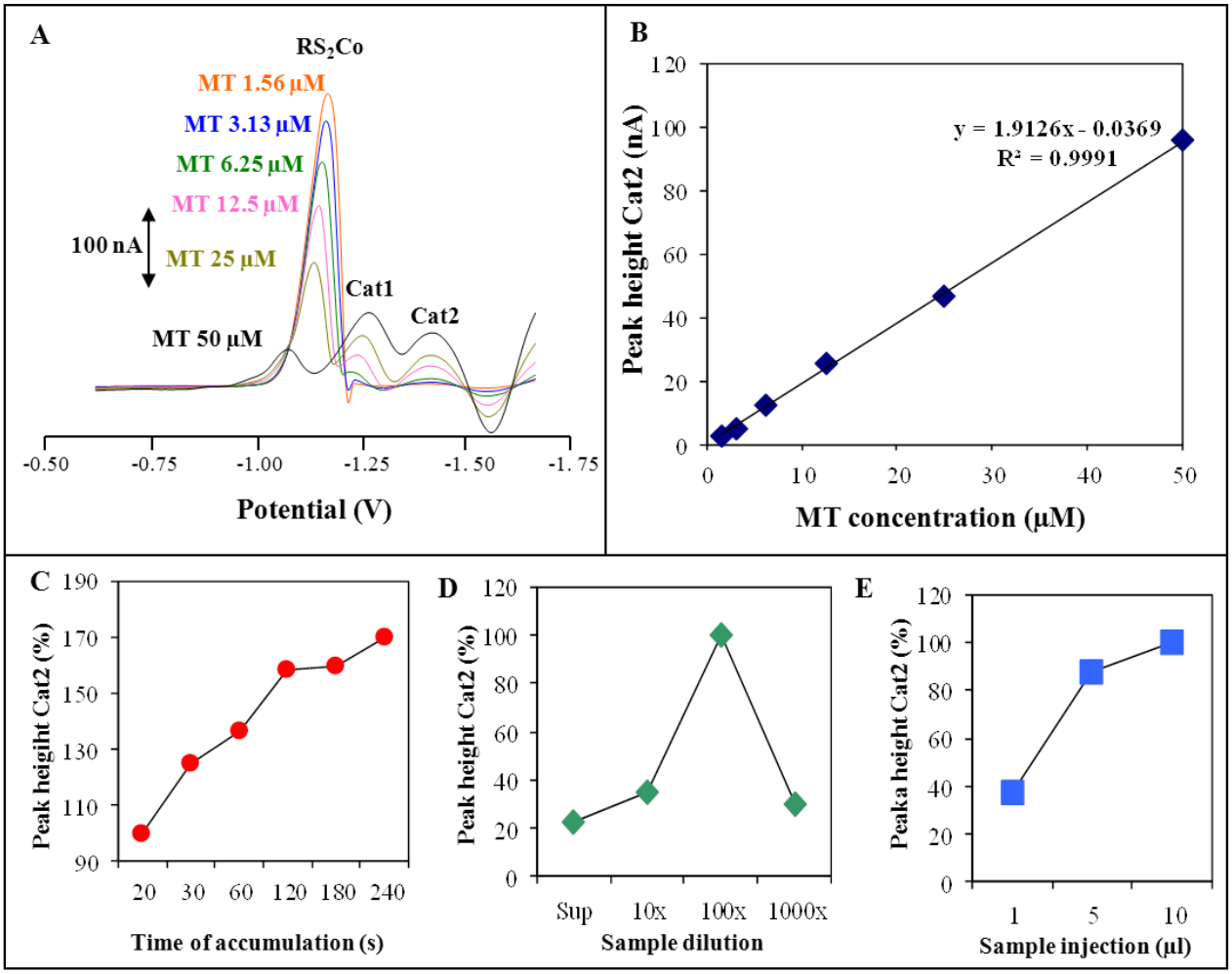

Figure 1. (**A**) Typical DP voltammograms of metallothionein in concentration of 1.56, 3.13, 6.25, 12.5, 25 and 50 µM. (**B**) Dependence of Cat2 peak height on MT concentration within the concentration interval from 1 to 50 µM. (**C**) Effect of time of accumulation on MT Cat2 blood sample signal. The influence of (**D**) blood samples dilution and (E) sample volume on Cat2 peak height. Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer ($NH_3(aq)$ + NH_4Cl, pH = 9.6) was used. The supporting electrolyte was changed after each analysis. The parameters of the measurement were as follows: initial potential of –0.7 V, end potential of –1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude -250 mV, Eads = 0 V, time of accumulation 240 s, volume of injected sample: 10 µl.

