Increased vesiculability of platelets in 24 patients with gastrointestinal cancer

Povečana vezikulabilnost trombocitov pri 24 bolnikih z rakom prebavil

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Izvleček

Izhodišča: Celični nanovezikli (NV) so majhni (velikostnega reda nanometrov), z membrano obdani celični fragmenti, ki se odcepijo od celice kot posledica prerazporeditve njenih sestavin. NV najdemo v izolatih iz različnih telesnih tekočin. V skladu s predlagano hipotezo je koncentracija NV v izolatih iz krvi pri bolnikih z rakom večja kot pri zdravih osebah, kar je posledica spremenjenih biofizikalnih lastnosti membrane trombocitov in krvne plazme pri bolnikih z rakom. Da bi preverili to hipotezo, smo določili koncentracijo NV v populacijah bolnikov z rakom prebavil, bolnikov z drugimi boleznimi prebavil in zdravih oseb. Da bi ugotovili, ali NV v izolatih izvirajo iz trombocitov, smo iskali korelacijo med koncentracijo trombocitov v krvi in koncentracijo NV v izolatih iz krvi ter korelacijo med vezikulabilnostjo trombocitov in koncentracijo NV v izolatih iz krvi.

Metode: Vzorce krvi je darovalo 24 bolnikov z rakom prebavil, 28 bolnikov z drugimi bolezni prebavil in 49 zdravih prostovoljcev. NVs smo izolirali z diferencialnim centrifugiranjem in izpiranjem vzorcev ter šteli s pretočno citometrijo. Izolacijo smo izvedli pri 37 °C. Trombocite smo šteli z impedančno metodo. Populacije smo primerjali z metodami opisne statistike.

Rezultati: Pri bolnikih z rakom prebavil je bila koncentracija NV v izolatih krvi znatno (44 %) in statistično pomembno (p = 0,0005) večja kot pri zdravih osebah ter znatno (34 %) in statistično pomembno (p = 0,025) večja kot pri bolnikih z drugimi boleznimi prebavil. Med koncentracijo NV v izolatih iz krvi in koncentracijo trombocitov v krvi ni bilo statistično pomembne korelacije (r = 0,1820, p = 0,0801). Ugotovili pa smo statistično pomembno pozitivno korelacijo med vezikulabilnostjo trombocitov in koncentracijo NV v izolatih iz krvi (r = 0,5690, p = 0,0000).

Zaključki: Pri bolnikih z rakom prebavil je povečana krhkost trombocitne membrane.

Abstract

Background: Cell nanovesicles (NVs) are small (mostly nano-sized) membrane-enclosed cell fragments that are pinched off from the cell due to rearrangement of its constituents. We have determined concentration of NVs in isolates from blood in populations of patients with gastrointestinal cancer, patients with other gastrointestinal diseases and in healthy subjects. To study whether NVs derive from platelets, we considered correlations between the concentration of platelets in blood and the concentration of NVs in isolates from blood, and between vesiculability of platelets and concentration of NVs in isolates from blood.

Methods: Blood samples were collected from 24 patients with gastrointestinal cancer, 28 patients with other gastrointestinal diseases and 49 healthy volunteers. NVs were isolated by centrifugation and washing of samples and counted by flow cytometry. Isolation was performed at 37 °C. Platelets were counted by impedance method. Populations were compared by methods of descriptive statistics.

Results: In patients with gastrointestinal cancer the concentration of NVs in isolates from blood was considerably (44 %) and statistically significantly (p = 0.0005) higher than in healthy subjects and considerably (34 %) and statsitically significantly (p = 0.025) higher than in patients with other gastrointestinal diseases. There was no statistically significant correlation between the concentration of NVs in isolates from blood and the concentration of platelets in blood (r = 0.1820, p = 0.0801). We found a statistically significant positive correlation between vesiculability of platelets and concentration of NVs in isolates from blood (r = 0.5690, p = 0,0000).

