Supplemental methods

Cryo-Electron microscopy

For cryo-electron microscopy (cryo-EM), human milk (HM) was diluted with annexin V-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4), supplemented with 0.1% ultracentrifuged bovine serum albumin (BSA). For annexin V labeling, 10 µL sample was incubated with 1 µL annexin V-conjugated gold nanoparticles at 2-4×10^{16} particles/L and 1 µL of 20 mM $CaCl_2$ at room temperature for 1hour, as previously described [1]. Moreover, gold-conjugated anti-TF antibodies 5B7, 5G9 and 10H10 were used. For cryo-EM, a 4 µL aliquot was deposited on a grid coated with a perforated carbon film. After draining the excess liquid with a filter paper, grids were plunge-frozen into liquid ethane cooled by liquid nitrogen using a Leica EMCPC cryo-chamber. For cryo-EM observation, grids were mounted onto a Gatan 626 cryoholder and transferred to a Tecnai F20 microscope (ThermoFisher, Waltham, MA) operated at 200 kV. Images were recorded with an Eagle 2k CCD camera (FEI, Hillsboro, OR).

Flow cytometry of extracellular vesicles

Samples were diluted to ~2 000 events/s to avoid swarm detection. Then 20 µL of the sample was labeled with CD9-PE, CD63-PE, or IgG_1-PE (clone X40; last three antibodies from BD Bioscience; San Jose, CA). Final concentration antibodies used were CD9-PE (2.5 µg/mL), CD63-PE (7.5 µg/mL), and IgG_1-PE (9 µg/mL). To investigate the cellular origin of EVs, SEC-isolated EVs were labeled with EpCAM-PE (CD326; ThermoFisher, Waltham, MA) at a final concentration of 12 µg/mL, CD66b-FITC (Beckman Coulter, Indianapolis, IN) at 50 µg/mL, CD61-PE (eBioscience, San Diego, CA) at 3 µg/mL, CD14-APC (eBioscience) at 12.5 µg/mL, CD20-PE (eBioscience) at 2.4 µg/mL, and CD4-PE (eBioscience) at 5 µg/mL. Used isotype controls were IgG1-FITC (BD bioscience, San Jose, CA) for CD66b-FITC, IgG2b-PE

(eBioscience) for CD20-PE, IgG1-APC (BD bioscience) for CD14-APC, and IgG1-PE (BD bioscience) for the remaining antibodies. Before labeling, antibody aggregates were removed by centrifugation at 18 890g for five minutes at 20 °C. The samples were labeled in the dark for two hours at room temperature. The incubation was stopped by adding 200 µL citrate-phosphated buffered saline. Samples were analyzed by Apogee A60 Micro Flow Cytometer (Hemel Hempstead, Hertfordshire, UK) for 1 minute with a flow rate of 3.01 µL/minute. The results were analyzed by the software FlowJo (Version 10, FlowJo LLC, Ashland, OR).

Surface plasmon resonance imaging

CD9, and CD63, and IgG_1 (BD Bioscience; San Jose, CA) were printed on a SPRi sensor (Easy2Spot G-type, Ssens; Enschede, the Netherlands) using a microfluidic printer (CFM 2.0, Wasatch Microfluidics, Salt Lake City, UT). Coupling buffer was prepared in acetic acid buffer (pH 4.5, Merck; Darmstadt, Germany) supplemented with 0.05% (v/v) Tween 80 (Sigma Aldrich, St. Louis, MO). Antibodies were prediluted in coupling buffer to 5 µg/mL. Blocking was performed with 1 M ethanolamine followed by blocking with 1% (v/v) bovine serum albumin (both Sigma Aldrich; St. Louis, MO). Then the printed spots were incubated three times 15 minutes with regeneration buffer: 0.1 M glycin-HCl (pH 3.0,Merck; Kenilworth, NJ) supplemented with 0.3% Triton-X100 (Sigma Aldrich; St. Louis, MO), to remove unbound material. Based on the results of the Western blot of the SEC fractions, the EV-rich fractions 8 and 9 were selected and pooled, and fractions 25 and 26 were pooled as negative control. EVs were concentrated by ultracentrifugation at 154 000g at 4 °C for 60 minutes. The pellets were re-suspended into 150 µL. Then the EV-containing samples were incubated for 60 minutes on the sensor surface and material capture was monitored in real time with the MX96 SPRi device (IBIS Technologies; Enschede, the Netherlands). SPRi signals were detected and processed using home-made software in Matlab R2016b (Mathworks, Natick, MA). Antibody spots were measured in parallel with isotype controls on the same sensor to correct for antibody release

from the surface and nonspecific binding. For data processing, the SPRi response was defined as the difference between the mean SPRi signal of the antibody and mean SPRi of the matched isotype control during the last 50 seconds of the measurement.