In the following experiment, real sample of blood obtained from healthy volunteer was prepared according to protocol mentioned in Experimental section. Due to sample processing, we were able to separate thermostable part of blood (metallothionein and other heat shock proteins and stabile peptides) from high-molecular mass thermolabile proteins. Prepared sample of volume of 10 μl was applied into the supporting basic electrolyte. Subsequently, on the influence of the time of accumulation on Cat2 peak height was investigated (Fig. 1C). Gradual increase of Cat2 peak is well evident from the obtained dependence and the increase of Cat2 peak for 90 % at the accumulation time of 240 s compared to non-accumulated one is obvious. Therefore, we used 240 s long accumulation at open circuit in the following part of this study. In addition, dilution of a sample had interesting effect on catalytic signals too. The experiment was carried out that we added heat treated sample of blood into the supporting electrolyte in the following dilutions 10, 100 and 1,000 times. We detected considerable differences in the range of tens of % with the increasing dilution of a sample (Fig. 1E). The most concentrated sample probably contains a large number of ballast compounds, which block electrochemical reaction on HMDE. The tested dilution (100 times) leads to decrease of concentration of these compounds, probably to change in MT structure and its better access to electrode. Therefore, we detected higher signal compared to lower dilutions. On the other hand, it is well evident that overdilution (more than 1,000 times) lowered concentration of analyte and lead to the reduction of Cat2 signal of MT (Fig. 1D). In conclusion, application of 10 μl to 1,990 μl of supporting electrolyte, i.e. 100 times dilution, gives the best results.

3.2. MT levels in healthy volunteers

Further, we were focused on determination of MT levels in blood of healthy volunteers (Fig. 2). All volunteers ranked in this study underwent health examination; subsequently, blood was sampled. Fifty eight blood samples were obtained from 2007 to 2009. Average age of analysed group was 22±5 years. Samples were 100 times diluted with the application volume of 10 μl and accumulation time of 240 s for their analysis. Typical DP voltammograms with well developed signals were obtained (Fig. 2B). MT concentrations derived based on Cat2 peak height varied from 0.17 to 0.90 μM with the average concentration of 0.51 μM (Fig. 2A). Distribution of MT values within various concentration intervals is shown in Fig. 2C. The highest occurrence numbers were detected in 0.20-0.49 μM interval. The increase of MT levels to 0.5-0.9 μM is closely connected with the using of food supplements containing heavy metal ions (zinc, chromium) and metalloids (selenium), increased physical activity and intensive metabolic activity of young organism. The obtained data enabled us to suggest reference MT level in blood of healthy human within the interval from 0.2 to 0.8 μM.

3.3. MT levels in patients suffering from malignant disease

During the years 2006-2009, 145 patients with newly diagnosed malignant tumour disease in the head and neck area were obtained. The most extend group was represented by patients suffering from oropharyngeal cancer (n = 69, 47.5 %), laryngeal cancer (n = 36, 24.8 %), hypopharyngeal cancer

(n = 14, 9.6 %), oral cavity cancer (n = 18, 12.4 %) and rarely occurring nasal cavity and paranasal sinus cancer (n = 4, 2.7 %) and parotid carcinoma (n = 4, 2.7%). Age median of the studied group was 60 years (Fig. 3).