Ključne besede:
celični mikropartikli; rak prebavil; metastaze; tromboza; celična membrana

Key words:
cell-derived microparticles; gastrointestinal neoplasms; neoplasm metastasis; thrombosis; cell membrane

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Conclusions: Fragility of platelet membrane in blood is increased in patients with gastrointestinal cancer.

Introduction

Budding of membrane is a general biophysical phenomenon and is common in all cell types. In the process, membrane becomes strongly curved to form nanostructures[1] which may eventually pinch off from the cell membrane to become NVs. NVs can move within the surrounding solution[2-4] and thereby convey proteins, nucleic acids, infectious agents and even organelles to distant cells where these molecules become functional and therefore affect physiological and pathopysiological processes within the organism.[5-8] Platelet-derived NVs carry necessary enzymes to catalyze reactions for blood clot formation and exhibit negatively charged lipid in the outer membrane layer, so they may contribute to blood clot formation in blood vessels.[9] Also, it was indicated that NVs have an impact on tumor microenvironment[10] and on cancer development and progression–by facilitating tumor growth and invasion,[12,13] escape from cell death[13] and by inducing angiogenesis,[14] metastasis,[14] chemoresistance[15,16] and immune evasion.[17] Thus, NVs play an important role in widespread diseases with considerable socio-economic consequences.[18,19]

It is yet unclear how nanovesicles interact with other cells and in which way material that is enclosed in them travels within body. It was believed that a NV, after pinching off, circulates with blood and reaches a distant cell with which it interacts. In accordance with this hypothesis, it was suggested that NVs can be harvested from body fluids and that their analysis with respect to concentration and composition would reveal the presence of disease, in particular cancer. This would enable early diagnostics of cancer by using relatively small samples of peripheral blood and present a method complementary or alternative to biopsies, as a great benefit to patients. For this purpose, methods were developed to harvest NVs from blood, consisting essentially from centrifugation and washing of samples.[20] After dividing blood cells from plasma by centrifugation at a relatively low speed (around 1000 g) for minutes, plasma is centrifuged at a higher speed (between 10000 g and 200000 g) for minutes to hours. Visualization of isolates revealed small globular structures that could correspond to NVs.[21,22] NVs were found in isolates from different body fluids, e.g. blood,[4,5] urine,[23,24] ascites,[25,22] synovial fluid,[26,21] malignant pleural effusions,[27,22] human semen,[28] breast milk,[29] pregnancy associated sera,[30] amniotic fluid,[31] ocular fluids[32] and human saliva.[33] To support attempts for a thorough analysis of the composition of peripheral blood,[34] NVs isolated from blood are especially interesting as biomarkers for different diseases.

Isolates from peripheral blood were analyzed by different microscopic techniques[21] and by flow cytometry.[35] They exhibited a heterogeneous population of sub-micron sized particles.[35] Labeling by antibodies showed that a majority of NVs in blood isolates carry receptors that are typically found in platelets.[20] This indicates that subjects with a larger concentration of blood platelets should have a larger concentration of NVs in isolates from blood. However, considering a population of healthy subjects, it was reported that no correlation between the concentration of platelets in blood and the concentration of NVs in isolates from blood could be found.[4] Poor repeatability and accuracy of methods for harvesting NVs from blood could be the reason for the absence of correlation; therefore, refined studies should be performed to pursue this issue.

Elevated numbers of NVs were found in isolates from blood of patients with gastrointestinal cancer when compared to control populations.[35-37] In our previous study,[35] we compared concentration of NVs in isolates from blood in a test group of 5 pati-

