LA-ICP-MS analysis reveals differences in chemotherapeutic drug distribution in surgically resected pleural mesothelioma

Anna Tisza¹, Thomas Klikovits², Szilvia Torok³, Beata Szeitz⁴, Zsuzsanna Valko², Zsolt Megyesfalvi¹, Mir Hoda², Balazs Hegedus⁵, Maximilian Bonta⁶, Winfried Nischkauer⁶, Konrad Hoetzenecker², Andreas Limbeck⁶, Karin Schelch², Viktoria Laszlo¹, and Balazs Dome²

¹National Koranyi Institute of Pulmonology
²Medical University of Vienna
³National Korányi Institute of Pulmonology
⁴Semmelweis University
⁵University of Duisburg-Essen Faculty of Medicine
⁶Technische Universität Wien
⁷Technische Universitat Wien

January 3, 2023

Abstract

Background and Purpose: Pleural mesothelioma (PM) is a highly aggressive thoracic tumor with poor prognosis. Although reduced tissue drug accumulation is one of the key features of platinum (Pt) resistance, little is known about Pt distribution in human PM. Experimental Approach: We assessed Pt levels of blood samples and surgically resected specimens from 25 PM patients who had received neoadjuvant Pt-based chemotherapy (CHT). Pt levels and tissue distributions were measured by laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) and correlated with clinicopathological features.

Key Results: In surgically resected PM specimens, mean Pt levels were significantly higher in the non-tumorous (fibrotic) areas (vs. tumorous regions, p<0.001). No major heterogeneity of Pt distribution was seen within the tumorous areas. Pt levels correlated neither with microvessel area (MVA) nor with apoptosis rate in the tumorous or in the non-tumorous regions. A significant positive correlation was found between serum and both full tissue section and tumorous area average Pt levels (r=0.415, p=0.039 and r=0.532, p=0.006, respectively). Furthermore, a significant negative correlation was detected between serum Pt concentrations and elapsed time from the last cycle of CHT (r=-0.474, p=0.017). Serum Pt levels correlated negatively with overall survival (OS) (p=0.029). Conclusion and Implications: There are major differences in the drug distribution between tumorous and non-tumorous areas of PM specimens. Serum Pt levels significantly correlate with full section- and tumorous areas average Pt levels, elapsed time from the last CHT cycle, and OS. Further studies investigating clinicopathological factors that modulate tissue Pt concentration and distribution are warranted.

Anna Tisza (ORCID ID: 0000-0001-5871-2930)
Viktoria Laszlo (ORCID ID: 0000-0002-6567-3965)
Balazs Dome (ORCID ID: 0000-0001-8799-8624)

LA-ICP-MS analysis reveals differences in chemotherapeutic drug distribution in surgically resected pleural mesothelioma

Running: Title: Platinum based chemotherapeutic drug distribution in PM
Anna Tisza1,2, Thomas Klikovits3, Szilvia Torok1, Beata Szeitz4, Zsuzsanna Valko3, Zsolt Megyesfalvi1,3,5, Mir Alireza Hoda1, Balazs Hegedus6,7, Maximilian Bonta8, Winfried Nischkauer8, Konrad Hoetzenecker3, Andreas Limbeck8, Karin Schelch3,9, Viktoria Laszlo1,3, Balazs Dome1,3,5,10

1Department of Tumor Biology, National Korányi Institute of Pulmonology, Budapest, Hungary.
2Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary.
3Department of Thoracic Surgery, Comprehensive Cancer Center, Medical University of Vienna, Austria.
4Division of Oncology, Department of Internal Medicine and Oncology, Semmelweis University, Budapest, Hungary.
5Department of Thoracic Surgery, National Institute of Oncology-Semmelweis University, Budapest, Hungary.
6Department of Thoracic Surgery, University Medicine Essen - Ruhelandklinik, University Duisburg-Essen, Essen, Germany.
7Department of Pathology, Forensic and Insurance Medicine, Semmelweis University, Budapest, Hungary.
8Institute of Chemical Technologies and Analytics, Division of Instrumental Analytical Chemistry, TU Wien, Vienna, Austria.
9Center for Cancer Research, Medical University of Vienna, Austria.
10Department of Translational Medicine, Lund University, Sweden.