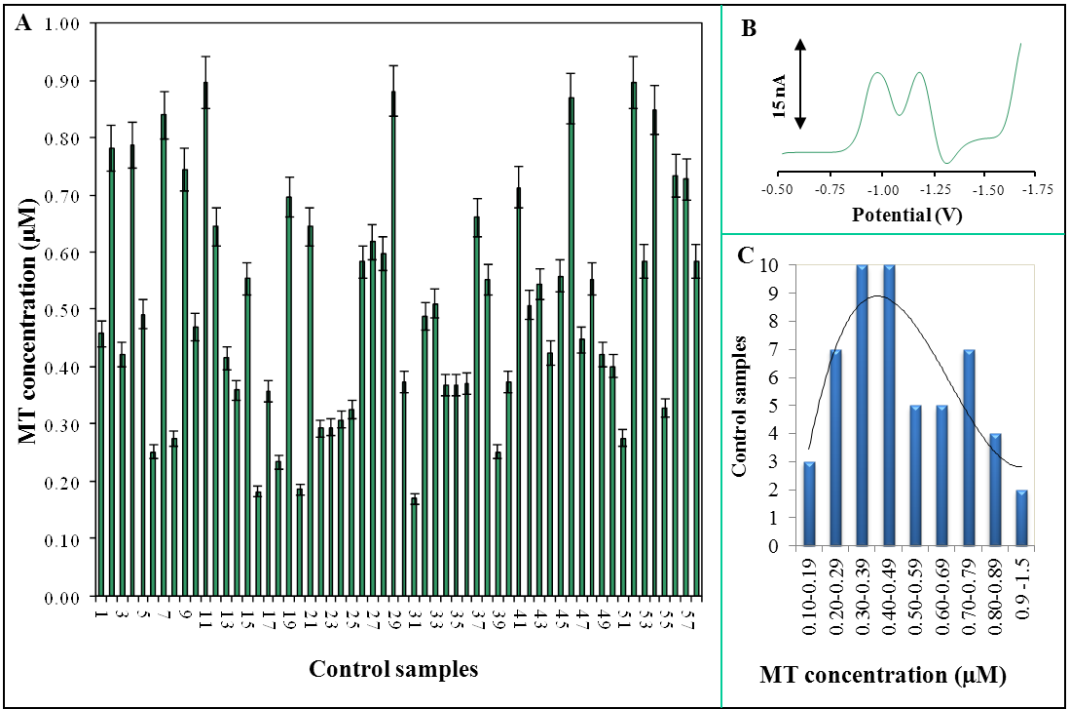

Figure 2. (**A**) MT concentration in blood of healthy volunteers (n = 58 (36 men, 22 women), age 22±5 years). (**B**) Typical DP voltammogram of blood control sample, Cat2 peak was detected at -1.45 V. (**C**) Histogram of MT distribution (per 0.2 μM intervals) in control blood samples. For other experimental details see Fig. 1.

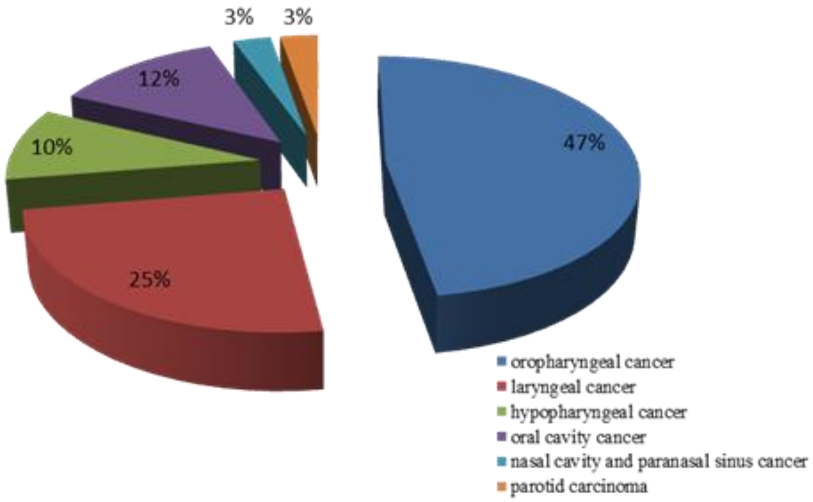

Figure 3. Distribution diagram of blood samples of newly diagnosed patients suffering from head and neck tumours obtained from 2006 to 2009 in South Moravian region. The patients are divided according to their diagnoses.

