Cell specific microvesicles vary with season and disease predisposition in healthy and previously laminitic ponies

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Highlights

- Antibodies were selected to detect equine platelets, leucocytes and endothelial cells.

- These antibodies detected cell specific microvesicles in equine plasma samples.

- Total numbers and proportions of cell specific microvesicles varied with season.

- Non laminitic ponies had higher concentrations of endothelial microvesicles than previously laminitic ponies.

Abstract

Microvesicles are small (up to 1µm) vesicles found in plasma and other bodily fluids. They are recognised as part of the normal system of inter-cellular communication but altered numbers are also used as biomarkers of disease. Microvesicles have not been studied in detail in the horse but may be relevant to diseases such as laminitis. Identification of equine cell specific microvesicles was performed by developing a panel of cross reactive antibodies to use in flow cytometry to detect microvesicles of platelet, leucocyte and endothelial origin in plasma from healthy ponies and those predisposed to laminitis. The total number and proportion of microvesicles from the different cell types varied with season and there were more annexin V positive endothelial MV in non laminitic ponies compared to previously laminitic ponies. Development of this antibody panel and the technique for measuring microvesicles in the horse opens a new field for further investigation of these important structures in equine health and disease.

Abbreviations

MV   Microvesicle

EDVEC   Equine digital vein endothelial cell

HBSS   Hank's buffered salt solution

PBS   Phosphate buffered saline

TBS   Tris buffered saline

HRP   Horseradish peroxidase

Introduction

Microvesicles (MV) are small (up to 1µm in diameter) vesicles released from the surface of various cell types into different bodily fluids including plasma, and are formed by budding and shedding of the cell membrane (Lawson et al., 2016). During budding, there is a loss of membrane asymmetry and phosphatidyl serine can be detected on the outer surface of the MV (Lawson et al., 2016). MV and other smaller extracellular vesicles such as exosomes, are becoming increasingly recognised as part of the normal communication system between cells (Tkach and Théry, 2016).

In people increased numbers of circulating endothelial MV have been shown to be associated with hypertension (Chen et al., 2012), and have been identified as markers of endothelial dysfunction in individuals with type 2 diabetes (Feng et al., 2010), coronary artery disease (Werner et al., 2006) and end stage renal failure (Amabile et al., 2005). MV have long been associated with coagulopathies, with both increased and decreased MV numbers being associated with abnormal coagulation (Eleftheriou et al., 2011; Toti et al., 1996). Additionally, MV are independently associated with disseminated intravascular coagulation in human septic shock (Delabranche et al., 2013).

Laminitis manifests as a painful condition of the hoof, one form of which typically affects ponies at pasture (Hinckley and Henderson, 1996). Some of these affected animals are predisposed to recurrent laminitis episodes and the phenotypic as well as the metabolic characteristics of this population are similar to those of people at increased risk of various cardiovascular diseases (Bailey et al., 2008; Menzies-Gow et al., 2016; Treiber et al., 2006). Endothelial dysfunction is one feature of the human cardiovascular cohort (Brunner et al., 2005) and may also play a role in laminitis pathophysiology. Since MV are markers of endothelial dysfunction in people, they may also be useful markers of endothelial dysfunction in horses.

Microvesicles can be detected in various different ways, the most common of which is flow cytometry (Lawson et al., 2016). Flow cytometry allows counting of MV, some assessment of size and the expression of phosphatidyl serine on the outer surface, via binding of fluorophore labelled annexin V (Lawson et al., 2016). Phosphatidyl serine is normally found on the inner leaflet of the cell membrane, but expression on the outer surface occurs during formation of microvesicles in some but not all instances (Connor et al., 2010; Larson et al., 2012) and may represent apoptosis of the cell of origin (Hamon et al., 2000). The phenotype (cell of origin) can also be determined with the use of fluorophores conjugated to antibodies binding to cell specific surface markers (Macey et al., 2011). Flow cytometry has been used to assess the populations of MV derived from platelets, leukocytes, erythrocytes and endothelial cells in human blood samples, and to investigate how these populations vary in health and disease (Jy et al., 2004). These methods have also been used in the dog (Helmond et al., 2013; Herring et al., 2013; Lopez-Alvarez, 2015), rat (Heinrich et al., 2015) and mouse (El-Assaad et al., 2014). Cell specific antibody markers have been used in the horse for flow cytomety and other purposes. The platelet marker CD41/CD61 (Lalko et al., 2003), leucocyte marker CD18 (Ibrahim et al., 2007) and endothelial marker CD62E (Hedges et al., 2001) have been used successfully to identify the relevant equine cells. The erythrocyte marker CD147 has not been assessed using flow cytometry but has been successfully used in Western blotting (Koho et al., 2002). To date, only platelet derived MV have been assessed in the horse in a single study; more platelet MV were detected in whole blood samples than platelet poor plasma from normal horses (Springer et al., 2014).

Thus, the aims of this study were to: 1) validate the antibody markers that have been used previously, as well as other alternatives, to create an antibody panel capable of identifying the different phenotypes of circulating equine MV, namely those derived from platelets, leucocytes, erythrocytes and endothelial cells; and 2) use this antibody panel to investigate the effect of season and history of laminitis on total MV number and number of the different phenotypes in healthy and previously laminitic ponies.