BD and VL share the last authorship

Address all correspondence to:
Balazs Dome, MD, PhD
Department of Thoracic Surgery, Medical University of Vienna
Waehringer Guertel 18-20, A-1090 Vienna, Austria
Tel: +43-1-40400-73529, Email: balazs.dome@meduniwien.ac.at

Word count: 3782

Acknowledgements

The authors would like to thank the members of Department of Thoracic Surgery, Comprehensive Cancer Center of Medical University of Vienna for their assistance in conducting this study.

Conflicts of Interest statement:

The authors declare no conflict of interest.

Bullet point summary

What is already known?
Reduced tissue drug accumulation is one of the key features of CHT resistance.
Little is known about Pt distribution in human PM.

What does this study add?
Pt accumulates in the non-tumorous (fibrotic) compartments of PM tissue specimens.
Serum Pt concentrations positively correlate with full tissue section and tumorous area average Pt levels.

What is the clinical relevance?
These findings might deepen our understanding of intratumoral drug penetration and distribution. These findings might lead to the development of optimized treatment protocols in PM.

Abstract

**Background and Purpose**: Pleural mesothelioma (PM) is a highly aggressive thoracic tumor with poor prognosis. Although reduced tissue drug accumulation is one of the key features of platinum (Pt) resistance, little is known about Pt distribution in human PM.

**Experimental Approach**: We assessed Pt levels of blood samples and surgically resected specimens from 25 PM patients who had received neoadjuvant Pt-based chemotherapy (CHT). Pt levels and tissue distributions were measured by laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) and correlated with clinicopathological features.

**Key Results**: In surgically resected PM specimens, mean Pt levels were significantly higher in the non-tumorous (fibrotic) areas (vs. tumorous regions, p<0.001). No major heterogeneity of Pt distribution was seen within the tumorous areas. Pt levels correlated neither with microvessel area (MVA) nor with apoptosis rate in the tumorous or in the non-tumorous regions. A significant positive correlation was found between serum and both full tissue section and tumorous area average Pt levels (r=0.415, p=0.039 and r=0.532, p=0.006, respectively). Furthermore, a significant negative correlation was detected between serum Pt concentrations and elapsed time from the last cycle of CHT (r=-0.474, p=0.017). Serum Pt levels correlated negatively with overall survival (OS) (p=0.029).

**Conclusion and Implications**: There are major differences in the drug distribution between tumorous and non-tumorous areas of PM specimens. Serum Pt levels significantly correlate with full section- and tumorous areas average Pt levels, elapsed time from the last CHT cycle, and OS. Further studies investigating clinicopathological factors that modulate tissue Pt concentration and distribution are warranted.

**Keywords**: pleural mesothelioma, neoadjuvant chemotherapy, platinum, laser ablation-inductively coupled plasma-mass spectrometry, intratumoral drug imaging

**Abbreviations**: Ar, argon; Au, gold; C, carbon; CHT, chemotherapy; eGFR, estimated glomerular filtration rate; EPP, extrapleural pneumonectomy; He, helium; H&E, hematoxylin and eosin staining; LA-ICP-MS, laser ablation inductively coupled plasma mass spectrometry; PM, pleural mesothelioma; MVA, microvessel area; Ni, nickel; NSCLC, non-small cell lung cancer; NCCN, National Comprehensive Cancer Network; OS, overall survival; S, sulphur; P, phosphorus; Pt, platinum; RT, room temperature.
cellular responses, including replication and cell-cycle arrest, transcription inhibition, DNA damage response and apoptosis. Pt resistance can be the result of changes in any of these molecular machineries as well as of alterations in chemotherapeutic agent’s cellular uptake or export[11]. Furthermore, the phenomenon of limited intratumoral drug penetration also has to be taken into account; abnormal tumor vasculature might result in reduced blood supply, leading to inadequate chemotherapeutic drug delivery[12]. Therefore, attempts to measure intratumoral drug concentrations and efforts to correlate their levels with clinicopathological and vascular parameters are justified.

Studies in several tumor types emphasized the importance of intratumoral Pt levels. When treating different tumor cell lines with cisplatin, DNA platination significantly correlated with therapy response[13]. In non-small-cell lung cancer (NSCLC), patients with higher Pt concentration of the homogenized tumor tissue had longer progression-free survival and OS[14]. Moreover, in an NSCLC ex vivo explant model, high Pt uptake correlated with improved therapeutic response[15]. However, most of the studies investigated cells or tissue homogenates and, therefore, were unable to consider the importance of spatial drug distribution with regards to therapeutic efficacy.

