DOI: 10.1111/eci.12733

ORIGINAL ARTICLE

Applicability of extracellular vesicles in clinical studies

Domen Vozel, Bojana Uršič, Judita Lea Krek, Roman Štukelj and Veronika Kralj-Iglič

Laboratory of Clinical Biophysics, Faculty of Health Sciences, University of Ljubljana, Ljubljana, Slovenia

ABSTRACT

Background Extracellular vesicles (EVs) are submicron cellular fragments that mediate intercellular communication. EVs have in the last decade attracted major interest as biomarkers or platforms for biomarkers of health and disease. To better understand the reasons why despite great expectations and considerable effort, EV-based methods have not yet been introduced into clinical practice, we present a systematic analysis of published results of clinical studies.

Materials and methods Clinical studies on populations of body fluid samples, published from 2010 to including 2015, applying centrifugation of fluid human samples with centrifuge accelerations up to about 25 000 *g* and flow cytometry for detection of EVs were analysed with respect to statistical significance (*p*), statistical power (*P*), clinical significance (CS), defined as the difference between the means divided by the sum of standard deviations, and size of the populations (N_{min}), defined as the number of samples in the smaller group.

Results Final analysis included 65 publications with 716 comparisons reporting 308 (43%) statistically significant differences ($P < 0{\cdot}05$), 242 (34%) had statistical power $P > 0{\cdot}8$ and 88 (12%) had clinical importance CS > 1·96. None of comparison with CS > 1·96 included populations in which the smaller group consisted of 50 or more samples.

Conclusions To fulfil claimed expectations for EV-based methods as promising diagnostic tools, more evidence on EV-based mechanisms of diseases should be gathered. Also, the methods of EV harvesting and assessment should be improved to yield better repeatability and thus allow clinical studies with larger number of samples.

Keywords Exosomes, intercellular communication, microparticles, microvesicles, nanovesicles, noninvasive diagnostics.

Eur J Clin Invest 2017; 47 (4): 305–313

Introduction

Due to their small (submicron) size and fragility, extracellular vesicles (EVs) have only recently attracted wide interest in medicine and biology. Their mediating role in intercellular communication was observed within *in vitro* studies, and fundamental processes of redistribution of membrane constituents and concomitant functionalization of particular molecules are being studied and manipulated. However, ultimate goal in medicine is to use EV-based methods in clinical practice. Many studies are motivated by a promising role of EVs as biomarkers or their platforms, for assessment of clinical status in health and disease [1,2]. However, the breakthrough to routine clinical applicability of EVs as biomarkers or their platforms has not yet taken place. The present review of published clinical studies is an attempt to contribute to better knowledge on the reasons for such outcome. Previous reviews of clinical studies [3,4] have exposed the findings of the authors of the published studies, for example mostly reports on statistically significant differences between populations of samples pertaining to patients and controls. To show whether the size of the populations was statistically appropriate, statistical power *P* could be calculated. A criterion $P > 0{\cdot}8$ is generally accepted, meaning that in 80% or more, the type 2 error (β) is avoided, or, rejecting the null hypothesis when there is a difference between groups, fails. However, analyses of statistical power are rare in the literature concerning EV-based clinical studies. Furthermore, statistically significant differences alone should not be the primary origin of clinical interpretation of the study results as they may not provide clinical insight into important variables, magnitude of possible differences and information on study design [5,6]. A study outcome can be statistically, but not clinically, significant and vice versa. From the beginning of evidence-based medicine, biomedical studies have induced progress of appropriate analytical approaches. In this view, a criterion (or criteria) can be introduced to indicate the extent to which the method succeeds in differentiating health from disease and not reflect findings by chance or imprecise

measurement [7]. Also, it is important how large (in absolute numbers) are the compared populations of samples. Larger number of samples is needed to achieve a true representation of clinical populations of interest; that is, it is more likely expected that the results of the study will be reproducible if it is repeated in a similar population of samples. Large populations will comply also to the requirements regarding sufficient statistical power. Large populations are therefore necessary to ensure that the results are valid.

In order to get insight into the state of the art regarding EVs as biomarkers or their platforms, the studies found in the literature that consider differences between populations of samples of isolated EVs (unmarked or marked by different markers) were analysed regarding statistical significance, statistical power, clinical significance and size of sample populations.

There are different protocols for harvesting EVs, depending on the technique of capture and manipulation of samples. Isolation of EVs from plasma by centrifugation and their counting by flow cytometry (which enables detection of EVs sized above some threshold, e.g. 100 nm) is often used in studies that consider cohorts of patients and controls. In this work, we have focused on such studies. We have excluded studies considering smaller EVs (exosomes) that require different equipment and protocols for harvesting and assessment (ultracentrifugation at high accelerations, analysis of molecular content).

Design

Search strategy, literature selection and study inclusion

We used the search key (http://www.ncbi.nlm.nih.gov/pmc) ('2010/01/01' [PmcLiveDate]: '2015/4/16' [PmcLiveDate]) AND ((((Circulating microvesicles [Abstract] OR Circulating microparticles [Abstract]) OR Microparticles[Abstract]) OR Microvesicles [Abstract]) AND flow cytometry[abstract]) (Fig. 1). Search on PubMed Central (PMC) from PMC live date 1 January 2010 to 16 April 2015 (performed on 30 July 2016) yielded 153 results (publications). Publications reporting on clinical studies involving *ex vivo* human samples subjected to centrifugation of fluid samples up to about 25 000 *g* prior to flow cytometry were included into final analysis.