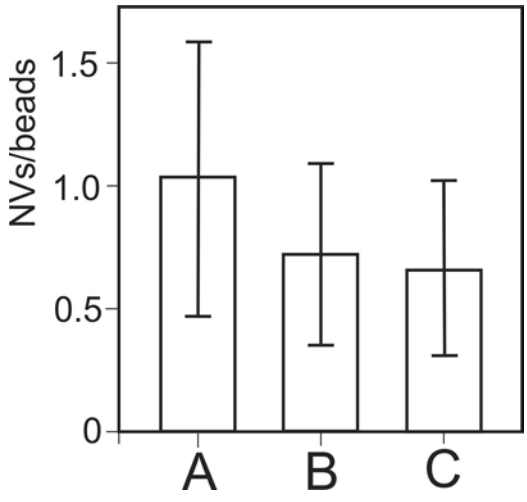

Figure 1: The average concentration of nanovesicles in isolates from blood of patients with gastrointestinal cancer (A), patients with noncancerous gastrointestinal diseases (B) and healthy volunteers (C). * stands for $p < 0.05$. Bars denote standard deviations.

ents with cancer and a control group of 14 patients with other gastrointestinal diseases. We observed that patients with cancer had a considerably elevated concentration of NVs in isolates from blood.[35] As the number of patients included in the study was rather small and as the control group consisted of patients with other gastrointestinal diseases (and not healthy subjects), we found these results to be preliminary.

Recently, on the basis of new insights into the mechanisms of vesiculation, it was suggested that a large pool of NVs found in isolates from blood can be created after blood sampling due to the exposure of blood cells to thermal and mechanical stress during harvesting.[4] Consequently, concentration, identity and size of NVs in isolates depend on external conditions such as temperature.[4] It was found that concentration of NVs in blood isolates is lower at higher temperatures during isolation, while measurements are less scattered at higher temperatures.[4] In search for parameters that are relevant for assessment of blood samples regarding NVs, vesiculability of platelets (f = concentration of NVs in blood isolates/concentration of platelets in blood) was suggested[38] and proved to correlate stronger with ability of plasma to suppress nanovesiculation.[35]

Using these recent findings, we designed a refined study in which we compared the concentration of NVs in isolates from blood of patients with gastrointestinal cancer, patients with other gastrointestinal diseases and from healthy subjects. We included a larger number of patients (24 patients with gastrointestinal cancer and 28 patients with other gastrointestinal diseases). The isolation was performed at body temperature (37 °C) to yield better accuracy of measured concentration of NVs than in the previous study.[34] Also, we studied the expected correlations between the concentration of platelets in blood and the concentration of NVs in isolates from blood and between vesiculability of platelets and concentration of NVs in isolates from blood.

Materials and methods

Subjects

Blood samples were collected from 24 patients with gastrointestinal cancer (pancreatic cancer, hepatocellular carcinoma and colorectal cancer) and 28 patients with other gastrointestinal diseases (liver cirrhosis, ulcerative changes of gastrointestinal organs, gallstones) who were treated at the Department of Gatroenetrology, Ljubljana University Medical Centre, from November 2009 to January 2010, and from 49 volunteers with no record of a disease, after 12 hours of fasting. The group of healthy volunteers consisted of students of the Faculty of Veterinary Medicine, University of Ljubljana, staff and authors. Students donated blood within sampling for regular check-up in the first year of study.

Written consent was given by patients and volunteers with no record of a disease. The study was conducted according to the principles of the Helsinki Declaratioon and approved by the National Ethics Committee, No 117/02/10. As in a previous study, no difference in post-fasting concentrations of NVs between female and male populations and no corelation between concentration of NVs and age of the donors[39] was found; populations were not stratified with respect to sex and age.

Blood sampling

Blood was collected into 2.7 ml evacuated tubes containing 270 µl trisodium citrate at a concentration of 0.109 mol/l. A 21-gauge needle (length 70 mm, inner radius 0.4 mm) (Microlance, Becton Dickinson, NJ, USA)