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Materials and methods

The studies were approved by the Royal Veterinary College Ethics and Welfare Committee and conducted under Home Office Licences PPL numbers 70/8195 and 70/6981.

Reagents

Annexin V binding buffer (Annexin V Apoptosis Detection Set PE-Cy7; VWR International Ltd) was diluted 1:10 in sterile filtered water and passed through a 0.2µm filter (Fisher Scientific UK Ltd) prior to use. Sizing beads (Latex beads, polystyrene, 1.1µm mean particle size; Sigma-Aldrich) were diluted 1:1000 in annexin V binding buffer prior to use. All fluorophore conjugated antibodies were purchased in the conjugated form except for FITC conjugated anti-sheep CD41/CD61 which was conjugated using the Lightening-Link Conjugation System (Innova Biosciences) as per the manufacturer's instructions.

Animals

Blood samples were obtained from non-laminitic (NL; n=6 mares; age 19.8 ± 4.8 years (mean ± SD)) and previously laminitic (PL; n=6; 5 mares, 1 gelding; age 16.7 ± 2.9 years (mean ± SD)) native breed ponies. PL ponies had all experienced at least 2 episodes of laminitis in their life but no episodes within 12 months of the study commencing. Laminitis is a seasonal disease and many risk factors for laminitis vary seasonally (Bailey et al., 2008; Treiber et al., 2008), therefore measurement of factors related to the risk of laminitis should be performed seasonally. Blood samples were therefore obtained at 4 seasonal time points (April/May, July/August, October, December/January) for MV measurement and from a subset of the ponies at a single time point for antibody validation. All of the ponies were kept at pasture and fed supplementary haylage during winter, but received no additional energy providing complementary feed or supplements. None of the ponies were exercised.

Blood collection and preparation

Samples for MV measurement were obtained by jugular venepuncture, immediately transferred to tubes containing citrate (final concentration 0.38% sodium citrate; Sigma-Aldrich Co Ltd) and stored

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on ice until centrifugation (4165 x g, 15 min, 4°C) within 15 min of collection. The plasma was harvested, transferred to microcentrifuge tubes and stored at -80°C until flow cytometric analysis. Whole blood samples for antibody validation were similarly obtained and stored on ice until flow cytometric analysis (within 6 h). Blood samples for cell isolations were also obtained as above, transferred into chilled citrate tubes and allowed to settle (30 min, room temperature) prior to separating the leucocyte and platelet rich plasma for cell isolation.

Cell isolation, activation and antibody labelling

Details of all antibodies used are provided in Table 1. Antibody labelling was performed on ice and protected from light. Appropriate isotype controls were used for all fluorescently labelled antibodies and secondary only controls were used when performing indirect labelling. Optimal antibody concentrations were determined in preliminary experiments.

Preparation of plasma samples for MV measurement

Plasma samples were centrifuged (17000g, 15 min, 4°C) and the pellet re-suspended in annexin V binding buffer prior to antibody labelling.

Preparation of whole blood samples for MV measurement

Whole blood was labelled with antibodies prior to flow cytometry.

Preparation of platelets for flow cytometry

Platelets were separated from the leucocyte and platelet rich plasma by centrifugation (350 x g, 10 min, 22°C) to obtain platelet rich plasma and further centrifugation of this supernatant (1200 x g, 15 min, 22°C) to obtain a platelet pellet. The platelet pellet was re-suspended in Hank's buffered salt solution (HBSS; Fisher Scientific UK Ltd) with 10mM HEPES (Fisher Scientific UK Ltd) and 0.1% bovine serum albumin (Sigma-Aldrich Co Ltd). The platelets were fixed by addition of an equal volume of 0.4% paraformaldehyde prior to antibody labelling.

Preparation of neutrophil and mononuclear cells for flow cytometry

Cells were separated from the leucocyte and platelet rich plasma by centrifugation (300 x g, 10 min, 4°C) and the pellet re-suspended in 15ml of plasma supernatant. This was layered onto a 60%/80% Percol (GE Healthcare) gradient before centrifugation (350 x g, 15 min, 4°C) (Brooks et al., 2009). Mononuclear cells were obtained from the plasma/60% Percol interface and neutrophils from the 60%/80% Percol interface. Cell pellets were re-suspended in HBSS and washed once in HBSS prior to antibody labelling.

Preparation of erythrocytes for flow cytometry

Cells were obtained from the settled whole blood after removal of the leucocyte and platelet rich plasma for platelet isolation and re-suspended in annexin V binding buffer (Annexin V Apoptosis Detection Set, VWR International Ltd) prior to antibody labelling.