Inductively coupled plasma mass spectrometry (ICP-MS) is an analytical technique used to identify elements[16]. Laser ablation devices, which are also commonly used in combination with ICP-MS instruments (LA-ICP-MS), can be used to investigate solid samples too[17].

Although reduced intracellular drug accumulation is consistently identified feature of Pt-resistant cells[13], to date, little is known about the tissue and serum Pt distribution in PM. Therefore, in our study, we measured the Pt level of blood samples and surgically resected tissue specimens from 25 PM patients who had received neoadjuvant Pt-based CHT. Moreover, we investigated if these Pt levels correlate with clinicopathological parameters of our PM patient cohort.

Methods

Study population and sample collection

Patients with histologically confirmed PM treated with neoadjuvant CHT at the Department of Thoracic Surgery, Medical University of Vienna, Austria between 2011 and 2017 were included in this study. Tissue samples (n=25) were collected during extrapleural pneumonectomy (EPP) and snap frozen in liquid nitrogen. Clinical data regarding patient age, gender, clinical stage, histological subtype, blood test parameters, treatment and survival were retrospectively collected from medical records and from the Austrian Public Health Insurance (ÖGK) Office.

All diagnostic and therapeutic approaches were conducted in accordance with the current National Comprehensive Cancer Network (NCCN) guidelines [18, 19]. Accordingly, based on predefined study aims, all patients received Pt-based CHT prior to surgery. After EPP, patients were treated with either CHT, palliative radiotherapy, combined chemoradiotherapy or best supportive care.

Blood samples (n=25) were collected by venous puncture from PM patients prior to surgery into serum separator tubes (BD, Cat No 367985). Centrifugation was performed after a 45-minute clotting time at 1200 x g for 10 minutes at room temperature, aliquoted and then snap frozen in liquid nitrogen. Both blood and tumor samples were stored at -80°C.

Estimated glomerular filtration rate (eGFR) was calculated based on serum creatinine levels according to Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) Creatinine Equation.

Tissue sample preparation and staining

Five consecutive 10 μm sections were cut from frozen tissues at -20 °C. The samples were analyzed consecutively with the following methods: #1 TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay (to examine apoptosis of PM cells); #2 anti-CD31 staining (to measure microvessel areas (MVAs); #3 Collagen I labeling (to localize collagen rich tissues); #4 LA-ICP-MS (to analyze Pt distribution and Pt levels); #5 Hematoxylin & Eosin staining (H&E).
Tissue slides were then digitalized by TissueFAXS (TissueGnostics GmbH, Vienna, Austria) with a 20x objective. The TissueFAX Viewer (TissueGnostics GmbH, Vienna, Austria) software was used to export the scanned images.

The histological composition of our sample cohort was heterogeneous. Some samples only contained tumorous areas (n=7), while others were mixed, containing both malignant and non-malignant tissue compartments (n=18). The proportion of tumorous and non-tumorous areas was measured on H&E-stained samples using the ImageJ software and confirmed by collagen staining. The Pt levels of the whole tissue section and the tumorous and non-tumorous regions were also analyzed.

MVA and TUNEL signals were evaluated by ImageJ software on the stained (CD31, TUNEL) tissue sections using entire sections and excluding unspecific signals. The observed parameters were expressed in percentage (area of positive pixels/total area x 100).

Further methods and protocols are described in the Supplementary Materials. The primary and secondary antibodies used for immunofluorescence labeling are listed in the Supplementary Materials’ Table 1.

Tissue and serum Pt measurements

A quadrupole ICP-MS instrumentation (Thermo iCAP Qc, ThermoFisher Scientific, Bremen, Germany) was used for the measurements of different elements. For solid sampling experiments, a commercially available laser ablation system with a frequency quintupled 213 nm Nd:YAG laser was employed (New Wave 213, ESI, Fremont, CA). To avoid higher risk of signal distortions, the used washout cell was held above the actual ablation site to guarantee fast washout times (below one second). As a carrier gas, helium was used to wash out the cell. Next, helium and the make-up gas, argon were mixed upon introduction into the plasma.\(^{13}\)C, \(^{31}\)P, \(^{34}\)S, \(^{194}\)Pt, \(^{195}\)Pt and \(^{197}\)Au were detected with ICP-MS. For data acquisition, the Qtegra software (ThermoFisher Scientific, Bremen, Germany) was used which was provided by the manufacturer. The calibration of the instrument and the optimization of the method were described previously [17].