A total of 113 of 153 publications did not meet inclusion criteria as follows: 31 reported on *in vitro* studies, 21 were performed on laboratory animals, two did not use centrifugation prior to flow cytometry, nine used too high centrifugation (exosome isolation protocol), five did not contain enough details regarding microparticles isolation protocol, two did not use body fluid samples, in 1, the units were unclear and 42 did not consider populations of body fluids (either considered pre-analytical issues of isolation protocols or presented case reports), or did not contain the necessary data required for our analysis as given below. The remaining 40 of 153 publications

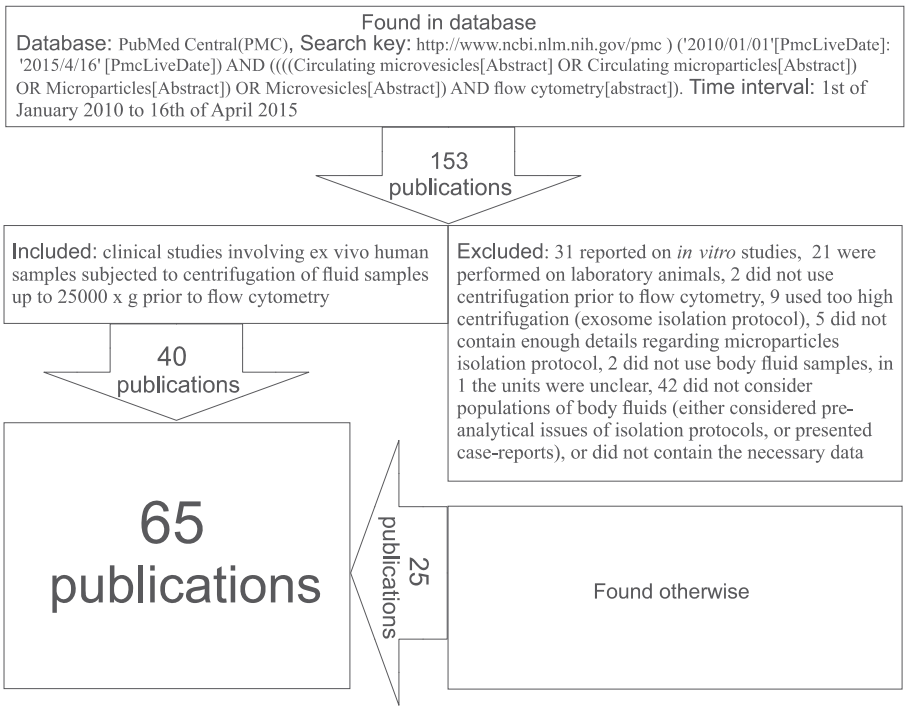

Figure 1 Flow diagram illustrating search and selection processes.

have met the inclusion criteria. Additionally, we included 25 publications that fulfilled inclusion criteria but were found otherwise. Final analysis was performed on 65 publications [8–72] (Fig. 1).

About 75% studies used protocols in which the sample was centrifuged at least twice before EVs were assessed by flow cytometry. Most of the studies used a two-step centrifugation. The first step ranged from 10 min at 160 *g* to 45 min at 20 500 *g*, and the second step ranged from 1 min at 6000 *g* to 45 min at 20 500 *g*. In the first centrifugation, larger cells are divided from the medium (e.g. plasma). The medium is differently named by authors as the 'platelet-poor plasma', 'platelet-free plasma' or 'cell-free plasma'.

We analysed comparisons with reported statistically significant differences between representative values of EV samples of both populations. The number of samples in the populations (the size of the populations), the median or mean values and the corresponding ranges of measurements or standard deviations were assessed. To unify the data, we approximated the mean values (μ) by the median values and standard deviations (σ) by a part of the range (ρ); $\sigma = \rho/4$ [73]. We estimated statistical power of the difference P, from the mean values (μ_1 and μ_2, where the indexes 1 and 2 denote the two respective populations being compared) and the larger of the two standard deviations (σ_{max}), by using software Power & Sample Size Calculator: http://powerandsamplesize.com for one-tailed test. Clinical significance (CS) was estimated by a modified Reliable Change Index [6],

$$CS = \frac{|\mu_1 - \mu_2|}{\sigma_1 + \sigma_2}, \quad (1)$$

where the values larger than 1·96 indicated clinical relevance. This criterion expresses that the populations (representing for example samples of healthy subjects and patients) should be sufficiently alienated to render a random sample of a healthy subject to likely fall within two standard deviations interval from the mean of the healthy population and a random sample of a patient to fall within two standard deviations interval from the mean of the diseased population. In other words, we considered the test clinically significant if we expect less than about 5% false-positive and/or false-negative results (grey area in Fig. 2). To assess the samples size, we used the number of samples in the smaller population (N_{min}).

To sum up, for each comparison with $p < 0{\cdot}05$, we assessed the available or estimated the effective mean values of the EV-based parameter (μ_1, μ_2), corresponding standard deviations (σ_1, σ_2) and the numbers of samples in the smaller and the larger population (N_{min} and N_{max}, respectively). By using these data, we calculated statistical power (P) and clinical significance (CS) of the differences. The assessed data and the results of the analysis are given in the Table S1, and in a companion document with explanation of respective contents.

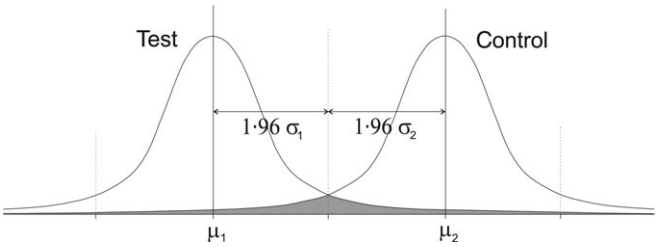

Figure 2 Presentation of the criterion of clinical significance. Here, μ_2 and μ_1 are the mean values of the two groups of samples subjected to comparison and σ_1 and σ_2 are the corresponding standard deviations. Integration of the curve from 1·96 σ to infinity yields 2·5% of the entire area under the curve. It is taken as a rough approximation that both distributions can be represented by normal distributions and that both standard deviations of these distributions are approximately equal.