Preparation of endothelial cells for flow cytometry

Equine digital vein endothelial cells (EDVEC) were isolated from the hindlimbs of horses euthanased at an abattoir not for research purposes as previously described (Bailey and Cunningham, 2001). Once confluent in 24 well plates, cells were made quiescent in serum free medium for 45 min and then incubated with cell culture medium containing 1% foetal calf serum with or without lipopolysaccharide (LPS; 1µg/ml; Sigma-Aldrich Co Ltd) for 18 h. Both groups of cells were washed in phosphate buffered saline (PBS; Fisher Scientific UK Ltd), lifted from the plate using trypsin/EDTA solution (1mg/ml and 0.25mg/ml respectively; Fisher Scientific UK Ltd) and re-suspended in PBS prior to antibody labelling. Cells were washed in PBS and fixed with 0.4% paraformaldehyde prior to analysis.

Flow cytometry

All samples were diluted to 500µl in annexin V binding buffer prior to analysis. Sizing beads (1µl) and enumeration beads (10µl; Flow Cytometry Absolute Count Standard; Bangs Laboratories) were added to plasma samples immediately prior to analysis. Analysis was performed within 6 h of sample

collection for fresh samples or within 36 h for fixed samples. Flow cytometry was performed using a Canto II flow cytometer (BD) with Diva software version 6.1. The instrument was calibrated on a daily basis with Cell Tracker Beads (BD). The instrument had 3 lasers and a standard optical filter setup with a window extension of 7 µsec and with forward scatter threshold set at 200 and side scatter threshold set low. The limit of detection of the instrument has previously been shown to be below 0.1µm (Macey et al., 2011). Colour compensation was performed using Comp Beads (BD) according to the manufacturer's instructions.

Flow cytometric analysis

MV or cells were first identified based on size determined by the sizing beads in a plot of side scatter (logarithmic scale; y-axis) and forward scatter (logarithmic scale; x-axis) (Figure 1)(Macey et al., 2011). Gated MV or cells were then displayed on a histogram for each type of fluorescence to allow gating of the negative population based on 98% events from the isotype control tube (Heinrich et al., 2015). The number of positive events for each antibody or Annexin V was calculated and converted to events per µl based on the number of counting beads detected (Heinrich et al., 2015).

$$Events\ per\ \mu l\ =\ \frac{number\ of\ enumeration\ beads\ added\ per\ \mu l\ \times\ events\ \times\ dilution\ factor}{number\ of\ beads\ counted}$$

Western blotting

Since none of the antibodies investigated could identify erythrocytes using flow cytometry, Western blotting of erythrocyte cell lysates was performed as part of the attempt to validate erythrocyte antibodies. Erythrocyte ghosts were obtained using a protocol modified from (Takeeuchi et al., 1998) used in our laboratory for isolation of human erythrocye ghosts for Western blotting (unpublished observations). Packed erythrocytes were washed twice in PBS, centrifuging between washes (3000 x g, 5 min, 4°C) prior to re-suspension (1:10, v:v) in sodium phosphate buffer (5mM pH 8) to achieve haemolysis. After 30 min at room temperature the erythrocyte cell lysates were separated by 4 rounds

of centrifugation (6000 x g, 10 min, 4°C) and washing in sodium phosphate buffer followed by further centrifugation (15000 x g, 5 min, 4°C).

The pellet was re-suspended in Laemlli loading buffer (Sigma-Aldrich). The erythrocyte antibody markers anti-mouse CD147, anti-horse CD147, anti-human CD235 or monoclonal anti-human CD238 were assessed by Western blotting of the erythrocyte cell lysates. Beta-actin was used as an internal control. Equal volumes of lysates in Laemlli loading buffer were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis using a Power Pac 300 (Bio-Rad) at 120 volts for 2 h. Proteins were transferred onto a polyvinylidene difluoride membrane (GE Healthcare) at 24 volts for 1 h. Membranes were blocked with 5% milk in Tris buffered saline (0.1% Tween®; TBS-Tween®; Sigma-Aldrich) at 4°C overnight. Immunodetection was performed with the antibodies of interest (Table 4.2) for 2 h at room temperature and isotype matched secondary horseradish peroxidase (HRP) conjugated antibodies for 45 min at room temperature. All antibodies were diluted in TBS-Tween® and 5% dry powdered skimmed milk. Membranes were washed in TBS-Tween® (3 x 2 min) after each antibody incubation. Immunoreactive proteins were detected using enhanced chemiluminescence (GE Health Care). The molecular weight of bands was determined by comparing to bands generated by a ladder (Prestained Protein Marker (7-175 kDa); New England Biolabs).

Statistical analysis

Statistical analysis was performed using R version 3.1.1 (The R Project for Statistical Computing; https://www.r-project.org/). Normality of the distribution of data was assessed by visual inspection of histograms. Summary data were displayed as median and interquartile range. The effects of season and predisposition to laminitis on microvesicle numbers were assessed using linear mixed effects models following square root transformation to achieve normality. Statistical significance was accepted at $p \leq 0.05$.

Results

Identification of cell specific antibody markers by flow cytometry

Summary results are provided in Table 2.