Typical DP voltammograms of various types of head and neck carcinomas unambiguously demonstrate differences between them and DP voltammogram of obtained from healthy volunteer (Figs. 2, 4, 5 and 6). Cat2 peak enhancement, which is characteristic for high level of free –SH groups and thereby increased amount of MT, is well evident. Determined MT levels in blood of patients varied from 1.08 to 6.39 μM, whereas average values differed in the accordance with tumour localization. MT levels were higher in all cases compared to control group (healthy volunteers). The minimal difference between MT level determined in healthy volunteer and tumour patient was 0.57 μM and the maximal 5.88 μM. Due to the fact that all samples originated from newly diagnosed patients without any treatment, MT levels were not affected by other conditions including chemotherapy.

3.3.1. Oropharynx

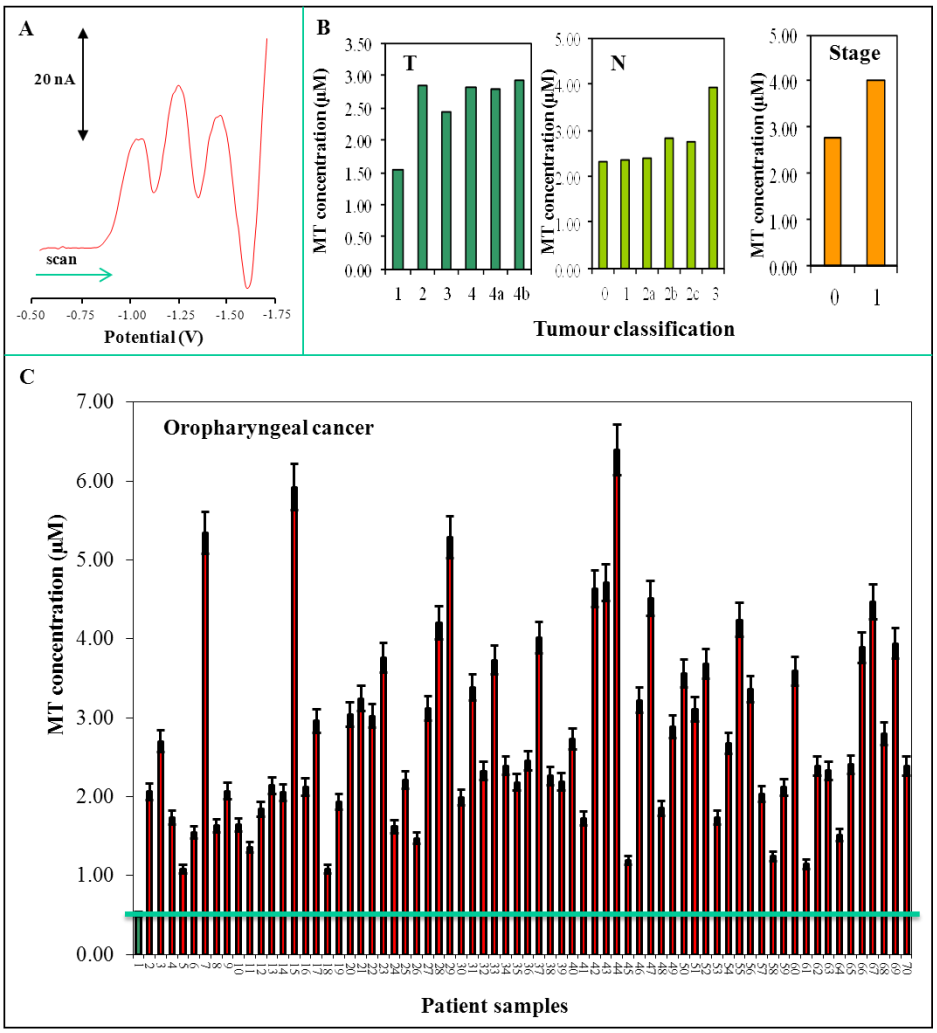

Figure 4. (**A**) Typical blood sample DP voltammogram of patient suffering from oropharyngeal cancer. (**B**) MT concentration determined in blood samples obtained from the same patients divided according to tumour classification as T, N and Stage. (**C**) Individual variability in blood MT content (n = 69, mean, average 68 years) in patients samples. For other details see Fig. 1.