Results and discussion

Table 1 shows that from 716 (100%) comparisons found in the literature, 157 (22%) did not report statistical significance of the difference. In 559 (78%) comparisons that reported statistical significance, it was found insufficient ($p > 0{\cdot}05$) in 251 (35%). The remaining 308 (43%) comparisons were further analysed with respect to statistical power and clinical significance; statistical power was insufficient ($P \leq 0{\cdot}8$) in 66 (9%), indicating that the populations of samples in these comparisons were not large enough. Clinical significance was insufficient ($CS \leq 1{\cdot}96$)

Table 1 Number of comparisons between sample populations sorted by the statistical significance of the difference between mean values (p), statistical power of the difference (P) and clinical significance (CS)

Number of comparisons			
716			
$p < 0{\cdot}05$		$p \geq 0{\cdot}05$	p not found
308		251	157
$P > 0{\cdot}8$	$P \leq 0{\cdot}8$		
242	66		
$CS > 1{\cdot}96$	$CS \leq 1{\cdot}96$		
88	154		

in 154 (22%). All per cents are given with respect to 716 comparisons.

To sum up, in 43% comparisons, the authors reported that they detected a statistically significant difference, while only few publications included power analysis. Our statistical criteria (statistical significance p and statistical power P) showed difference between health and disease in 34% of the differences, but considering clinical significance (CS), this portion was lowered to only 12%. In the remaining 88%, either there really were no indications for difference, or these indications existed, but could not be detected and the risk of false-positive or false-negative outcome was too high. There were very few studies that considered comparisons between (moderately) larger populations of samples (Fig. 3b–d); in only six comparisons (of 308 that claimed statistically significant difference), the smaller group contained more than 50 samples, and neither of these

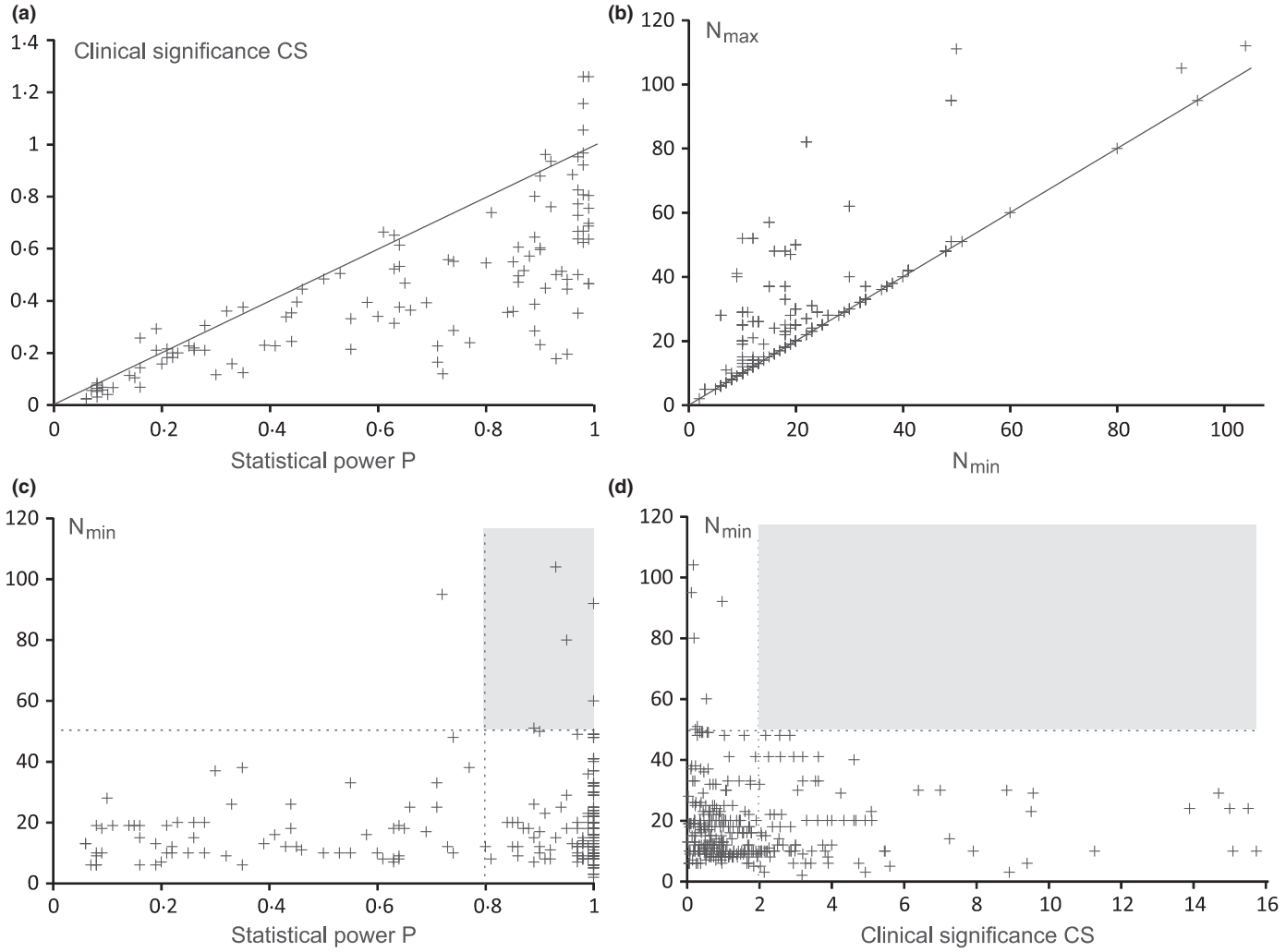

Figure 3 Interdependences between parameters of statistical and clinical relevance. (a) interdependence between clinical significance of the difference CS and statistical power of the difference P, (b) interdependence between the numbers of samples in the larger and the smaller populations compared, N_{max} and N_{min}, respectively, (c) interdependence between the number of samples in the smaller population N_{min} and statistical power of the difference P, (d) interdependence between the number of samples in the smaller population N_{min} and clinical significance of the difference CS. The lines in panels (a) and (b) indicate the ideal one-to-one relation. The dotted lines refer to threshold for criteria imposed upon statistical power ($P = 0{\cdot}8$) (a, c), clinical significance (CS = 1·96) (d) and the size of the populations ($N_{min} = 50$) (c, d). The data refer to 308 comparisons that reported statistically significant differences ($p < 0{\cdot}05$).

met our criterion for clinical significance. On the other hand, in majority of comparisons (260 of 308), the number of the samples in the smaller population was < 30 (Fig. 3b–d). Those studies that exceptionally included larger number of samples did not show clinically significant differences.