Platelets

Initial experiments performed with the FITC-conjugated CD41/CD61 antibody resulted in 37% binding of isolated platelets and 62% binding of the candidate population (based on forward and side scatter characteristics consistent with platelets) in whole blood. However, in subsequent samples only 5% binding of isolated platelets was achieved. To investigate this change, binding to platelets in whole blood samples was explored, using either freshly conjugated primary antibody or unconjugated primary antibody with a commercially available fluorophore-conjugated secondary antibody. The conjugated primary antibody resulted in 2% binding whereas the unconjugated primary antibody combined with conjugated secondary antibody resulted in 55% binding. This combination of unconjugated primary antibody with conjugated secondary antibody was therefore chosen for platelet MV detection. There was no antibody binding of CD31 (platelet and endothelial cell marker), CD62E/CD62P (activated platelet and activated endothelial cell marker; fluorescent intensity less than the corresponding isotype control) in the same population in either whole blood or isolated platelets.

Neutrophils and mononuclear cells

In isolated cell suspensions, 99% of neutrophils and 81% of mononuclear cells expressed the leucocyte marker CD18. There was no antibody binding of CD45 (mononuclear cell marker; fluorescence intensity less than the corresponding isotype control).

Erythrocytes

Erythrocytes were identified via flow cytometry when viewed on a scatter plot of forward versus side scatter, however they could not be identified via positive antibody staining. There was no consistent antibody binding of the erythrocyte markers CD71, anti-mouse CD147, anti-horse CD147, CD235,

OkA2 or CD238 in the selected erythrocyte population (based on forward and side scatter characteristics) in either whole blood or isolated cells.

Endothelial cells

Expression of the endothelial cell marker CD105 (antibody clone SN6) by quiescent cells was 90%. There was no antibody binding of CD31 (platelet and endothelial cell marker), CD62E/CD62P (activated platelet and activated endothelial cell marker) or CD105 (antibody clone MJ7/18) in quiescent cells. Expression of the activated endothelial cell marker CD62E/CD62P by LPS-activated cells ranged from 65% to 86%.

Western blotting of erythrocyte cell lysates

Protein expression was detected using anti-human CD238 at the appropriate molecular weight, indistinct bands were detected using anti-horse CD147 (Figure 2). Protein expression could not be detected using anti-mouse CD147 or anti-human CD235.

Measurement of plasma microvesicle populations

Microvesicles (MV) originating from platelets, leucocytes (neutrophils and mononuclear cells) and endothelial cells (quiescent or activated) could be detected in equine plasma samples using the specific antibody markers previously identified (i.e. CD41/CD61, CD18, CD105, CD62E/CD62P). A large proportion (22 ± 13 %; mean ± SD %) of MV in most individuals were not labelled with a specific antibody marker.

There were significantly (p = 0.008) more annexin V positive endothelial MV (EMV) in NL than PL ponies (Table 4). There were no other differences between the groups in either the total number of MV or the cell specific populations (annexin V positive or total in each case).

The largest population of cell specific MV in each season was platelet MV (PMV), except in winter samples when leucocyte MV (LMV) were most common. When all seasons were combined there were

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26 ± 25 % PMV, 28 ± 27 % LMV, 8 ± 10 % EMV and 0.3 ± 0.6 % activated endothelial MV (aEMV) (mean ± SD %). If only annexin V positive MV were assessed, there were 17 ± 13 % PMV, 17 ± 18 % LMV, 9 ± 9 % EMV and 0.7 ± 1.5 % aEMV (mean ± SD %). The overall mean proportion of MV that were annexin V positive was 47 ± 19 %.

The total number of MV, as well as of LMV and EMV (both annexin V positive and the total population), was significantly higher in winter than all other seasons (except spring for the total number of annexin V positive and negative MV combined; Tables 3 and 4). There were no seasonal differences in the number of PMV or aEMV. Annexin V status could not be determined for EMV in 2 ponies due to experimental error (1 PL and 1 NL).

Discussion

This present study demonstrated that equine MV can be measured using flow cytomety and that the phenotype can be identified for MV originating from platelets, leucocytes and endothelial cells, but not erythrocytes, using the currently available panel of cross reactive and equine specific antibodies. There was a significant effect of season and disease on the equine MV population. The total number of MV, as well as the number of leucocyte MV and endothelial MV, varied with season and there were significantly more annexin V positive endothelial MV in NL ponies compared to PL ponies, independent of season.

There were considerable difficulties in developing an antibody panel to detect all the MV phenotypes of interest. Most commercially available antibodies for flow cytometry are developed to identify human, rat or mouse cells. There is variable cross-reactivity between these antibodies and equine cells. The antibodies most commonly used for MV identification in previous recent studies in dogs (Lopez-Alvarez, 2015) were tested in the present study, as were those antibodies previously used in equine studies of MV (Springer et al., 2014) and intact cells (Hedges et al., 2001; Ibrahim et al., 2007;

Lalko et al., 2003). An adequate panel was developed to detect MV from platelets, leucocytes and endothelial cells.

The binding of the CD41/CD61 antibody to platelets was low in some preliminary experiments. This antibody was not available in the conjugated form, so it was conjugated using a commercially available kit designed for that purpose. The results obtained using the conjugated antibody also varied over time in preliminary experiments so the conjugated antibody was compared to using the unconjugated antibody with a fluorescently labelled secondary antibody. There was greater platelet labelling using the unconjugated CD41/CD61 with fluorescent secondary antibody, thus this was chosen for the final antibody panel. Further investigation of the relatively low binding of platelets (37-62%) in comparison to previous studies (85% (Lalko et al., 2003)) should ideally be undertaken by assessing antibody binding using other methods such as immunocytochemistry.