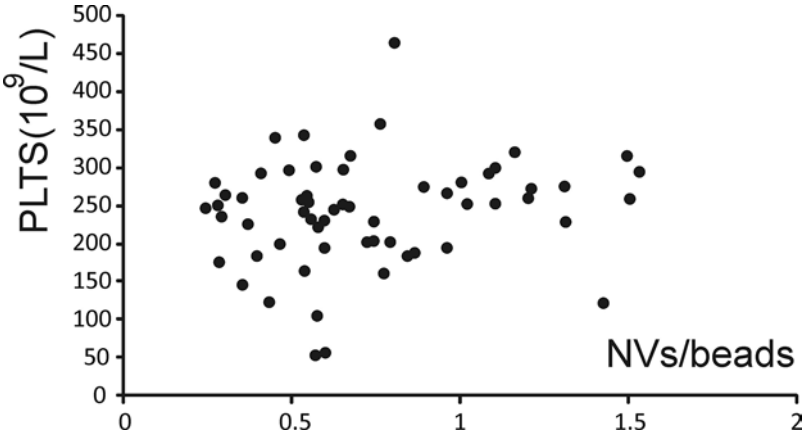

Figure 2: Concentration of platelets in blood as a function of concentration of nanovesicles in isolates from blood.

was used for blood sampling in all experiments. The tubes were previously incubated at 37 °C up to 30 minutes and kept in a water bath during the handling of the samples.

Isolation of nanovesicles

The isolation procedure was performed at a constant temperature of 37 °C. In order to separate cells from plasma, centrifugation at 1550 × g for 20 minutes was performed in a temperature regulated Centric 400/R centrifuge (Domel d.o.o., Železniki, Slovenia). The upper 250 µl of plasma was removed and placed in a 1.5 ml Eppendorf tube. The samples were then centrifuged at 17570 × g for 30 minutes in a Centric 200/R centrifuge (Domel d.o.o., Železniki, Slovenia). 225 µl of supernatant was discarded and the pellet (25 µl) was resuspended in citrated phosphate buffer saline. Samples were centrifuged again at 17570 × g for 30 minutes, superatant (225 µl) was discarded and pellet (25 µl) resuspended in 75 µl of phosphate buffer saline.

Determination of concentration of nanovesicles–flow cytometric analysis

After the isolation, NVs in isolates were counted and measured by flow cytometry, using an Altra Flow Cytometer (Beckman Coulter Inc., Fullerton, CA, USA) with a 448 nm water-cooled laser. For data aquisition and analysis of the results the Coulter software EXPO32 was used. The presence of NVs was determined by forward and side scatter (FS/SS) parameters. The flow-count beads (10 µm, Beckman Coulter) at a known concentration (1×10^6/ml) were used to determine concentration of NVs. At least 10000 events were recorded in each sample analysis. Results are presented relative to concentration of fluorospheres.

Determination of blood platelet concentration

Platelets in blood were counted with Pentra 120 analyzer (Horiba, ABX Diagnostics) by impendance measurement. Concentration of platelets was measured in 23 patients and in 38 healthy subjects.

Statistical analysis

Methods of descriptive statistics were used. To compare the groups, two tailed Student t-test with equal variance was calculated by Microsoft Excel software (Microsoft Office XP Professional 2003, Microsoft® Corporation, Washington, USA). Correlations were assessed by the Pearson coefficient and the corresponding statistical significance (one tailed p value), using SPSS software (version 20.0, IBM Corporation, Armonk, NY, USA).

Results and discussion

The concentration of NVs in isolates from blood was considerably (44 %) and statistically significantly (p = 0.0005) higher in patients with gastrointestinal cancer than in healthy subjects and considerably (34 %) and statistically significantly (p = 0.025) higher in patients with gastrointestinal cancer than in patients with noncancerous gastrointestinal diseases. The concentration of MVs was higher in patients with noncancerous gastrointestinal diseases than in healthy subjects (10 %), but the difference was not statistically significant (p = 0.385) (Figure 1).

There was no statistically significant correlation between the concentration of NVs in isolates from blood and the concentration of platelets in blood (r = 0.1820, p = 0.0801) (Figure 2), however, we found a statistically significant positive correlation between vesiculability of platelets *f* and concentration of NVs in isolates from blood (r = 0.5690, p = 0,0000) (Figure 3).