After MS data export, serum sample analysis was performed using the Microsoft Excel software. Raw data of solid samples were transformed into 2D images and analyzed by the ImageLab 2.18 (Epina GmbH, Retz, Austria) software.

Further ICP-MS settings for serum and tissue analyses are described in the Supplementary Materials. The typical measurement settings of solid samples are summarized in Supplementary Materials’ Table 2.

Statistical analysis

To compare serum and tissue \(^{195}\)Pt signals with clinicopathological data, statistical analysis was performed with GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA), Microsoft Excel and R version 4.0.4 ( R Foundation for Statistical Computing, Vienna, Austria). Data distribution was verified by the Kolmogorov-Smirnov normality test. Tissue and serum Pt levels were analyzed with regards to clinicopathological variables by the Wilcoxon signed rank test, the Mann-Whitney U test, the Student’s t-test or the Kruskal–Wallis test (and Dunn’s test). The association between serum and tissue Pt levels and other continuous variables were evaluated by using the Spearman’s rank correlation. Statistical analysis of survival was done by the Log-rank (Mantel-Cox) test. Differences were considered statistically significant at a p value < 0.05.

Ethics statement

This study was conducted in accordance with the guidelines of the Helsinki Declaration (as revised in 2013) of the World Medical Association and the Good Scientific Practice guidelines of the Medical University of Vienna with the approval of the national level ethics committee (Medical University of Vienna; EK#: 904/2009). All patients provided written informed consent and were listed anonymously in securely handled databases.
Results

Clinicopathological characteristics and Pt levels

The full cohort comprised 21 (84%) epithelioid and 4 (16%) non-epithelioid (i.e., biphasic or sarcomatoid) PMs. Median age of all cases was 64.5 years (range, 33–78 years) and patients were predominantly male (76%). At the time of sampling, 16 (64%) and 9 (36%) patients had IMIG/TNM stage I-II and stage III-IV disease, respectively. Data are shown in Table S1.

Serum Pt concentrations ranged between 50.7 and 408.4 μg/L, with a mean value of 205.1 μg/L. The mean full tissue section Pt level was 1.04 μg/g (range: 0.2-3.28 μg/g). A statistically significant positive correlation was found between serum and full tissue section average Pt levels (Spearman r=0.532, p=0.006; Figure 1).

Pt accumulates in the fibrotic, non-tumorous regions of surgical PM tissue specimens

The histological composition of surgically resected PM specimens was rather heterogeneous. H&E and collagen staining revealed that, while some samples only contained tumorous areas (n=7), others were mixed in terms of histological composition, with variable amount of collagen rich fibrotic tissue mixed with viable tumor compartments (n=18).

When analyzing the Pt distribution in tumorous vs. non-tumorous regions, we found that Pt accumulated in the fibrotic (i.e. non-tumorous) areas. Accordingly, the mean Pt level was significantly higher in non-tumorous areas as compared to tumorous regions (1.23 μg/g vs. 0.83 μg/g, respectively; Mann-Whitney U test, p=0.0031; Figure 2).

Moreover, as shown in Figure S1, when analyzing the paired tumorous and non-tumorous compartments within the same tissue specimen, we also found a statistically significant difference in mean Pt levels (Wilcoxon matched-pairs signed-rank test, p=0.0003). Representative images demonstrating tissue heterogeneity by H&E staining, collagen, P and Pt distribution are shown in Figure 3.

Tumor tissue Pt levels do not correlate with angiogenesis and apoptosis in human PM

Preclinical studies suggest that apoptosis defects and increased tumor vascularization are characteristic features of PM progression and chemo-resistance [20, 21]. In our cohort, the mean MVA of tumorous and non-tumorous areas were 3.66% and 3.78%, respectively, and they did not differ statistically (Spearman r=0.083, p=0.751). As shown in Table S2, no significant correlation was found between tumor tissue Pt levels and MVA either in the tumorous (Spearman r=-0.122, p=0.56) or in the non-tumorous (Spearman r=-0.222, p=0.392) regions.

To determine the rate of apoptotic cells, we performed a TUNEL assay. However, we did not detect any correlations between Pt levels and apoptotic rate in tissue samples (tumor areas: Spearman r=-0.144, p=0.501; non-tumorous areas: Spearman r=-0.163, p=0.546).