Despite investigating many different potential erythrocyte antibodies, none were found to identify erythrocytes using flow cytometry. Two antibodies were trialled which have previously been used to study equine erythrocytes using Western blotting (Koho et al., 2012), but neither of these bound erythrocytes by flow cytometry. Since there is variable expression of these antigens in horses (Koho et al., 2012), Western blotting was performed on erythrocytes from one pony in this study to investigate whether the lack of binding was due to low antigen expression in the study population. One of these antibodies (anti-horse CD147) detected indistinct bands in the region of the appropriate molecular weights; another antibody trialled that was commercially available for Western blotting of human cells (anti-human CD238) clearly detected protein expression at the appropriate molecular weight. The conclusion, therefore, is that these proteins are expressed by erythrocytes in the study population but are not accessible for antibody binding of the intact cell by flow cytometry. Further work is necessary to develop an antibody that can detect equine erythrocytes and therefore MV of erythrocyte lineage by flow cytometry. Erythrocyte MV, along with platelet MV and endothelial MV,

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are increased in human patients with metabolic syndrome (Helal et al., 2011), therefore these MV should not be discounted in equine studies which may include animals with similar metabolic disease.

Binding of the leucocyte antibody CD18 and the endothelial antibodies CD105 and CD62E/CD62P all exceeded 65% and in the case of CD18 binding in neutrophils was as high as 99%. These antibodies were therefore considered suitable for detection of MV from these cell types.

There is debate as to whether phosphatidyl serine expression, as detected by annexin V binding, is an absolute requirement to classify a MV as such (Connor et al., 2010; Hou et al., 2014; Larson et al., 2012). Annexin V has also been well described as a reagent to identify apoptotic cells and circulating apoptotic bodies (AB). AB are much larger (typically >2 micron) and so were not measured in this study. This does not rule out the possibility that some cells were undergoing apoptosis. Without co-staining with a vitality stain such as propidium iodide, annexin V status cannot distinguish between apoptotic and necrotic cells, therefore annexin V positive MV may have originated from either apoptotic or necrotic cells. In the present study, populations of both total MV (annexin V positive and negative) and annexin V positive MV alone are reported. The proportion of annexin V positive MV varied across the individual samples, however when investigating differences between groups and seasons the same results were obtained whether total or positive MV were compared, except when comparing endothelial MV. In this case there was a difference in annexin V positive endothelial MV between NL and PL ponies, but no difference in total endothelial MV between NL and PL ponies.

In comparison to one study in people (Macey et al., 2011), the number (median across all seasons and all individuals) of annexin V positive MVs in the present study was much lower (487/µl vs 2817/µl). This may be due to species differences or to differences in the centrifugation protocol used between studies, since differences in sample preparation can have significant effects on MV numbers (Lacroix et al., 2012). The proportion of platelet MV was also much lower than in people (17% vs 75%) (Macey et al., 2011). This may be due to low antibody binding efficacy since only 55% of platelets were detected as expressing CD41/CD61. In comparison to the same human study, the proportion of

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leucocyte MV was higher in ponies (17% vs 8.9-10.6%) (Macey et al., 2011). The ponies were all older individuals so may have underlying chronic inflammation which is associated with age in equids (Adams et al., 2009). Neutrophil derived MV have been shown to be increased in a variety of inflammatory conditions in people (Johnson et al., 2014); this may be the reason for the greater proportion of leucocyte derived MV in this equine study.

In dogs the majority of labelled MV were of erythrocyte origin and the second most numerous were of platelet origin (Lopez-Alvarez, 2015). Since an erythrocyte marker could not be identified in the present study, it is not possible to make a direct comparison with the canine study. It may be that a large proportion of the unlabelled MV were of erythrocyte origin but until an equine specific erythrocyte antibody marker can be identified to label MV in flow cytometry, this cannot be tested. The canine study was also performed using whole blood samples rather than plasma samples therefore it is difficult to make comparisons between the studies.

The only difference found between NL and PL ponies was that the number of annexin V positive endothelial MV was significantly greater in the NL ponies compared to PL. This finding was unexpected and not consistent with the hypothesis that endothelial cell dysfunction is a risk factor for equine laminitis. In human studies, a higher concentration of endothelial MV has been associated with reduced endothelial function (Chen et al., 2012; Feng et al., 2010; Werner et al., 2006) therefore we expected to see higher numbers in PL ponies rather than NL, if PL ponies do indeed have impaired endothelial function. This difference did not reach statistical significance when total rather than annexin V positive endothelial MV were compared. Phosphatidyl serine expression on MV (assessed by annexin V binding) is associated, in some studies, with apoptosis of the cells producing the MV (Diamant et al., 2004), therefore the presence of more annexin V positive endothelial MV in NL ponies may indicate increased endothelial cell apoptosis. The significance of this is unclear and warrants further investigation, potentially by assessing *in vivo* the function of endothelial cells from these groups of ponies.