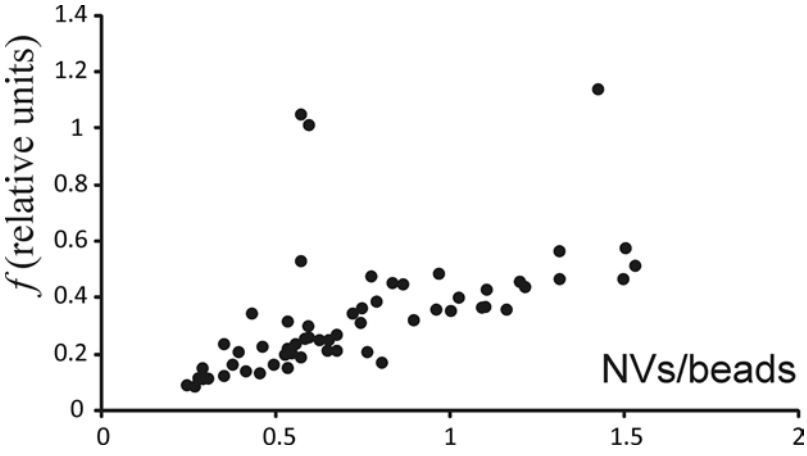

Figure 3: Vesiculability of platelets *f* as a function of concentration of nanovesicles in isolates from blood.

In agreement with our previous study,[35] we observed that the concentration of NVs in isolates from blood is considerably higher in patients with gastrointestinal cancer than in patients with other gastrointestinal diseases. In the present study we also included healthy subjects. We found that in the group of patients with other gastrointestinal diseases the concentration of NVs was neither considerably nor statistically significantly higher than in the group of healthy subjects indicating that the group of patients with noncancerous gastrointestinal diseases was, within the present accuracy of the method for harvesting NVs from blood, a convenient control group in the previous study. The present study contributes additional evidence to support the hypothesis that an increased concentration of NVs in isolates from blood is found in patients with gastrointestinal cancer.

In the previous study[35] the isolation was performed at room temperature. As it was recently found that the temperature during the isolation process importantly influenced the concentration of NVs in blood isolates due to an increased viscosity of plasma at lower temperatures and due to activation of platelets at room temperature,[4] we performed isolation at body temperature (37 °C) to minimize these effects. Higher temperature during the isolation yields lower concentration of NVs in isolates, so that in the present study the difference between the concentration of NVs in the group of patients with gastrointestinal cancer and the group of patients with other gastrointestinal diseases was smaller (but still considerable) than in the previous study (48 % vs. 140 %). However, the statistical significance was higher in the present study ($p = 0.025$ vs. $p = 0.033$).

Staining by antibodies indicates that the pool of NVs in isolates from peripheral blood is largely composed of the platelet-derived NVs.[20] This implies that the concentration of NVs in isolates from blood should be larger for higher concentrations of platelets in blood. However, to our best knowledge no positive correlation between concentration of NVs and concentration of platelets in blood has hitherto been reported. The absence of correlation could be a consequence of poor accuracy and repeatability of the method for harvesting NVs. We have made some improvements in the isolation procedure (better control of temperature, choice of higher temperature) and obtained a correlation coefficient of 0.1820 ($p = 0.08$) which is better than that obtained in previous studies[4] and close to the border of statistical significance $p = 0.05$ for a positive correlation. We think that further improvements of the method for isolation and further attempts will eventually reveal the expected correlation.

The concentration of NVs in isolates from blood was expected to strongly depend on the properties of platelet membranes (reflected in the vesiculability of platelet membranes *f*), which was confirmed by statistical significance of the correlation between the two parameters ($p < 10^{-7}$). This indicates that the concentration and composition of NVs in blood isolates reflect the clinical status. NVs are therefore a promising system for finding the answers regarding characteristics and prognosis of gastrointestinal diseases.[40,41]

Besides on membrane properties, nanovesiculation also depends on the composition of the solution that surrounds the membrane, both *in vivo* and *in vitro*. Especially relevant is mediated attractive interaction between membranous structures which can suppress vesiculation by causing adhesion of buds to the mother membrane.[4,42-46] Mediated interaction can be explained by orientational ordering of molecules (e.g. proteins, ions) in the solution between the interac-

ting membranes, which is a non-specific biophysical mechanism.