Association between serum and tissue Pt levels and clinicopathological parameters

Serum Pt concentration correlated with Pt levels in tumorous areas to a greater degree than non-tumorous regions (Spearman r=0.415, p=0.039 vs r=0.406, p=0.095, respectively). Importantly, only the correlation between serum and tumorous area Pt levels remained statistically significant in these subgroup analyses (Figure 4).

No significant differences were found in Pt levels with regards to age, gender, histological subtype and tumor pathological stage either in tissue samples or in serum (Table S1).

Both serum and tumor tissue Pt levels might depend on the time passed between the last cycle of CHT and sampling (i.e., surgery). As expected, a statistically significant negative correlation was found between serum Pt levels and elapsed time from the last CHT (Spearman r=-0.474, p=0.017). In contrast, no such correlation was found between the elapsed time and the full tissue section mean Pt levels (Spearman r=-0.24,
p=0.249). Similarly, the elapsed time from the last CHT correlated neither with the tumorous (Spearman r=-0.261, p=0.208) nor with the non-tumorous tissue Pt levels (Spearman r=-0.12, p=0.636).

Next, to examine whether kidney function influences Pt levels, calculated eGFR (both pre-CHT and post-CHT/pre-surgery) values were compared to serum Pt concentrations, and full-, tumorous- and non-tumorous PM tissue Pt levels, but no correlations were found (data not shown). Interestingly, a mild negative trend was visible in case of pre-operative eGFR levels and non-tumorous Pt levels (Spearman r=-0.457, p=0.065).

With regards to the number of CHT cycles, the vast majority of patients (n=19) received three cycles of neoadjuvant CHT, thus the case numbers in the other groups were too low to adequately address the question whether the number of CHT cycles influence serum or tissue Pt levels.

As for the type of chemotherapeutic agents, most patients included in our study received cisplatin (n=16), whereas others were treated with carboplatin (n=7). We found that PM patients treated with carboplatin had comparable serum (Mann-Whitney, p=0.3) and tissue Pt levels (Mann-Whitney, p=0.867, 0.442, 0.571 for full-, tumorous- and non-tumorous tissue compartments, respectively) to those receiving cisplatin (of note, 2 patients received both; data is shown in Table S3).

Serum but not tumor tissue Pt levels correlate with survival of PM patients

Next, we examined if serum or tissue Pt levels have prognostic significance in PM. Using the median serum concentration (177.78 μg/L) as a cut-off, we found that patients with low serum Pt concentration had significantly longer OS than those with high serum Pt concentration (median OSs were 873 days vs. 476 days, respectively, p=0.029; Figure 5A). Nevertheless, as shown in Figures 5B-D, no significant differences were found in OS with regards to the tissue Pt levels of full-, tumorous- and non-tumorous- areas (Log-rank test, median cut-offs: 0.826 μg/g, 0.51 μg/g, 1.08 μg/g; p=0.492, p=0.773 and p=0.796 respectively).

Lastly, we compared the apoptotic rate and MVA of tumorous areas with OS, but no significant correlations were revealed (Spearman r=-0.354, p=0.09, and r=0.298 p=0.157, respectively; Figure S2).

Discussion

PM is a rare but devastating disease, which is largely caused by asbestos exposure [22]. Pt compounds have been used for cancer treatment since the 1970s and even nowadays, in the era of targeted therapies and immunotherapies, they form the backbone of systemic therapy in several solid tumors including PM [10]. Nevertheless, their contribution to OS improvement remains moderate [23]. Importantly, a pivotal cause of therapeutic resistance may be the insufficient drug penetration into the tumor tissue itself [12]. In the current study, we assessed the Pt levels of blood and surgically resected tissue samples of PM patients treated with neoadjuvant Pt-based CHT and correlated the measured Pt levels with clinicopathological parameters.