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Alternatively, the reduced number of circulating annexin V positive endothelial MV may reflect increased clearance from the circulation rather than decreased production. Platelet MV have been shown to be cleared from the circulation by endothelial cells (Dasgupta et al., 2012), whether a similar mechanism may affect numbers of circulating endothelial MV is not known.

There was a highly significant effect of season on the total number of MV, leucocyte MV and endothelial MV (for both annexin V positive only or total), with higher concentrations in winter than any other season. The effect of season on MV concentration has not been investigated before in any species. The increase in MV in the winter may relate to changes in haemodynamics relating to the requirement for hypothermic vasoconstriction. Animals which are adapted to extremes of temperature have various techniques for conserving or losing heat (Ivanov, 1997). This could be investigated by repeating the study in animals housed indoors at a constant temperature or in animals from tropical climates which are not acclimatised to seasonal temperature changes. The effect of outdoor temperature on MV concentration has not been studied before.

An alternative explanation for the increased MV in winter may be related to exercise. In winter, the ponies received supplemental forage feeding in the form of haylage from a static feeder in the field. Informal observations by EJTF suggested that when haylage was available, the ponies spent a large amount of time standing at or close to the feeder, rather than walking and grazing pasture. Although it was not measured, the level of activity in winter may therefore have been less than in other seasons. In previously active humans, endothelial MV increased after 5 days of reduced exercise (Boyle et al., 2013). Increased exercise has also been shown in many studies to affect MV populations, but the level of exercise in these studies is not comparable to the limited level of voluntary exercise performed by the ponies when grazing in the present study. The relationship between season, exercise level and MV in ponies could be investigated by monitoring their exercise level in different seasons using GPS tracking devices (de Laat et al., 2016) and also by instigating an increased exercise regimen and investigating the effect of increased exercise on MV.

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Dietary changes can also influence MV numbers; a low carbohydrate diet decreased endothelial MV in overweight women (Wekesa et al., 2016) and high fat feeding increased total MV, leucocyte MV, endothelial MV and platelet MV in rats (Heinrich et al., 2015). Neither of these diets represent the seasonal variation in dietary intake of pasture in ponies; therefore the role of diet in the seasonal variation of MV is unknown. Measuring MV after experimental dietary interventions would help to determine this.

Leucocyte MV were significantly increased in winter; this may be related to seasonal increases in inflammatory biomarkers as previously identified in ponies (Wray et al., 2013). As discussed earlier, neutrophil derived MV are increased in inflammatory conditions in people (Johnson et al., 2014).

In addition to the constraints placed on the present study by the difficulties in identifying cross-reactive antibodies, there are other limitations to the present study that should be acknowledged. The flow cytometer used to identify the MV does not provide a visual image of the particles so it is possible that some of the events identified were actually debris. This possibility was excluded as much as possible by using the gating strategy to exclude all events that were present in the diluent. Using a technique with a greater sensitivity for the smallest vesicles, such as nanoparticle tracking analysis, in conjunction with flow cytometry, would give greater confidence in the interpretation of these smallest events. In addition, electron microscopy would allow visualisation of the MV and confirm their structure. Neither technique was available for the present study.

The development of guidelines for measuring circulating MV has been a gradual process (Chandler, 2016). When the present study was undertaken, the sample processing was performed in line with recent guidelines (Lacroix et al., 2012) except for the centrifugation step. Rather than the recommendation of two centrifugations at 2500 x g for 15 minutes prior to storage, blood was only centrifuged once at 4165 x g (maximum speed for the centrifuge) for 15 min, due to logistical factors. Therefore, the degree of platelet contamination and also artefactual increases in MV count after freeze-thawing may have been slightly higher. The influence of this difference should be investigated

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in blood samples from ponies. Since there are many pre-analytical differences between all the published studies (Chandler, 2016) it is difficult to compare these results with results in previously published studies, or compare between published studies.

In conclusion, there were significant effects of laminitis predisposition and season on different MV populations. The development of this antibody panel will facilitate further studies of equine MV and enable determination of their role in other equine diseases.