Further analysis showed that statistical power P and clinical significance CS were interdependent (Fig. 3a, Pearson coefficient 0·77). Only comparisons with P different from 1 were considered here because statistical power cannot be larger than 1, while clinical significance has no upper bound which technically limits the correlation if the data with $P = 1$ are included. It can be seen that none of the comparisons shown in Fig. 3a had sufficient clinical significance (CS = 1·96). However, all comparisons with sufficient clinical significance had also sufficient statistical power. We have used the milder, one-tailed test (assuming that the authors expected that the concentration of EV-based markers are going to be higher/lower in the test population) to calculate statistical power, which yielded the upper bound of statistical power P. On the other hand, following Jacobson and Truax [6], our criterion for clinical significance CS > 1·96 requiring on the average less than about 5% false results was more stringent. Considering the size of the compared populations, we show asymmetry in the numbers of the two populations compared (Fig. 3b) and add the size of the smaller population N_{min} to the analysis of statistical power P (Fig. 3c) and clinical significance CS (Fig. 3d). Figurs 3c and d present regions (shaded) that meet the chosen criteria. In panel C, the parameters are the statistical power P and the size of the smaller population N_{min}, while in panel D, the parameters are the clinical significance CS and the size of the smaller population N_{min}. It can be seen that in panel D, the quadrant defined by our criterion $P > 0{\cdot}8$ and an arbitrary choice $N_{min} > 50$ is empty; that is, we found no reported comparison to fulfil statistical significance and clinical significance of the difference for $N_{min} > 50$. In panel C, however, there are five comparisons that fulfilled statistical significance, statistical power and size requirement (but not clinical significance).

A small sample size limits statistical power; while larger sample sizes provide more power to detect statistically significant differences. However, it was previously found that while larger sample sizes are obviously preferred in a clinical study to capture a true representation of the clinical population being studied, larger samples sizes can lead to statistically significant differences that remain clinically insignificant [74], which is in agreement with our results (Fig. 3d).

Clinical significance, also named 'effect size' is not defined 'generally' and should take into account a particular perspective [75]. Jacobson and Truax [6] used an effect size criterion for the effect of psychotherapy (reliable change index $RCI = |\mu_1 - \mu_2|/(2\sigma_E^2)^{1/2}$), where σ_E is the standard error of the measurement. Values of RCI larger than 1·96 were considered as sufficient [6]. Applying RCI to our data, where the larger of the two standard deviations was taken to approximate σ_E, yielded 87 comparisons that fulfil the criterion (comparing to 88 obtained with CS (Table 1)), whereas likewise, none of these fulfilled the criterion $N_{min} > 50$. Also frequently used effect size is Cohen's d ($d = |\mu_1 - \mu_2|/\sigma$), where μ_1 and μ_2 are the mean values and σ is the assumed common standard deviation [74]. Values of d larger than 0·8 are considered as strong effect (but allowing more than 30% of false-positive and/or false-negative findings). Applying d to our data where the larger of the two standard deviations was taken to approximate σ, yielded 214 (30%) fulfilling comparisons, however, in only one of them $N_{min} > 50$. As clinical significance (i.e., effect size) is poorly defined, there is evidently freedom to adjust its stringency. However, as regards the size of the populations, large populations should be considered in order to assure repeatability of the results and relevance of the applied methods in clinical practice, in particular to avoid false-positive claims [76].

As claims for statistical and clinical significance and need for large populations have been considered by many authors and over a long period of time, and the problems have been largely acknowledged, a question can be raised why most EV-based clinical studies do not report statistical power and why clinical significance is not assessed. Obviously, assessment of statistical power and clinical significance of the results renders positive results more moderate, which could contribute to a higher probability that the work would be rejected by the reviewers and editors of scientific journals. As clinical studies require considerable effort and in spite of negative results bring important and sometimes even pioneering information in the field, it may seem inevitable to the authors not to show all the results, else the manuscripts are likely rejected and valuable information is thus kept from the interested society [77]. In some diseases, collecting material that finally presents about 30 patients takes many months during which external parameters that are important for EV harvesting may change. Yet, the experience of the researchers may be of important help to others. It should be noted that at the present stage of knowledge on EV-based clinical methods, it is of great interest to learn of the experiences of the researchers on EV-based mechanisms of various diseases, even though they find no statistically or clinically significant differences. This should be taken into account by the editorial staffs in processing of the manuscripts and as to optimally advice the authors how to reasonably present their findings.

Even more relevant is the question why most studies consider small populations of samples and why to our best knowledge there are no studies including large populations (thousands of samples). In our opinion, repeatability of EV harvesting connected to knowledge on mechanisms of EV formation, especially during the process of harvesting, is key to

clinical relevance of EV-based methods [78]. It is not yet established how a subject should be prepared for blood sampling as regards previous intake of substances and physical activity. Also, the effects of blood sampling (such as passing of blood through the needle) are not yet elaborated. The parameters regarding the centrifugation of samples, the effects of the material that comes in contact with samples, the parameters of keeping the samples during the processing, the yield of dying the samples with markers, etc., are not yet explored to sufficient level. All this prevents effective standardization of procedures because it is poorly known which parameters should be standardized and how a particular standardization may considerably affect the yield and identity of isolated EVs. To avoid dissipation of results due to different harvesting and assessment protocols, in this study we focused on experiments in which body fluid and body fluid-derived samples were centrifuged up to about 25 000 *g* and assessed by flow cytometry, as these procedures were hitherto most frequently used in assessment of EV samples in clinical studies.