Poor repeatability and accuracy of methods for harvesting NVs is a bottleneck to any method based on the assessment of NVs from blood. As the results obtained cannot be given in absolute units, but must be callibrated, accuracy of the results obtained on larger populations is rather poor. Also, singular measurements may deviate considerably, which yet prevents the use of these methods in clinical practice. However, as better understanding of nanovesiculation may lead to manipulation of the disease at its origin, further studies on these issues are encouraged.

Conclusions

Concentration of NVs in isolates from blood reflects vesiculability of platelets and is increased in cancer patients. The accuracy and repeatability of the methods for isolation of NVs should be further improved to render them repeatable and quantitative, which is necessary if they are to reach a predictive clinical value.

Abbreviation

NVs nanovesicles

References

1. Kralj-Iglič V, Iglič A, Hagerstrand H, Bobrowska--Hagerstrand M, Hypothesis on nanostructures of cell and phospholipid membranes as cell infrastructure. Med Razgl 2005; 44(2): 155–69.
2. Kralj-Iglič V, Batista U, Hagerstrand H, Iglič A, Majhenc J, Sok M. On mechanisms of cell plasma membrane vesiculation. Radiol Oncol 1998; 32: 119–23.
3. Kralj-Iglič V, Iglič A, Hagerstrand H, Peterlin P. Stable tubular microexovesicles of the erythrocyte membrane induced by dimeric amphiphiles. Phys Rev E 2000; 61: 4230–4.
4. Šuštar V, Bedina-Zavec A, Štukelj R, Frank M, Bobojević G, Janša R, et al. Nanoparticles isolated from blood – a reflection of vesiculability of blood cells during the isolation process. Int J Nanomed; 2011; 6: 2737–48.
5. Ratajczak J, Wysoczynski M, Hayek F, Janowska--Wieczorek A, Ratajczak MZ. Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. Leukemia 2006; 20(9): 1487–95.
6. Camussi G, Deregibus MC, Bruno S, Cantaluppi V, Biancone L. Exosomes/microvesicles as a mechanism of cell-to-cell communication. Kidney Int 2010; 78(9): 838–48.
7. Pisetsky DS. Microparticles as biomarkers in autoimmunity: from dust bin to center stage. Arthritis Res Ther 2009; 11: 135
8. Schara K, Janša V, Šuštar V, Dolinar D, Pavlič JI, Lokar M, et al. Mechanisms for the formation of membranous nanostructures in cell-to-cell communication. Cell Mol Biol Lett 2009; 14: 636–56.
9. Müller I, Klocke A, Alex M, Kotzsch, M, Luther T, Morgenstern E, et al. Intravascular tissue factor initiates coagulation via circulating microvesicles and platelets. FASEB J 2000; 17: 476–8.
10. Lee TH, D'Asti E, Magnus N, Al-Nedawi K, Meehan B, Rak J. Microvesicles as mediators of intercellular communication in cancer–the emerging science of cellular 'debris'. Semin Immunopathol 2011; 33(5): 455–67.
11. Ginestra A, La Placa MD, Saladino F, Cassara D, Nagase H, Vittorelli ML. The amount and proteolytic content of vesicles shed by human cancer cell lines correlates with their in vitro invasiveness. Anticancer Res 1998; 18(5A): 3433–7.
12. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol 2008; 10(12): 1470-U209.
13. Abid Hussein MN, Boing AN, Sturk A, Hau CM, Nieuwland R. Inhibition of microparticle release triggers endothelial cell apoptosis and detachment. Thromb Haemost 2007; 98 (5): 1096–107.
14. Janowska-Wieczorek A, Wysoczynski M, Kijowski J, Marquez-Curtis L, Machalinski B, Ratajczak J, Ratajczak MZ. Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. Int J Cancer 2005; 113 (5): 752–60.
15. Safaei R, Larson BJ, Cheng TC, Gibson MA, Otani S, Naerdemann W, Howell SB. Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells. Mol Cancer Ther 2005; 4 (10): 1595–604.
16. Shedden K, Xie XT, Chandaroy P, Chang YT, Rosania GR. Expulsion of small molecules in vesicles shed by cancer cells: association with gene expression and chemosensitivity profiles. Cancer Res 2003; 63 (15): 4331–7.
17. Valenti R, Huber V, Filipazzi P, Pilla L, Sovena G, Villa A et al. Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. Cancer Res 2006; 66 (18): 9290–8.
18. Kralj-Iglič V. Stability of membranous nanostructures: a possible key mechanism in cancer progression. Int J Nanomed 2012; 7: 3579–96.
19. Atanasijevič-Kunc M, Drinovec J, Guštin B, Mrhar A. Simulation analysis of economic burden in hypertension and myocardial infarction treatment with beta blockers. Slov Med J 2012; 81 (2): 105–18.