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Figure 1. Plasma samples were centrifuged (17000g, 15 minutes, 4°C) and the pellet re-suspended in annexin V binding buffer prior to antibody labelling and flow cytometric analysis. Representative forward versus side scatter plot illustrating gating protocol for plasma samples. Firstly enumeration beads and sizing beads were analysed individually to enable selection of gate P1 (enumeration beads), gate P2 (sizing beads) and gate P3 (microvesicles) based on forward scatter (FSC-A) less than sizing beads and to exclude debris present in the reagents (side scatter (SSC-A) below gate P3). This plot is from a sample containing enumeration beads (red), sizing beads (green) and resuspended plasma pellet (microparticle fraction in blue).
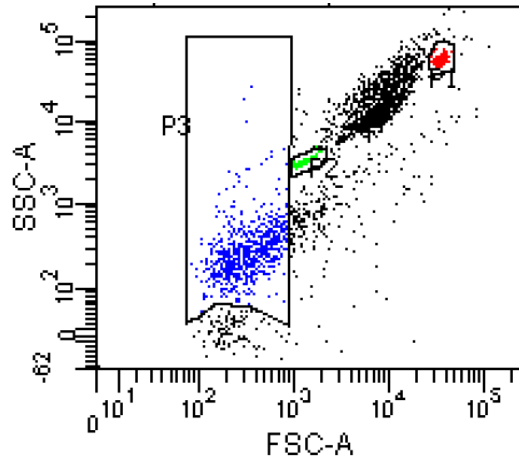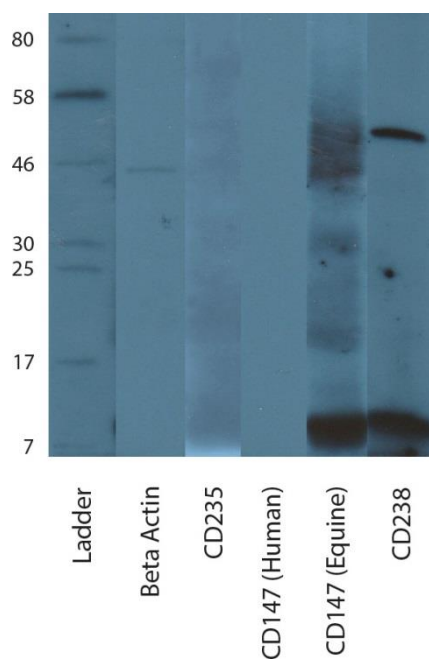
Figure 2. Evaluation of the ability of previously validated antibodies to detect the proteins CD147, CD235 and CD238 in equine erythrocytes using Western blotting.

Table 1. Antibodies used for development of antibody panel (references included where previously validated in the horse)

Antibody	Target	Clone	Concentration	Manufacturer
Anti-rat CD31-PE	Platelets and endothelial cells	TLD-3A12	1:25	BD Pharmingen
Anti-sheep CD41/CD61	Platelets (Lalko et al., 2003)	CO.35E4	1:12.5	AbD Serotec
Anti-human CD62E/CD62P-APC	Activated platelets and activated endothelial cells (Hedges et al., 2001)	BBIG-E6	1:25	R&D systems
Anti-mouse CD147-PE	Erythrocytes	RL73	1:20	eBioscience
Anti-human CD18-PE	Leukocytes (Ibrahim et al., 2007)	IB4	1:25	Ancell
Anti-human CD45-FITC	Mononuclear cells	MEM-55	1:20	AbD Serotec
Anti-human CD71-APC	Erythrocytes	OKT9	1:25	eBioscience
Anti-human CD235-FITC	Erythrocytes	HIR2	1:25	eBioscience
Anti-horse CD147	Erythrocytes (Koho et al., 2002; Western blotting only)	Purified antibody against c-terminus CD147	1:20	gift, Dr N Koho
Anti-human OkA2	Erythrocytes	Unpurified rabbit serum	1:20	gift, Dr N Koho
Anti-human CD238	Erythrocytes	MM0435-12X3	1:20	abcam
Polyclonal anti-human CD238	Erythrocytes	Polyclonal	1:20	abcam
Anti-human CD105-eFluor 450	Endothelial cells	SN6	1:25	eBioscience
Anti-mouse IgG-PerCP	Mouse primary antibody	Polyclonal	1:12.5	eBioscience
Anti-rabbit IgG-FITC	Rabbit primary antibody	Polyclonal	1:12.5	eBioscience
Anti-rabbit-HRP	Rabbit primary antibody (Western blotting)	Polyclonal	1:2000	Dako

Table 2. Percentage binding of selected antibodies to different cell types (mean ± SD where indicated, otherwise results from 1 experiment, NT = not tested, neg = less than isotype control). Cells or whole blood were incubated with either fluorophore conjugated primary antibody or primary unlabelled antibody followed by fluorophore conjugated secondary antibody and the proportion of cells positive for that antibody tested using flow cytometry.

	Cell type tested					
Primary antibody	Isolated platelets	Isolated neutrophils	Isolated mononuclear cells	Isolated erythrocytes	EDVECs	Whole blood
Anti-rat CD31 (PE conjugated)	neg	NT	NT	NT	NT	Platelets neg RBC neg
Anti-sheep CD41/CD61 (FITC conjugated)	37%, 5%	NT	NT	NT	Quiescent 6%	Platelets 62%, neg RBC neg
Anti-sheep CD41/CD61 (used with secondary)	NT	NT	NT	NT	NT	Platelets 55% RBC neg
Anti-human CD62E/CD62P (APC conjugated)	neg	NT	NT	NT	Quiescent neg LPS-activated 65%, 83%, 86%	Platelets neg RBC neg
Anti-mouse CD147 (PE conjugated)	neg	NT	NT	neg	NT	NT
Anti-human CD18 (PE conjugated)	NT	99%	81%	NT	NT	Platelets neg RBC neg
Anti-human CD45 (FITC conjugated)	NT	neg	neg	NT	Quiescent neg	Platelets neg RBC neg
Anti-human CD71 (APC conjugated)	NT	NT	NT	neg	NT	Platelets neg RBC neg
Anti-human CD235 (FITC conjugated)	NT	NT	NT	NT	NT	Platelets neg RBC neg
Anti-horse CD147 (used with secondary)	NT	NT	NT	3.4 ± 1.4% (n=12)	NT	Platelets 75% RBC 10%
Anti-human OkA2 (used with secondary)	NT	NT	NT	3.1 ± 1.8% (n=12)	NT	Platelets 11% RBC 15%
Anti-human CD238 (used with secondary)	NT	NT	NT	neg	NT	NT
Polyclonal anti-human CD238 (used with secondary)	NT	NT	NT	8%, 7%, 15%	NT	NT
Anti-human CD105 (eFluor 450 conjugated)	NT	NT	NT	NT	Quiescent 90%	NT