Western blot analysis

For the Western blots shown in Figures 2 and 3, human milk was diluted 1:1 (vol/vol) with saline, and loaded on 10 mL Sepharose CL-2B column as described in the Methods section (Isolation of extracellular vesicles from human milk), and 26 fractions were collected. The total protein of each fraction was precipitated, mixed with 10% of β-mercaptoethanol reducing laemmli buffer and then boiled at 100 °C for 10 minutes. Samples (15 µL) were loaded on a 4-12% gradient gel (PAGEr EX gel, Lonza, Rockland, ME). Proteins were blotted onto polyvinylidene difluoride (PVDF) membrane (Millipore; Amsterdam, The Netherlands). Following blocking with 5% Protifar (low fat milk, Nutrition) to avoid non-specific staining, the PVDF membranes were incubated with anti-TF (clone HTF-1, 1:1000), anti-CD63 (1:500) and anti-CD9 (1:250) at 4 °C overnight. After washing the PVDF membrane, a secondary antibody goat-anti-mouse (GAM)-horseradish peroxidase (HRP) (1:3000, Dako, Glostrup, Denmark) was used. Bands were visualized by incubating the PVDF membrane with two-fold-diluted Lumi-LightPLUS Western Blotting Substrate (Sigma-Aldrich, St. Louis, MO) for two minutes, and subsequently the bands were analyzed by Image Quant LAS 4000 (GE life science, Eindhoven, The Netherlands).

Digestion of human milk

We applied a simulated gastric fluid model, that was introduced by Liu et al [2]. To prepare the simulated gastric fluid (SGF), a gradient of HCl was dissolved in 0.2% NaCl. The resulting solutions were further adjusted until a gradient of pH-value from 1 to 7 at 37 °C was reached. Before the measurement, pepsin (P7000, from porcine gastric mucosa, >250 units/mg, Sigma

Aldrich, Germany) was added to the SGF to reach 0.032 mg/mL. Then SGF was pre-warmed and maintained at 37°C. 100 µL pre-diluted HM (20% pre-dilution in 0.9% NaCl) of all probands was incubated with 300 µL SGF at 37 °C for 1 hour. After incubation, the samples were centrifuged at 154 000 g for 1 hour at 4°C. Then the acid containing supernatant was removed,the pellet was re-suspended in 400 µl 0.9% NaCl and the clotting assay was performed.

We prepared simulated intestinal fluid (SIF) as described by Mikenus et al [3] without addition of bile acids due to very low concentrations in newborns [4]. SIF electrolyte solution contained 6.8 mM KCl, 0.8 mM KH_2PO_4, 85 mM $NaHCO_3$, 38.4 mM NaCl, 0.33 mM and $MgCl_2(H_2O)_6$. 200 µL human milk (10% pre-dilution in 0.9% NaCl) of all probands was mixed with 110 µL SGF electrolyte solution. 50 µL pancreatin (P1750, Pancreatin from porcine pancreas, Sigma Aldrich, Germany) made up in SIF electrolyte solution was added to reach the final concentrations of 4 mg/mL, 0.8 mg/mL, 0.16 mg/mL, 0.032 mg/mL. Thereafter the pH value of the resulting SIF solution was adjusted into 7 at 37 °C by adding NaOH or HCl. $CaCl_2(H_2O)_2$ was added into the final mixture of milk and SIF to reach 0.3 mM. Total volume was adjusted into 400 µL by adding water. All the chemicals, if not specific mentioned, were purchased from Merck KGaA, Darmstadt, Germany. The mixture was incubated at 37°C for one hour. After incubation, the mixture was centrifuged at 154 000 g for one hour. After pipetting off the supernatant, the pellet was re-suspended in 400 µL and the clotting assay was performed.

Collection of bovine milk and blood

Fresh unprocessed bovine milk (breed *Fleckvieh*) was obtained from a local farmer and processed the same way as HM. Bovine blood was collected at the University of Veterinary Medicine Vienna (Department for Farm Animals and Veterinary Public Health) with approval of the institutional Ethics Committee in citrate vacuum tubes (Vacuette; Greiner-Bio One, Kremsmuenster, Austria), centrifuged at 3000 g for 10 minutes at 18 °C and at 12 000 g for 2

minutes at 20 °C to obtain platelet-free plasma. Bovine plasma samples were stored at -80 °C until measurements were performed. For the fibrin generation assay, "EV-depleted" bovine plasma was generated by centrifugation at 18 890g at 20°C for 1 hour.