Some of the procedures (such as blood sampling and interaction of samples with different interfaces) are practiced in harvesting larger EVs and in harvesting exosomes, as well as in other methods of harvesting and assessment (such as recently introduced nanosight track assay or nanoscale flow cytometry), and can be a source of similar problems thereof. As different protocols for harvesting EVs led to different results, it was previously suggested that protocols need standardization. The International Society on Thrombosis and Haemostasis (ISTH) Vascular Biology Standardisation Subcommittee (VB SSC) took steps towards standardization of platelet-derived MP (PMP) measurement by flow cytometry [79]; it was indicated that the fluorescent calibrated beads could be used to standardize the protocol for measurement of EVs [79,80]. Elaboration of the protocol for harvesting and assessment of EVs showed that the delay before the first centrifugation, agitation of the tubes during transportation of blood and the centrifugation protocol have important effects on the isolate [81]. An optimal protocol was suggested [81]. Then, several laboratories prepared EV isolates from platelet-free plasma of healthy donors according to the same protocol and according to their own protocol. It was found that the interlaboratory variability of flow cytometric measurement was smaller if the same protocol was applied [82]; however, the fluctuations found in spite of following the same protocol indicated that further studies are needed to identify more parameters that are important in determining the isolates [82]. Poor repeatability of the harvesting and assessment of EVs prevents comparison of samples processed in different lots. Furthermore, the methods for keeping and transporting EV samples have to our best knowledge not yet enabled multicentric clinical studies reporting significant differences between health and disease. Better understanding of the mechanisms of EV formation, in particular during the process of isolation, is necessary to achieve clinical applicability of the EV-based methods.

It was indicated that the research findings are less likely to be true for smaller populations studied, smaller effect sizes and poorly defined relationships to be tested, furthermore, this situation is aggravated in 'hot' scientific fields that promise greater financial and other interests [77]. Introduction of EV-based methods in clinical practice is indeed 'hot' and interesting from medical and financial point of view and our analysis of the results found in the literature showed that the studies that we found consider small populations with small portion of large effect size. However, vast laboratory research motivated by potential clinical applicability of EV-based methods [83] presents a sound foundation to continue studies directed towards clinical applicability of EVs. To achieve that EV-based methods could be widely used in clinical practice [84], further research on the basic mechanisms should be inevitably invested also in clinical study design and especially in implementation of nanoscale research in advanced technologies of EV harvesting and assessment. Standardization of protocols should be consistent with increased understanding of relevant mechanisms, else key parameters could be missed. Also, methods for keeping EVs should be elaborated to allow clinical studies with larger numbers of included samples in order to first see whether there is an effect at the population level. EVs are very small particles of transient identity, and they are essentially remodelled by manipulation and observation, meaning that all these processes should be considered also in designing the protocols and interpretation of results.

Acknowledgements

Availability of data and materials: The data sets acquired, generated and/or analysed during the current study are available in supplementary files (Table S1 in Excel and Companion to Table S2).

Competing interests

The authors declare that they have no competing interests.

Funding

Authors acknowledge grants from Slovenian Research Agency P3-0388 and J5-7098 and support from European Commission COST initiative MEHAD.

Authors' contributions

DV, BU, JLK and RŠ collected the papers, assessed the data, contributed to interpretation of the results, to writing the manuscript and critically read the manuscript. VKI designed

the study, interpreted the data, contributed to writing the manuscript and critically read the manuscript. Statistical analysis was performed by VKI, DV and BU. DV and BU contributed equally to this work.

Address
Laboratory of Clinical Biophysics, Faculty of Health Sciences, University of Ljubljana, Zdravstvena 5, SI-1000 Ljubljana, Slovenia (D. Vozel, B. Uršič, J. L. Krek, R. Štukelj, V. Kralj-Iglič).

Correspondence to: Prof Veronika Kralj-Iglič, Laboratory of Clinical Biophysics, Faculty of Health Sciences, University of Ljubljana, Zdravstvena 5, SI-1000 Ljubljana, Slovenia. Tel.: +38641720766; fax: +38614768850; e-mail: veronika.kralj-iglic@fe.uni-lj.si