Typical DP voltammogram of blood obtained from newly diagnosed patient with head and neck carcinoma in oropharynx is shown in Fig. 4A. Due to a large number of blood samples of patients with malignant tumour localization in oropharynx, the results obtained may be considered to be the most exact amongst all studied tumour localizations. In accordance with distribution of MT levels based on T criterion, the enhancement of MT levels in blood is well evident with the tumours stage, i.e. size of primary carcinoma (Fig. 4B). Based on the regional lymph nodes (N) affection, tendency of MT level had increasing character with higher stage of tumour classification. In addition, similar trend is observable in the case of distant metastasis (Fig. 4B). However, situation is not so obvious due to low number of patients with primary tumours with distant metastases (n = 5). Occurrence of patients with distant metastases was limited only to this localization. In the comparison of MT blood levels in the same localization related to the rate of differentiation of tumour cells, tendency is reverse, i.e. MT level decreased in blood with the decreasing level of tumour cell differentiation. In conclusion, it is obvious that patients suffering from malignant tumours of higher stage of classification have relatively higher MT blood levels compared to patients with low-stage malignant tumours (Fig. 4C).

3.3.2. Hypopharynx

Typical DP voltammogram of blood obtained from newly diagnosed patient with hypopharyngeal carcinoma is shown in Fig. 5Aa. Trend of blood MT level changes in patients suffering from hypopharynx malignant tumour disease was increasing up to IV. stage in connection with size of carcinoma. However, individual sub-stages demonstrated lower blood MT concentrations (Fig. 5Ab). Similarly to oropharynx, concentration dependence of blood MT on the progression of tumour disease into regional lymph nodes is noticeable. Due to limited number of variously differentiated tumour cell types, it is untimely to deduce any conclusions. However, the increasing tendency of blood MT level with the decrease of tumour differentiation is well evident. Blood MT level decreased in the case of patients with late tumour states (IVa, IVb). Distribution of samples is not statistically significant in accordance with this criterion. In addition, sudden MT increase in IVa stage may be closely connected with the non-uniformity of distribution of MT levels (Fig. 5Ac).

3.3.3. Oral cavity

Typical DP voltammogram of blood obtained from newly diagnosed patient with oral cavity carcinoma is shown in Fig. 5Ba. Results obtained by analysis of blood samples from patients suffering from head and neck carcinoma in oral cavity are not convincing compared to previous localizations (Fig. 5Ba). No tendency in blood MT levels in connection with tumour size was observed. We can assume the decreasing blood MT levels in connection with the increasing metastatic affection of regional lymph nodes (Fig. 5Bb). Nevertheless, lower blood MT levels were demonstrated also in patients without distant metastases. Rate of differentiation significantly affects blood MT level in patients with oral cavity cancer. Increase of MT levels is observable in connection with lower tumour

cell differentiation. In conclusion, tendency of blood MT level is rather decreasing depending on the stage of tumour disease (Fig. 5Bb,c).

3.3.4. Larynx, nasal cavity and paranasal sinuses, and parotid glands

Group of patients suffering from malignant tumour in the laryngeal localization is the second most occurred (Fig. 3).