Collection and preparation of human milk from mothers of preterm infants at a neonatal intensive care unit

Human milk was collected from 5 healthy adult mothers (age [years]: median: 32, range: 25-41) of preterm infants at the neonatal intensive care unit of the Medical University of Vienna with approval of the institutional Ethics Committee (EC number 1721/2015). According to the study inclusion criteria, each participant had delivered a pre-term infant (median gestation age [weeks]: 29, range: 24-35). Infants received human milk for median 42 days (range: 8-80). The coagulant activity of fresh (untreated) HM and milk prepared according to routine preparations was investigated in a fibrin generation assay. Aliquots of each milk sample were fortified (Aptamil FMS fortifier, 4g/100ml, freeze-thawed (1 hour at -20 °C), and cooled (1 hour at 4 °C). Also the coagulant activity of pasteurized pooled donor milk from the institutional milk bank was investigated.

References

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2. Liu W, Ye A, Liu W, Liu C, Singh H. Stability during in vitro digestion of lactoferrin-loaded liposomes prepared from milk fat globule membrane-derived phospholipids. *J Dairy Sci*. 2013;96(4):2061-2070.
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4. Kamstrup D, Berthelsen R, Sassene PJ, Selen A, Müllertz A. In Vitro Model Simulating Gastro-Intestinal Digestion in the Pediatric Population (Neonates and Young Infants). *AAPS PharmSciTech*. 2017;18(2):317-329.

Supplemental Table 1. Effect of simulated gastric fluid on human milk-derived extracellular vesicles

	Plasma clotting time (s)	P
HM	753 ± 424	
+ SGF pH 7	607 ± 428	0.138
+ SGF pH 6	897 ± 766	0.485
+ SGF pH 5	820 ± 720	0 .727
+ SGF pH 4	787 ± 678	0.833
+ SGF pH 3	858 ± 722	0.513
+ SGF pH 2	2484 ± 1102	0 006
+ SGF pH 1	>3600	< 0.001
Buffer	>3600	< 0.001

SGF: simulated gastric fluid.

Supplemental Table 2. Effect of simulated intestinal fluid on human milk-derived extracellular vesicles

	Plasma clotting time (s)	P
HM	1014 ± 564	
+ SIF pancreatin 0.032 mg/mL	986 ± 526	0.591
+ SIF pancreatin 0.16 mg/mL	832 ± 524	0.206
+ SIF pancreatin 0.8 mg/mL	681 ± 406	0.151
+ SIF pancreatin 4.0 mg/mL	992 ± 643	0.920
Buffer	>3600	< 0.001

SIF: simulated intestinal fluid.

Supplemental Table 3. Comparison of the coagulant activity of human milk and bovine milk

	Plasma clotting time (s)	
	Human plasma	Bovine plasma
Human milk	555 ± 383	1743 ± 666
Bovine milk	>3600*	>3600*
Buffer	>3600*	>3600*

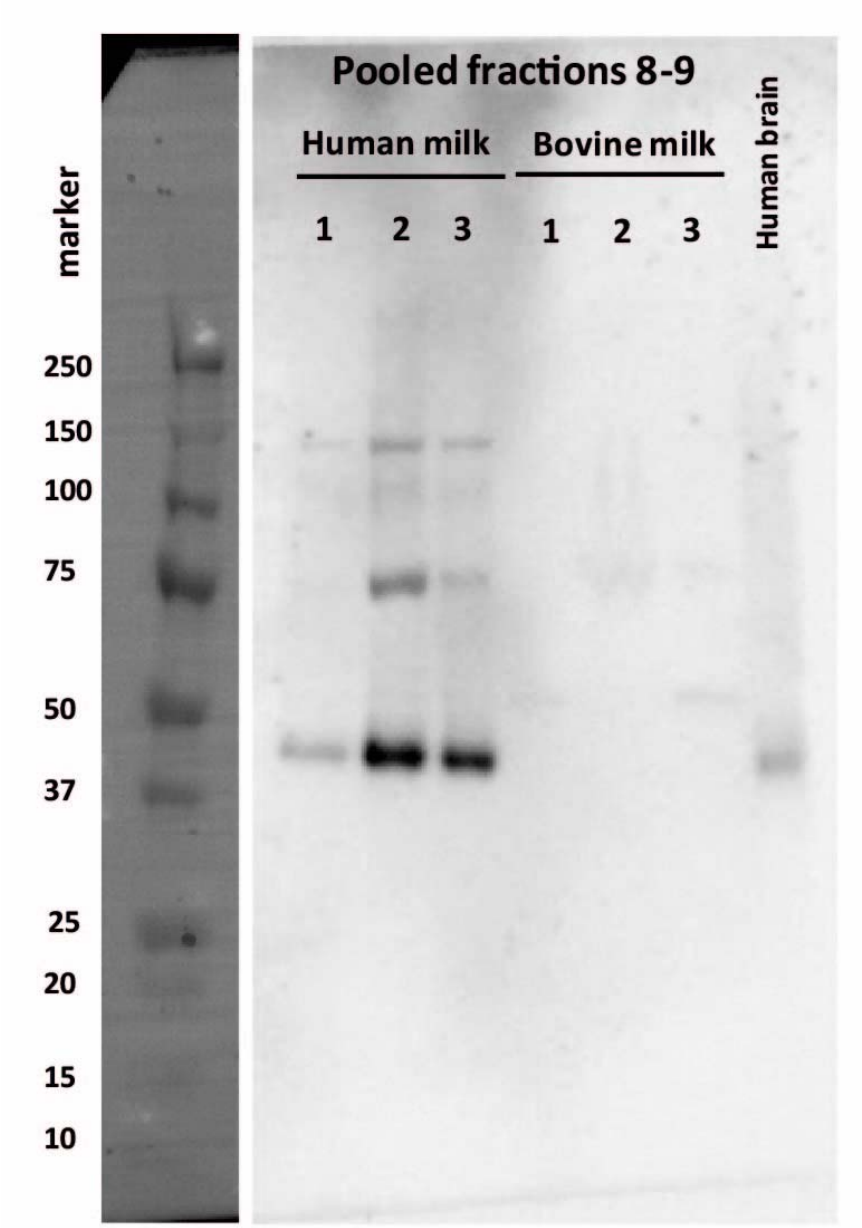
Human milk and bovine milk: 1% (vol/vol); *P<0.001