An adequate blood supply is regarded as essential for the growth of solid tumors [24]. To ensure the necessary amount of oxygen and nutrients, the tumor vascular network can be formed by various mechanisms [25]. Likewise, optimal drug delivery also relies strongly on tumor vascularization [12,26]. The beneficial effects of radio-, chemo- and immunotherapy depends on the intratumoral circulation as well [27,28]. Accordingly, we investigated the association between tissue MVA, and Pt penetration into the tumor, as well as patients' survival. Importantly, MVA influenced neither of these parameters in our study. It is important to mention, however, that increased intratumoral vascularity does not necessary improve oxygenation, drug delivery and therapeutic response, as tumoral blood vessels are structurally and functionally abnormal [29]. This may contribute to a hostile microenvironment and to the selection of more aggressive tumor cells [29]. Indeed, increased vascularization has been regarded as a negative prognostic factor in several tumor types [30] including PM [20,31]. This led Rakesh Jain to the elaboration of the "vessel normalization theory", whereby treatment with an anti-VEGF antibody (bevacizumab) normalizes the chaotic tumor blood vessel network, leading to increased drug delivery [32]. Based on this hypothesis, anti-angiogenic agents became widely studied and introduced to the clinical practice in PM and several other malignancies. However, clinical data has raised serious concern that bevacizumab can reduce the uptake of chemotherapeutic drugs by human tumors [33]. For the interpretation of our results, it is important to mention that only two patients received bevacizumab...
as part of their neoadjuvant treatment in our cohort, and no significant difference was detected between the MVA of tumorous and non-tumorous tissue areas. Of note, vascular tortuosity and permeability that may also influence drug delivery were not assessed in the current study.

Even if drug delivery to the tumor tissue itself is sufficient, several processes can lead to inadequate therapeutic response. We, therefore, investigated whether tissue Pt levels correlate with response to CHT. We found that tissue Pt levels (full, tumorous and non-tumorous areas) showed correlation neither with the apoptotic rate of tumor cells (as determined by TUNEL labeling) nor with the patients' OS. These findings are in contrast to the literature, as high intratumoral Pt level was identified as a favorable prognostic factor in different tumor types [14,15,34,35]. Moreover, in a recent study on cisplatin-treated NSCLC ex vivo explant cultures, Ki67 labeling (cell proliferation) and PARP expression (cell death) also correlated with intratumoral Pt levels [15]. Our results indicate that, beside inadequate penetration into the tumor tissue [13], other types of resistance mechanisms may also play a key role in the limited effectiveness of Pt agents in PM patients [11,36].

Although spatial drug distribution can affect therapeutic response, most studies investigated the level of chemotherapeutic agents only in cells or tissue homogenates [37]. As LA-ICP-MS is suitable for the visualization of Pt spatial distribution, we investigated whether there is a pattern in Pt tissue distribution that might affect therapeutic response in human PM. We found that Pt spatial distribution is rather heterogeneous in the tissue samples and non-malignant, collagen-rich fibrotic areas show higher Pt levels than the tumorous compartments. Similar findings were reported by Chang et al. in a patient-derived xenograft model of pancreatic cancer. In their study, Pt accumulated in the collagen fibers of both the tumor stroma and non-malignant tissues (skin, small intestine, and kidney) of cisplatin-treated tumor-bearing mice. In contrast, Pt levels remained relatively low in pancreatic cancer cells [38]. These authors suggested that the collagen-bound Pt may be slowly released, and as a result, the adjacent tumor cells are constantly exposed to metronomic CHT, leading to a therapeutic response. Similarly, Cao et al., demonstrated that Pt binds to the collagen compartment of gastric cancer tissue samples. In their cohort, both collagen content and binding of Pt to collagen fibers were significantly higher in those responding to therapy compared to the therapy-resistant cases [34]. Pt compounds interact with proteins containing free methionine- and cysteine side chains through the thiol groups of the amino acids[39]. Moreover, Pt also binds to the nitrogen of imidazole on the histidine residue of proteins[40]. Given that histidine and methionine also present in collagen fibers, it is likely that the Pt-collagen bond is realized through these amino acids[41]. On the other hand, it is well-established that cancer-associated fibroblasts can also decrease intratumoral Pt concentrations by altering the tumor microenvironment or releasing cysteine and glutathione, which bind Pt through their thiol groups [42,43]. Given that stromal collagen is produced by cancer-associated fibroblasts [44,45], co-localization of cysteine and glutathione with collagen could also contribute to the observed accumulation of Pt in the fibrotic collagen-rich tumor stroma. The possibility of this phenomenon needs to be further investigated.