Received 5 October 2016; accepted 29 January 2017

References

1 Witwer KW, Buzás EI, Bemis LT, Bora A, Lässer C, Lötvall J *et al.* Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles* 2013;**2**:20360.
2 Verma M, Lam TK, Hebert E, Divi RL. Extracellular vesicles: potential applications in cancer diagnosis, prognosis, and epidemiology. *BMC Clin Pathol* 2015;**15**:6.
3 Boulanger CM, Amabile N, Tedgui A. Circulating microparticles A potential prognostic marker for atherosclerotic vascular disease. *Hypertension* 2006;**48**:180–6.
4 György B, Szabó TG, Pásztói M, Pál Z, Misják P, Aradi B *et al.* Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol Life Sci* 2011;**68**:2667–88.
5 Sainani KL. Putting P values in perspective. *PM R* 2009;**1**:873–7.
6 Jacobson NS, Truax P. Clinical significance: a statistical approach to defining meaningful change in psychotherapy research. *J Consutl Clin Psychol* 1991;**59**:12–9.
7 Govani SM, Higgins PDR. How to read a clinical trial paper: a lesson in basic trial statistics. *Gastroenterol Hepatol (N Y)* 2012;**8**:241–8.
8 Al Kaabi A, Traupe T, Stutz M, Buchs N, Heller M. Cause or effect of arteriogenesis: compositional alterations of microparticles from CAD patients undergoing external counterpulsation therapy. *PLoS ONE* 2012;**7**:e46822.
9 Alkhatatbeh MJ, Enjeti AK, Acharya S, Thorne RF, Lincz LF. The origin of circulating CD36 in type 2 diabetes. *Nutr Diabetes* 2013;**3**:e59.
10 Amabile N, Guérin AP, Leroyer A, Mallat Z, Nguyen C, Boddaert J *et al.* Circulating endothelial microparticles are associated with vascular dysfunction in patients with end-stage renal failure. *J Am Soc Nephrol* 2005;**16**:3381–8.
11 Ayers L, Ferry B, Craig S, Nicoll D, Stradling JR, Kohler M. Circulating cell-derived microparticles in patients with minimally symptomatic obstructive sleep apnoea. *Eur Respir J* 2009;**33**:574–80.
12 Biró E, Nieuwland R, Tak PP, Pronk LM, Schaap MCL, Sturk A *et al.* Activated complement components and complement activator molecules on the surface of cell-derived microparticles in patients with rheumatoid arthritis and healthy individuals. *Ann Rheum Dis* 2007;**66**:1085–92.
13 Biró E, Sturk-Maquelin KN, Vogel GMT, Meuleman DG, Smit MJ, Hack CE *et al.* Human cell-derived microparticles promote thrombus formation in vivo in a tissue factor-dependent manner. *J Thromb Haemost* 2003;**1**:2561–8.
14 Brogan PA, Shah V, Brachet C, Harnden A, Mant D, Klein N *et al.* Endothelial and platelet microparticles in vasculitis of the young. *Arthritis Rheum* 2004;**50**:927–36.
15 Camargo LM, França CN, Izar MC, Bianco HT, Lins LS, Barbosa SP *et al.* Effects of simvastatin/ezetimibe on microparticles, endothelial progenitor cells and platelet aggregation in subjects with coronary heart disease under antiplatelet therapy. *Braz J Med Biol Res* 2014;**47**:432–7.
16 Campos FMF, Franklin BS, Teixeira-Carvalho A, Filho ALS, de Paula SCO, Fontes CJ *et al.* Augmented plasma microparticles during acute Plasmodium vivax infection. *Malar J* 2010;**9**:327.
17 Carp H, Dardik R, Lubetsky A, Salomon O, Eskaraev R, Rosenthal E *et al.* Prevalence of circulating procoagulant microparticles in women with recurrent miscarriage: a case-controlled study. *Hum Reprod* 2004;**19**:191–5.
18 Chaari M, Ayadi I, Rousseau A, Lefkou E, Van Dreden P, Sidibe F *et al.* Impact of breast cancer stage, time from diagnosis and chemotherapy on plasma and cellular biomarkers of hypercoagulability. *BMC Cancer* 2014;**14**:991.
19 Chahed S, Leroyer AS, Benzerroug M, Gaucher D, Georgescu A, Picaud S *et al.* Increased vitreous shedding of microparticles in proliferative diabetic retinopathy stimulates endothelial proliferation. *Diabetes* 2010;**59**:694–701.
20 Chalasani P, Marron M, Roe D, Clarke K, Iannone M, Livingston RB *et al.* A phase I clinical trial of bavituximab and paclitaxel in patients with HER2 negative metastatic breast cancer. *Cancer Med* 2015;**4**:1051–9.
21 Chirinos JA, Heresi GA, Velasquez H, Jy W, Jimenez JJ, Ahn E *et al.* Elevation of endothelial microparticles, platelets, and leukocyte activation in patients with venous thromboembolism. *J Am Coll Cardiol* 2005;**45**:1467–71.
22 Chironi G, Simon A, Hugel B, Pino MD, Gariepy J, Freyssinet J-M *et al.* Circulating leukocyte-derived microparticles predict subclinical atherosclerosis burden in asymptomatic subjects. *Arterioscler Thromb Vasc Biol* 2006;**26**:2775–80.
23 Cloutier N, Tan S, Boudreau LH, Cramb C, Subbaiah R, Lahey L *et al.* The exposure of autoantigens by microparticles underlies the formation of potent inflammatory components: the microparticle-associated immune complexes. *EMBO Mol Med* 2013;**5**:235–49.
24 Curtis AM, Zhang L, Medenilla E, Gui M, Wilkinson PF, Hu E *et al.* Relationship of microparticles to progenitor cells as a measure of vascular health in a diabetic population. *Cytometry B Clin Cytom* 2010;**78**:329–37.
25 Curry N, Raja A, Beavis J, Stanworth S, Harrison P. Levels of procoagulant microvesicles are elevated after traumatic injury and platelet microvesicles are negatively correlated with mortality. *J Extracell Vesicles* 2014;**3**:25625.
26 Dursun I, Poyrazoglu HM, Gunduz Z, Ulger H, Yykylmaz A, Dusunsel R *et al.* The relationship between circulating endothelial microparticles and arterial stiffness and atherosclerosis in children with chronic kidney disease. *Nephrol Dial Transplant* 2009;**24**:2511–8.
27 Eleftheriou D, Ganesan V, Hong Y, Klein NJ, Brogan PA. Endothelial injury in childhood stroke with cerebral arteriopathy: a cross-sectional study. *Neurology* 2012;**79**:2089–96.
28 Ferreira AC, Peter AA, Mendez AJ, Jimenez JJ, Mauro LM, Chirinos JA *et al.* Postprandial hypertriglyceridemia increases circulating