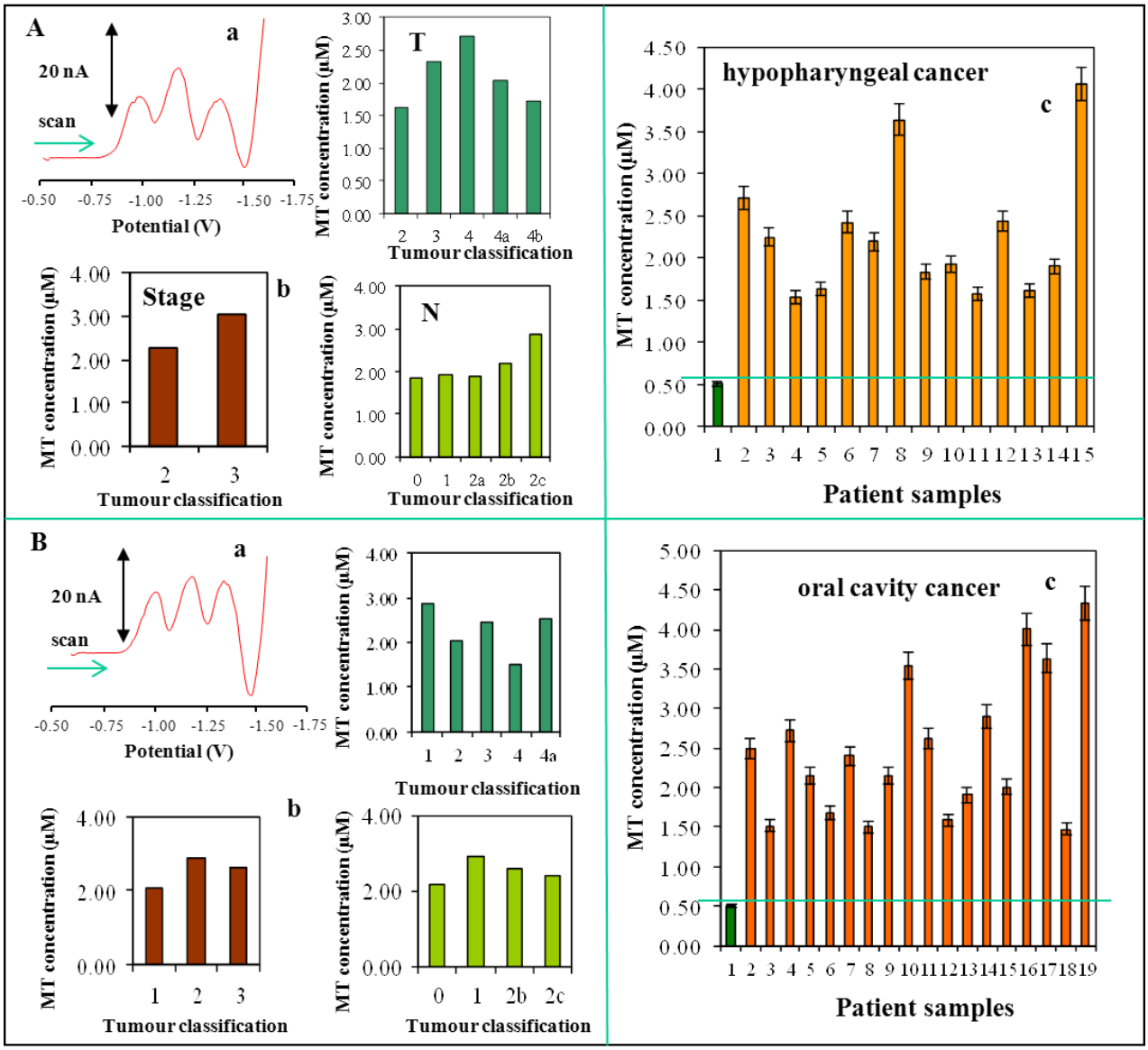

Figure 5. Typical DP voltammograms of blood samples of patients suffering from (**Aa**) hypopharyngeal cancer and (**Ba**) oral cavity cancer. MT concentration determined in blood samples obtained from the same patients divided according to tumour classification (T, N and Stage) in (**Ab**) hypopharyngeal cancer and (**Bb**) oral cavity cancer. (**Ac**) Individual variability in blood MT content (n = 14, 64 years at average) in patient samples suffering from hypopharyngeal cancer and (Bc) blood MT content (n = 18, 58 years at average) in patient samples suffering from oral cavity cancer. For other details see Fig. 1.