20. Diamant M, Nieuwland R, Pablo RF, Sturk A, Smit JW, Radder JK. Elevated numbers of tissue-factor exposing microparticles correlate with components of the metabolic syndrome in uncomplicated type 2 diabetes mellitus. Circulation 2002; 106: 2442–7.
21. Junkar I, Šuštar V, Frank M, Janša V, Bedina-Zavec A, Rozman B, et al. Blood and synovial microparticles as revealed by atomic force and scanning electron microscope. Open Autoimmun J 2009; 1:e50-e58.
22. Mrvar-Brečko A, Šuštar V, Janša V, Štukelj R, Janša R, Mujagić E, et al. Isolated microvesicles from peripheral blood and body fluids as observed by scanning electron microscope. Blood Cell Mol Dis 2010; 44: 307–12.
23. Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci USA 2004; 101 (36): 13368–73.
24. Gonzales P, Pisitkun T, Knepper MA. Urinary exosomes: is there a future? Nephrol Dial Transplant 2008; 23 (6): 1799–801.
25. Graves LE, Ariztia EV, Navari JR, Matzel HJ, Stack MS, Fishman DA. Proinvasive properties of ovarian cancer ascites-derived membrane vesicles. Cancer Res 2004; 64 (19): 7045–9.
26. Skriner K, Adolph K, Jungblut PR, Burmester GR. Association of citrullinated proteins with synovial exosomes. Arthritis Rheum 2006; 54 (12): 3809–14.
27. Bard MP, Hegmans JP, Hemmes A, Luider TM, Willemsen R, Severijnen LA, et al. Proteomic analysis of exosomes isolated from human malignant pleural effusions. Am J Respir Cell Mol Biol 2004; 31(1): 114–21.
28. Sullivan R, Saez F, Girouard J, Frenette G. Role of exosomes in sperm maturation during the transit along the male reproductive tract. Blood Cells Mol Dis 2005; 35(1): 1–10.
29. Admyre C, Johansson SM, Qazi KR, Filen JJ, Lahesmaa R, Norman M, et al. Exosomes with immune modulatory features are present in human breast milk. J Immunol 2007; 179 (3): 1969–78.
30. Taylor DD, Akyol S, Gercel-Taylor C. Pregnancy--associated exosomes and their modulation of T cell signaling. J Immunol 2006; 176 (3): 1534–42.
31. Asea A, Jean-Pierre C, Kaur P, Rao P, Linhares IM, Skupski D, Witkin SS. Heat shock protein-containing exosomes in mid-trimester amniotic fluids. J Reprod Immunol 2008; 79 (1): 12–17.
32. Perkumas KM, Hoffman EA, McKay BS, Allingham RR, Stamer WD. Myocilin-associated exosomes in human ocular samples. Exp Eye Res 2007; 84 (1): 209–12.
33. Ogawa Y, Kanai-Azuma M, Akimoto Y, Kawakami H, Yanoshita R. Exosome-like vesicles with dipeptidyl peptidase IV in human saliva. Biol Pharm Bull 2008; 31(6): 1059–62.
34. Gruden K, Hren M, Herman A, Blejec A, Albrecht T, Selbig J, et al. A »crossomics« study analysing variability of different components in peripheral blood of health caucasoid individuals. PloS ONE 2012; 7(1): e28761.