levels of endothelial cell microparticles. *Circulation* 2004;**110**:3599–603.
29 Fink K, Schwarz M, Feldbrügge L, Sunkomat JN, Schwab T, Bourgeois N *et al.* Severe endothelial injury and subsequent repair in patients after successful cardiopulmonary resuscitation. *Crit Care* 2010;**14**:R104.
30 Fleitas T, Martínez-Sales V, Vila V, Reganon E, Mesado D, Martín M *et al.* Circulating endothelial cells and microparticles as prognostic markers in advanced non-small cell lung cancer. *PLoS ONE* 2012;**7**:e47365.
31 Guervilly C, Lacroix R, Forel J-M, Roch A, Camoin-Jau L, Papazian L *et al.* High levels of circulating leukocyte microparticles are associated with better outcome in acute respiratory distress syndrome. *Crit Care* 2011;**15**:R31.
32 György B, Szabó TG, Turiák L, Wright M, Herczeg P, Lédeczi Z *et al.* Improved flow cytometric assessment reveals distinct microvesicle (cell-derived microparticle) signatures in joint diseases. *PLoS ONE* 2012;**7**:e49726.
33 Hu S-S, Zhang H-G, Zhang Q-J, Xiu R-J. Small-size circulating endothelial microparticles in coronary artery disease. *PLoS ONE* 2014;**9**:e104528.
34 Jy W, Minagar A, Jimenez JJ, Sheremata WA, Mauro LM, Horstman LL *et al.* Endothelial microparticles (EMP) bind and activate monocytes: elevated EMP-monocyte conjugates in multiple sclerosis. *Front Biosci* 2004;**9**:3137–44.
35 Kasar M, Boğa C, Yeral M, Asma S, Kozanoglu I, Ozdogu H. Clinical significance of circulating blood and endothelial cell microparticles in sickle-cell disease. *J Thromb Thrombolysis* 2013;**38**:167–75.
36 Khodeir SA, El Raouf YMA, Farouk G, EL-Bradey M. Detection of circulating microparticles in patients with proliferative diabetic retinopathy. *Life Sci J* 2012;**9**:204–9.
37 Kim SJ, Moon GJ, Cho YH, Kang HY, Hyung NK, Kim D *et al.* Circulating mesenchymal stem cells microparticles in patients with cerebrovascular disease. *PLoS ONE* 2012;**7**:e37036.
38 Knijff-Dutmer EAJ, Koerts J, Nieuwland R, Kalsbeek-Batenburg EM, van de Laar MA. Elevated levels of platelet microparticles are associated with disease activity in rheumatoid arthritis. *Arthritis Rheum* 2002;**46**:1498–503.
39 Koch CJ, Lustig RA, Yang X-Y, Jenkins WT, Wolf RL, Martinez-Lage M *et al.* Microvesicles as a biomarker for tumor progression versus treatment effect in radiation/temozolomide-treated glioblastoma patients. *Transl Oncol* 2014;**7**:752–8.
40 Koga H, Sugiyama S, Kugiyama K, Fukushima H, Watanabe K, Sakamoto T *et al.* Elevated levels of remnant lipoproteins are associated with plasma platelet microparticles in patients with type-2 diabetes mellitus without obstructive coronary artery disease. *Eur Heart J* 2006;**27**:817–23.
41 Kurtzman N, Zhang L, French B, Jonas R, Bantly A, Rogers WT *et al.* Personalized cytomic assessment of vascular health: evaluation of the vascular he alth profile in diabetes mellitus. *Cytometry B Clin Cytom* 2013;**84**:255–66.
42 Lowery-Nordberg M, Eaton E, Gonzalez-Toledo E, Harris MK, Chalamidas K, McGee-Brown J *et al.* The effects of high dose interferon-β1a on plasma microparticles: correlation with MRI parameters. *J Neuroinflammation* 2011;**8**:43.
43 Mallat Z, Benamer H, Hugel B, Benessiano J, Steg PG, Freyssinet JM *et al.* Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. *Circulation* 2000;**101**:841–3.
44 Marcos-Ramiro B, Oliva Nacarino P, Serrano-Pertierra E, Blanco-Gelaz MA, Weksler BB, Romero IA *et al.* Microparticles in multiple sclerosis and clinically isolated syndrome: effect on endothelial barrier function. *BMC Neurosci* 2014;**15**:110.
45 Matsumoto H, Yamakawa K, Ogura H, Koh T, Matsumoto N, Shimazu T. Enhanced expression of cell-specific surface antigens on endothelial microparticles in sepsis-induced disseminated intravascular coagulation. *Shock* 2015;**43**:443–9.
46 Mayne E, Funderburg NT, Sieg SF, Asaad R, Kalinowska M, Rodriguez B *et al.* Increased platelet and microparticle activation in HIV infection: upregulation of P-selectin and tissue factor expression. *J Acquir Immune Defic Syndr* 2012;**59**:340–6.
47 Merino A, Portolés J, Selgas R, Ojeda R, Buendia P, Ocaña J *et al.* Effect of different dialysis modalities on microinflammatory status and endothelial damage. *Clin J Am Soc Nephrol* 2010;**5**:227–34.
48 Nantakomol D, Dondorp AM, Krudsood S, Udomsangpetch R, Pattanapanyasat K, Combes V *et al.* Circulating red cell-derived microparticles in human malaria. *J Infect Dis* 2011;**203**:700–6.
49 Nascimbene A, Hernandez R, George JK, Parker A, Bergeron AL, Pradhan S *et al.* Association between cell-derived microparticles and adverse events in patients with nonpulsatile left ventricular assist devices. *J Heart Lung Transplant* 2014;**33**:470–7.
50 Nebor D, Bowers A, Connes P, Hardy-Dessources M-D, Knight-Madden J, Cumming V *et al.* Plasma concentration of platelet-derived microparticles is related to painful vaso-occlusive phenotype severity in sickle cell anemia. *PLoS ONE* 2014;**9**:e87243.
51 Nébor D, Romana M, Santiago R, Vachiery N, Picot J, Broquere C *et al.* Fetal hemoglobin and hydroxycarbamide moduate both plasma concentration and cellular origin of circulating microparticles in sickle cell anemia children. *Haematologica* 2013;**98**:862–7.
52 Ogura H, Kawasaki T, Tanaka H, Koh T, Tanaka R, Ozeki Y *et al.* Activated platelets enhance microparticle formation and platelet-leukocyte interaction in severe trauma and sepsis. *J Trauma* 2001;**50**:801–9.
53 Park MS, Owen BAL, Ballinger BA, Sarr MG, Schiller HJ, Zietlow SP *et al.* Quantification of hypercoagulable state after blunt trauma: microparticle and thrombin generation are increased relative to injury severity, while standard markers are not. *Surgery* 2012;**151**:831–6.
54 Parker B, Al-Husain A, Pemberton P, Yates AP, Ho P, Gorodkin R *et al.* Suppression of inflammation reduces endothelial microparticles in active systemic lupus erythematosus. *Ann Rheum Dis* 2014;**73**:1144–50.
55 Pérez-Casal M, Thompson V, Downey C, Welters I, Wyncoll D, Thachil J *et al.* The clinical and functional relevance of microparticles induced by activated protein C treatment in sepsis. *Crit Care* 2011;**15**:R195.
56 Petrozella L, Mahendroo M, Timmons B, Roberts S, McIntire D, Alexander JM. Endothelial microparticles and the antiangiogenic state in preeclampsia and the postpartum period. *Am J Obstet Gynecl.* 2012;**207**:140.e20–6.
57 Preston RA, Jy W, Jimenez JJ, Mauro LM, Horstman LL, Valle M *et al.* Effects of severe hypertension on endothelial and platelet microparticles. *Hypertension* 2003;**41**:211–7.
58 Priou P, Gagnadoux F, Tesse A, Mastronardi ML, Agouni A, Meslier N *et al.* Endothelial dysfunction and circulating microparticles from patients with obstructive sleep apnea. *Am J Pathol* 2010;**177**:974–83.
59 Rank A, Liebhardt S, Zwirner J, Burges A, Nieuwland R, Toth B. Circulating Microparticles in patients with benign and malignant ovarian tumors. *Anticancer Res* 2012;**32**:2009–14.