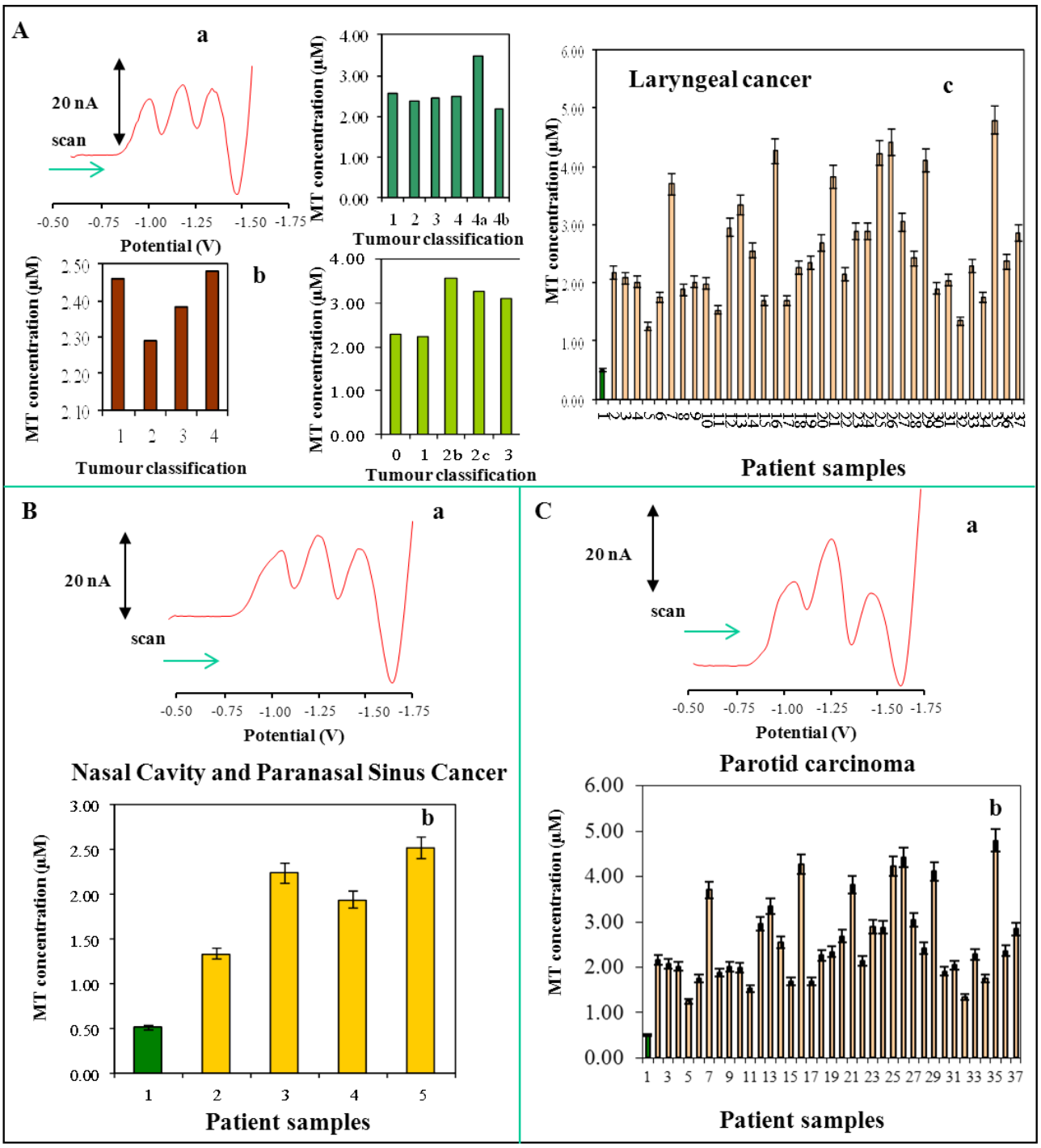

Figure 6. Typical DP voltammograms of blood samples of patients suffering from (**Aa**) laryngeal cancer, (**Ba**) nasal cavity and paranasal sinus cancer and (**Ca**) parotid carcinoma. (**Ab**) MT concentration determined in blood samples obtained from the same patients divided according to tumour classification (T, N and Stage) in laryngeal cancer patients. (**Ac**) Individual variability in blood MT levels in patients suffering from laryngeal cancer (n = 36, 66 years at average). (**Bb**) Blood MT content in patients suffering from nasal cavity cancer and paranasal sinuses cancer (n = 4, 63 years at average). (**Cb**) Blood MT content of patients suffering from parotid carcinoma (n = 4, 61 years at average). For other details see Fig. 1.