Table 3. Microvesicle (MV) numbers were measured in plasma from NL and PL ponies (n=6/group) at 4 seasonal time points using flow cytometry. MV were classified based on cell of origin. CD41/CD61 positive MV were classified as platelet MV. CD18 positive MV were classified as leucocyte MV. CD105 positive MV were classified as endothelial MV. CD62E/CD62P positive MV were classified as activated endothelial MV. Annexin V positive and negative populations were combined.

Season	Microvesicles (number/µl; median (interquartile range))							
	Total			Platelet		Leukocyte		
	NL	PL		NL	PL	NL	PL	
Spring	1060 (729 – 1898)	1236 (761 – 1394)		240 (124 – 326)	123 (68 – 214)	109 (31 – 173)	104 (30 – 457)	y
Summer	1134 (787 – 1420)	854 (741 – 928)	y	79 (60 – 331)	108 (89 – 207)	132 (64 – 303)	46 (13 – 182)	y
Autumn	1086 (824 – 1550)	843 (705 – 986)	y	219 (107 – 524)	172 (84 – 271)	115 (49 – 125)	16 (12 – 27)	y
Winter	2371 (1711 – 3445)	1383 (1167 – 1895)	z	181 (91 – 883)	177 (121 – 205)	878 (595 – 1008)	232 (95 – 383)	z

Season	Microvesicles (number/µl; median (interquartile range))				
	Endothelial			Activated endothelial	
	NL	PL		NL	PL
Spring	67 (18 – 409)	47 (19 – 58)	y	4 (0 – 7)	5 (2 – 10)
Summer	88 (64 – 90)	44 (19 – 96)	y	3 (0 – 11)	0 (0 – 1)
Autumn	48 (27 – 67)	17 (9 – 36)	y	6 (1 – 16)	1 (0 – 4)
Winter	486 (284 – 637)	75 (31 – 130)	z	7 (6 – 9)	9 (5 – 11)

y,z indicates significant difference (p≤0.05) between the two seasons with different letters determined using linear mixed effect model and Fisher's Least Significant Difference post hoc comparisons following square root transformation to achieve normality.

Table 4. Annexin V positive microvesicle (MV) numbers were measured in plasma from NL and PL ponies (n=6/group unless stated) at 4 seasonal time points using flow cytometry. MV were classified based on annexin V positivity and cell of origin. CD41/CD61 positive MV were classified as platelet MV. CD18 positive MV were classified as leucocyte MV. CD105 positive MV were classified as endothelial MV. CD62E/CD62P positive MV were classified as activated endothelial MV.

Season	Microvesicles (number/µl; median (interquartile range))							
	Total			Platelet			Leukocyte		
	NL	PL		NL	PL		NL	PL	
Spring	419 (333 – 1130)	490 (302 – 734)	y	166 (85 – 251)	71 (52 – 132)		93 (19 – 172)	79 (22 – 449)	y
Summer	525 (462 – 906)	416 (394 – 454)	y	52 (38 – 73)	82 (56 – 99)		115 (43 – 289)	42 (10 – 159)	y
Autumn	397 (336 – 470)	410 (218 – 532)	y	206 (93 – 338)	79 (44 – 151)		75 (18 – 105)	1 (0 – 4)	y
Winter	1116 (993 – 1721)	670 (527 – 724)	z	139 (87 – 542)	97 (82 – 120)		808 (539 – 946)	199 (46 – 351)	z

Season	Microvesicles (number/µl; median (interquartile range))					
	Endothelial*			Activated endothelial		
	NL (n=5)	PL (n=5)		NL	PL	
Spring	40 (2 – 75)	20 (11 – 37)	y	0 (0 – 0)	0 (0 – 2)	
Summer	51 (45 – 73)	13 (3 – 14)	y	1 (0 – 2)	0 (0 – 0)	
Autumn	24 (12 – 36)	3 (2 – 9)	y	1 (0 – 5)	0 (0 – 0)	
Winter	369 (236 – 570)	70 (29 – 127)	z	0 (0 – 0)	3 (0 – 4)	

y,z indicates significant difference (p≤0.05) between the two seasons with the same letter, * indicates significant difference between NL and PL, independent of season determined using linear mixed effect model and Fisher's Least Significant Difference post hoc comparisons following square root transformation to achieve normality.