60 Ren J, Zhang J, Xu N, Han G, Geng Q, Song J *et al.* Signature of circulating microRNAs as potential biomarkers in vulnerable coronary artery disease. *PLoS ONE* 2013;**8**:e80738.
61 Sabatier F, Darmon P, Hugel B, Combes V, Sanmarco M, Velut J-G *et al.* Type 1 and type 2 diabetic patients display different patterns of cellular microparticles. *Diabetes* 2002;**51**:2840–5.
62 Simak J, Holada K, Risitano AM, Zivny JH, Young NS, Vostal JG. Elevated circulating endothelial membrane microparticles in paroxysmal nocturnal haemoglobinuria. *Br J Haematol* 2004;**125**: 804–13.
63 Takeshita J, Mohler ER, Krishnamoorthy P, Moore J, Rogers WT, Zhang L *et al.* Endothelial cell-, platelet-, and monocyte/ macrophage-derived microparticles are elevated in psoriasis beyond cardiometabolic risk factors. *J Am Heart Assoc* 2014;**3**:e000507.
64 Tan KT, Tayebjee MH, Lim HS, Lip GYH. Clinically apparent atherosclerotic disease in diabetes is associated with an increase in platelet microparticle levels. *Diabet Med* 2005;**22**:1657–62.
65 Tramontano AF, Lyubarova R, Tsiakos J, Palaia T, DeLeon JR, Ragolia L. Circulating endothelial microparticles in diabetes mellitus. *Mediators Inflamm* 2010;**2010**:e250476.
66 Tsiantoulas D, Perkmann T, Afonyushkin T, Mangold A, Prohaska TA, Papac-Milicevic N *et al.* Circulating microparticles carry oxidation-specific epitopes and are recognized by natural IgM antibodies. *J Lipid Res* 2015;**56**:440–8.
67 van Ierssel SH, Van Craenenbroeck EM, Hoymans VY, Vrints CJ, Conraads VM, Jorens PG. Endothelium dependent vasomotion and in vitro markers of endothelial repair in patients with severe sepsis: an observational study. *PLoS ONE* 2013;**8**:e69499.
68 VanWijk MJ, Nieuwland R, Boer K, van der Post JAM, VanBavel E, Sturk A. Microparticle subpopulations are increased in preeclampsia: possible involvement in vascular dysfunction? *Am J Obstet Gynecol* 2002;**187**:450–6.
69 Vince RV, Chrismas B, Midgley AW, McNaughton LR, Madden LA. Hypoxia mediated release of endothelial microparticles and increased association of S100A12 with circulating neutrophils. *Oxid Med Cell Longev* 2009;**2**:2–6.
70 Wang C-C, Tseng C-C, Hsiao C-C, Chang H-C, Chang L-T, Fang W-F *et al.* Circulating endothelial-derived activated microparticle: a useful biomarker for predicting one-year mortality in patients with advanced non-small cell lung cancer. *Biomed Res Int* 2014;**2014**:173401.
71 Wang Y, Zeng X, Yao F, Wu F, Su C, Fan Z *et al.* Endurance capacity is not correlated with endothelial function in male university students. *PLoS ONE* 2014;**9**:e103814.
72 Yun C-H, Jung K-H, Chu K, Kim S-H, Ji K-H, Park H-K *et al.* Increased circulating endothelial microparticles and carotid atherosclerosis in obstructive sleep apnea. *J Clin Neurol* 2010;**6**:89–98.
73 Hozo SP, Djulbegovic B, Hozo I. Estimating the mean and variance from the median, range, and the size of a sample. *BMC Med Res Methodol* 2005;**5**:13.
74 Page P. Beyond statistical significance: clinical interpretation of rehabilitation research literature. *Int J Sports Phys Ther* 2014;**9**:726–36.
75 Keefe RSE, Kraemer HC, Epstein RS, Frank E, Haynes G, Laughren TP *et al.* Defining a clinically meaningful effect for the design and interpretation of randomized controlled trials. *Innov Clin Neurosci* 2013;**10**:4S–19S.
76 Taubes G. Epidemiology faces its limits. *Science* 1995;**269**:164–9.
77 Ioannidis JPA. Contradicted and initially stronger effects in highly cited clinical research. *JAMA* 2005;**294**:218–28.
78 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 Nanomedicine* 2011;**6**:2737–48.
79 Lacroix R, Robert S, Poncelet P, Kasthuri RS, Key NS, DignatGeorge F. Standardization of platelet-derived microparticle enumeration by flow cytometry using calibrated beads: results of ISTH SSC collaborative workshop. *J Thromb Haemost* 2010;**8**:2571–4.
80 Cointe S, Judicone C, Robert S, Mooberry MJ, Poncelet P, Wauben M *et al.* Standardization of microparticle enumeration across different flow cytometry platforms: results of a multicenter collaborative workshop. *J Thromb Haemost* 2017;**15**:187–93.
81 Lacroix R, Judicone C, Poncelet P, Robert S, Arnaud L, Sampol J *et al.* Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol. *J Thromb Haemost* 2012;**10**:437–46.
82 Lacroix R, Judicone C, Poncelet P, Boucekine M, Key NS, Dignat-George F *et al.* Standardization of pre-analytical variables in plasma microparticle determination: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. *J Thromb Haemost* 2013;**11**:1190–93.
83 Yáñez-Mó M, Siljander PR, Andreu Z, Bedina Zavec A, Borràs FE, Buzas EI *et al.* Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles* 2015;**4**:27066.
84 Fais S, O'Driscoll L, Borràs FE, Buzas EI, Camussi G, Cappello F et al. Evidence-based clinical use of nanoscale extracellular vesicles in nanomedicine. *ACS Nano* 2016;**10**:3886–99.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Assessed data and calculated parameters subject to statistical and clinical relevance of the comparisons between populations of EV-based samples.

Table S2. Companion document with explanation of Table S1.