Typical DP voltammogram of blood obtained from newly diagnosed patient with this type of carcinoma is shown in Fig. 6Aa. There is no significant dependence between blood MT level and T classification up to IVa stage, where its increase is highly significant (Fig. 6Ab,c). This finding is in agreement with other experimental data, where the increased blood MT levels were observed. Blood MT levels have similar tendency in the case of N classification detected in patients suffering from oral cancer. The highest levels were determined between I and II stage of classification. Blood MT levels had increasing tendencies in the oral and hypopharynx tumours up to the most progressive stages. There is still unclear dependence between blood MT levels and rate of tumour cell differentiation, nevertheless, highly differentiated cells are connected with higher blood MT levels compared to low differentiated tumour cells. Increasing cell dedifferentiation is accompanied by increase of blood MT level. Effect of the stage of malignant disease on blood MT level is not obvious; however, increasing tendency is observable between stages II and IV with exception of IVb stage with disruption of this tendency (Fig. 6Ab,c).

Typical DP voltammograms of blood obtained from newly diagnosed patient with nasal cavity and paranasal sinuses, and parotid glands carcinoma are shown in Figs. 6Ba and 6Ca. Due to only limited number of patients suffering from rare malignant diseases in head and neck are as paranasal sinuses and parotid glands it was not possible to find any statistical significant dependences between blood MT levels and TNM classification. Figures Fig. 6Bb and Fig. 6Cc show detected blood MT levels in monitored patients. In spite of the fact that we were not able to do any statistics, the increased blood MT levels were observable in all patients.

3.3.5. Differences between localisations

Individual DP voltammograms for given localisations significantly differ. In addition, differences can be observed also for individual patients. Clearly observable increase of Cat2 catalytic signal corresponding to MT concentration in samples obtained from tumour patients is evident in comparison with control samples, i.e. blood of healthy volunteers. Other differences in the shape of voltammetric curves are caused by now unknown factors. These factors, which are based especially on biological composition of sample, may probably be used in diagnostics of individual types of malignant diseases. Moreover, average levels of MT in blood of patients suffering from malignant disease in head and neck are (regardless of closer localisation) could reveal relations between this low-molecular protein and initiation, development and progression of malignant tumours [6]. With respect the size of primary tumour, there is no significant dependence on blood MT concentration. Highest blood MT level corresponds to the IVb stage; however, this level is not significantly different from other classification stages. This fact may be connected with high cell death rate in the central part of malignant tumours, where cells undergo the processes of apoptosis. These apoptosis-undergoing cells and death cells do not contribute to MT production and its level is predominantly constant. The increasing tendency in blood MT level was observed in connection with invasion of tumour into regional lymph nodes. Analogously, increasing number of tumour cells and number of mitotic cells may affect expression of metallothionein. However, in the case of Ia stage the differences are

significant. This effect is probably connected with tumour development. Finally, we can conclude that blood MT level increases with the rate of dedifferentiation. In the case of the last stage (with the least differentiated stage), blood MT level significantly decreases. Nevertheless, we had only limited number of samples of patients classified in this stage, thus, further investigation is necessary.

4. CONCLUSIONS

Increased level was observed in proliferating cells [41]. This fact shows evidence of the role of MT in the process of initiation, progression and development of malignant tumour disease. Due to fact that MT plays crucial role in storage and transport of zinc(II) ions, which are essential in regulation of cellular processes including gene expression and cell proliferation, MT levels are crucial during different stages of cell cycle and its control. It is not surprising that the increased MT levels were observed in many types of tumour diseases. On the other hand, usage of this low molecular mass protein for diagnostics purposes is still controversial, especially due to very difficult interpretation of the obtained results. In conclusion, MT levels are closely associated with the rate of tumour differentiation, stage of tumour disease and tumour cell characteristics [86]. Our study demonstrates not only changes in blood MT levels in patients suffering from malignant tumour disease in head and neck area, but also suitability of electrochemical techniques for blood analysis.

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