Serum Pt concentrations can fundamentally influence tissue Pt levels and may explain the differences in patient survival. Therefore, we correlated serum Pt levels with that of the tissues and, moreover, with the patients' OS and found that low serum Pt concentration was associated with lower full and tumor tissue Pt levels and, interestingly, with better OS. It is well-known that Pt can accumulate in non-malignant tissues, resulting in long-term treatment-related adverse events associated with CHT [46,47]. Notably, biologically active cisplatin can be detected in the peripheral blood of patients even years after CHT administration [48,49]. As a potential source, collagen of healthy tissues was speculated[38]. Based on the above findings, a possible explanation of our results might be that in patients with low serum Pt levels the drug accumulated in the collagen rich non-malignant tissues. This might subsequently lead to a constant low dose metronomic Pt exposure even after the removal of the tumor tissue, thus hindering tumor recurrence. However, this hypothesis clearly requires further investigations.

Since a wide range of additional factors can influence the adsorption, distribution, metabolism and excretion (ADME) of therapeutic agents, we examined whether the clinicopathological characteristics of our patients
affect serum and tissue Pt levels. However, we found no correlation between tissue or serum Pt levels and age, gender, histological subtype, tumor pathological stage, renal clearance or the type of Pt agent. These findings are consistent with the literature, as none of the aforementioned parameters had an impact on tissue Pt levels of NSCLC or gastric cancer patients [14,35]. Moreover, although cisplatin and carboplatin levels have not been directly compared in PM tissues yet, as they show equivalent survival rates, it is presumable that they have similar biological activities in PM[50].

As expected, serum Pt concentrations negatively correlated with the elapsed time between the last CHT cycle and sample collection. Interestingly, however, no such correlation could be detected in case of tissue Pt levels, suggesting that tissue-bound Pt is slowly released. Indeed, Tothill et al. demonstrated that non-tumorous tissue Pt levels remain comparable 1-17 month after the last CHT dosage[47]. Given that surgery was performed within 11 weeks after the completion of neoadjuvant CHT, our timescale might have been too short to significantly impact tissue Pt levels.

The present study has certain limitations that need to be addressed in future settings. First, although we collected a unique cohort of matched blood and surgically resected samples, the number of included patients remained relatively small. Nonetheless, given that PM is a relatively rare disease and only a fraction of patients is eligible for multimodality treatment including neoadjuvant Pt-based CHT, this can be regarded as a rather large cohort. Second, the heterogeneity of the study population’s clinicopathological characteristics made the subgroup analyses difficult to accomplish. Lastly, patients were subjected to surgery within a relatively large timescale from the last cycle of CHT. This might have influenced Pt levels, but given the heterogeneity of our cohort, multivariate statistical analyses were not feasible.

To the best of our knowledge, this is the first study investigating serum and tissue Pt levels and tissue distributions of surgically treated PM patients by using LA-ICP-MS. Our results revealed major differences in Pt distribution between tumorous and non-tumorous fibrotic areas of surgically resected PM specimens, with non-tumorous fibrotic areas showing significantly higher mean Pt levels. We also demonstrated that serum Pt levels significantly correlate with full tissue specimen and tumorous area Pt levels, with elapsed time from the last CHT cycle and with OS. These findings might represent a step forward in our understanding of intratumoral drug penetration and distribution, and might therefore lead to the development of optimized treatment protocols in PM.

Funding
BD and ZM acknowledge funding from the Hungarian National Research, Development and Innovation Office (KH130356 and KKP126790 to BD; 2020-1.1.6-JÖVÖ and TKP2021-EGA-33 to BD and ZM). BD was also supported by the Austrian Science Fund (FWF I3522, FWF I3977 and I4677). ZM was supported by the UNKP-20-3 and UNKP-21-3 New National Excellence Program of the Ministry for Innovation and Technology of Hungary, and by the Hungarian Respiratory Society (MPA #2020). BS was a recipient of the Semmelweis 250+ Excellence PhD Scholarship (EFOP-3.6.3-VEKOP-16-2017-00009). BH was supported by the Austrian Science Fund (FWF I2872-B28). KS was supported by the Austrian Science Fund (FWF No. T 1062-B33) and the City of Vienna Fund for Innovative Interdisciplinary Cancer Research. VL is a recipient of the Bolyai Research Scholarship of the Hungarian Academy of Sciences and the UNKP-19-4 New National Excellence Program of the Ministry for Innovation and Technology. AT was supported by the Erasmus+ and Campus Mundi EFOP-3.4.2-VEKOP-15-2015-00001 Traineeship program (CM-SMP-KA103/155607/2016), and the Ernst Mach Grant, Austrian-Hungarian scholarship (ICM-2019-13765; OeAD-GmbH, ICM).