35. Janša R, Šuštar V, Frank M, Sušanj P, Bešter J, Manček-Keber M, et al. Number of microvesicles in peripheral blood and ability of plasma to induce adhesion between phospholipid membranes in 19 patients with gastrointestinal diseases. Blood Cells Mol Dis 2008; 41(1): 124–32.
36. Kim HK, Song KS, Park YS, Kang YH, Lee YJ, Lee KR, et al. Elevated levels of circulating platelet microparticles, VEGF, IL-6 and RANTES in patients with gastric cancer: possible role of a metastasis predictor. Eur J Cancer 2003; 39(2): 184–91.
37. Baran J, Baj-Krzyworzeka M, Weglarczyk K, Szatanek R, Zembala M, Barbasz J, et al. Circulating tumour-derived microvesicles in plasma of gastric cancer patients. Cancer Immunol Immunother 2010; 59(6): 841–50.
38. Frank M, Manček-Keber M, Kržan M, Sodin-Šemrl S, Jerala R, Iglič A, et al. Prevention of microvesiculation by adhesion of buds to the mother cell membrane–A possible anticoagulant effect of healthy donor plasma. Autoimmun Rev 2008; 7(3): 240–5.
39. Šuštar V, Bedina-Zavec A, Štukelj R, Frank M, Ogorevc E, Janša R, et al. Post–prandial rise of microvesicles in peripheral blood of healthy human donors. Lipids Health Dis 2011; 10: 47.
40. Novak K, Štepec S, Hafner M, Ribnikar M, Markovič S. Characteristics of primary biliary cirrhosis in Slovenian patients. Analysis of 169 patients in the period from 1984 to 2010. Slov Med J 2012; 81(5): 372–82.
41. Ivanecz A, Jagrič T, Hazabent M, Horvat M, Potrč S. Characteristics and prognosis of young patients with gastric cancer in Slovenia. Slov Med J 2011; 80(1): 25–32.
42. Šuštar V, Janša R, Frank M, Hagerstrand H, Kržan M, Iglič A, et al. Suppression of membrane microvesiculation — A possible anticoagulant and anti--tumor progression effect of heparin. Blood Cells Mol Dis 2009; 42: 223–7.
43. Frank M, Sodin-Šemrl S, Rozman B, Potočnik M, Kralj-Iglič V. Effects of low-molecular-weight heparin on adhesion and vesiculation of phospholipid membranes–a possible mechanism for the treatment of hypercoagulability in antiphospholipid syndrome. Ann NY Acad Sci. Contemporary Chalanges in Autoimmunity 2009; 1173: 874–86.
44. Kralj-Iglič V, Šuštar V, Hagerstrand H, Frank M, Janša R. Suppression of membrane vesiculation: A possible anticoagulant, antimetastatic and anti-inflammatory effect of heparin. In: Piyathilake DE, Liang RH, eds. Heparin: Properties, Uses and Side Effects. Hauppauge, NY: Nova Science Publishers; 2011. p. 27–57.
45. Štukelj R, Šuštar V, Mrvar-Brečko A, Veranič P, Hägerstrand H, Kralj-Iglič V, et al. Suppression of membrane vesiculation as anticoagulant and anti--metastatic mechanism. Role of stability of narrow necks. Gen Physiol Biophys 2013; in press.
46. Urbanija J, Tomšič N, Lokar M, Ambrožič A, Čučnik S, Rozman B, et al. Coalescence of phospholipid membranes as a possible origin of anticoagulant effect of serum proteins. Chem Phys Lipids 2007; 150: 49–57.