Author contributions
Anna Tisza: Investigation, Formal analysis, Data curation, Visualization, Writing - Original Draft, Writing - Review & Editing; Thomas Klikovits: Investigation, Resources, Formal analysis, Data curation; Szilvia Torok: Visualization, Writing - Original Draft, Writing - Review & Editing; Beata Szeitz: Formal analysis, Writing - Review & Editing; Zsuzsanna Valko: Investigation; Zsolt Megyesfalvi: Writing - Review & Editing; Mir Alireza Hoda: Methodology, Resources; Balazs Hegedus: Conceptualization,
Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Declaration of Transparency and Scientific Rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

Ethics approval statement

All the research protocols were submitted and approved by their specific Institutional Ethical Review Board.

References:


Figure Legends:

**Figure 1.** Scatter plot showing statistically significant positive correlation between serum and full tissue section mean Pt levels (Spearman $r=0.532$, $p=0.006$). Pt, platinum.

**Figure 2.** Pt levels of tumorous and non-tumorous tissue compartments. The mean Pt level was significantly higher in non-tumorous areas as compared to tumorous regions (1.23 µg/g vs. 0.83 µg/g, respectively; Mann-Whitney U test, $p=0.0031$).

**Figure 3.** Representative images of collagen, P and Pt distribution in tumorous and non-tumorous compartments of PM specimens. H&E ($A$) and collagen ($B$) stainings of tumorous area reveal low collagen content. Meanwhile, the same area exhibits high P ($C$) and low Pt ($D$) signals as measured by LA-ICP-MS. Comparative analysis of tumorous and non-tumorous (i.e. fibrotic) areas of another PM sample ($E$-$H$) show considerable differences in collagen distribution according to H&E ($E$) and collagen ($F$) staining. Tumorous compartments are delineated with solid black lines ($E$). LA-ICP-MS revealed that tumorous areas contain P ($G$) and Pt ($H$) to a greater and lesser extent, respectively (vs. the collagen-rich non-tumorous compartments). Similar tendencies are seen in a third PM specimen ($I$, $J$), as Pt accumulates in the non-tumorous, collagen-rich fibrotic area of the tissue sample.

Blue, nuclear staining with DAPI; green, collagen staining with anti-collagen type I $\alpha$-1. The color bar scale represents the P and Pt signal intensities of a given compartment as measured by LA-ICP-MS. Cold colors, low signal intensities; warm colors, high signal intensities. H&E, Hematoxylin & Eosin; LA-ICP-MS, laser ablation-inductively coupled plasma-mass spectrometry; PM, pleural mesothelioma, P, phosphorous, Pt, platinum.

**Figure 4.** Correlation patterns between the Pt level of serum and different tissue compartments. ($A$) Scatter plot showing a statistically significant positive correlation between serum and tumoral Pt levels (Spearman $r=0.415$, $p=0.039$). ($B$) No statistically significant correlation was found between serum Pt concentrations and the Pt levels of the non-tumorous area (Spearman $r=0.406$, $p=0.095$). Pt, platinum.

**Figure 5.** Kaplan-Meier estimates for OS according to the serum and tissue mean Pt levels. ($A$) Patients with high serum Pt levels ($>177.78$ µg/L) exhibited significantly worse median OS than those with low serum Pt levels ($<177.78$ µg/L) (log rank test, $p=0.029$). The Pt level of the full tissue section, the tumorous area and the non-tumorous compartment did not have a significant impact on OS (log rank test, $p$ values were 0.492, 0.773 and 0.796, respectively). OS, overall survival; Pt, platinum. *$p<0.05$.
Figure 1

Spearman $r = 0.532$
$p = 0.006$

Full tissue mean Pt level (μg/D)
Serum Pt level (μg/L)
Figure 2

$p=0.001$

Mean Pr level (μg/L)

Tumorous area  Non-tumorous area
Figure 4
Figure 5

A. Serum Pi concentration

B. Full-thickness mean Pi levels

C. Tumour area mean Pi levels

D. Non-tumour area mean Pi levels

Legend:
- Low (< 0.774 mg/dL)
- High (> 0.774 mg/dL)

Statistical significance:
- p = 0.039
- p = 0.240
- p = 0.773
- p = 0.796