Supplemental Table 4. The coagulant activity of human milk preparations routinely used at neonatal ICUs

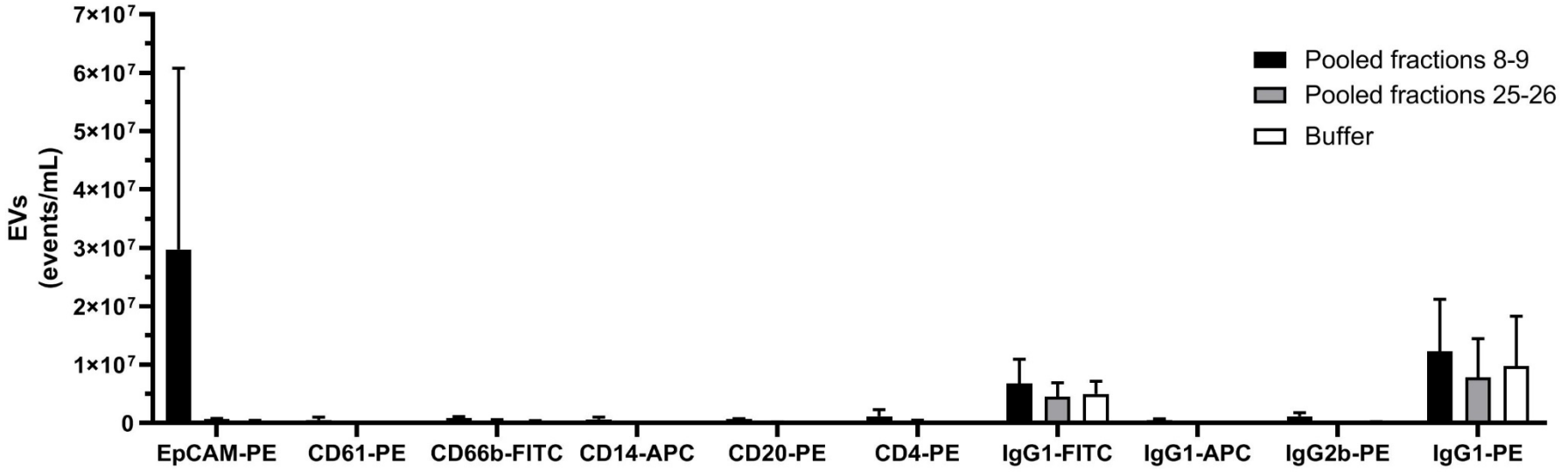
	Plasma clotting time (s)	P
Fresh human milk	141 ± 26	
+ fortifier	129 ± 33	0.121
after freeze/thaw	88 ± 25	0.002
after cooling	121 ± 28	0.091
Pasteurized pool donor human milk	1496 ± 305	< .001
Buffer	>3600	< .001

Supplemental figures

Supplementary Figure 1. Tissue factor associated with extracellular vesicles is not present in bovine milk. Bovine milk was fractionated by Sepharose 2B-size exclusion chromatography. The extracellular vesicle containing fractions were pooled and blotted for tissue factor (TF) protein. The blots also show human milk and human brain lysate as positive control.



Supplementary Figure 2. The cellular origin of extracellular vesicles from human milk. EVs were isolated from human milk by SEC as described in the methods section. The EV-containing fractions 8 and 9 were pooled. Pooled fractions 25 and 26 were used as negative and procedural control. EpCAM (CD326) was used for epithelial cells; CD66b for granulocytes; CD61 for platelets and megakaryocytes; CD14 for monocytes; CD20 for B cells; and CD4 for T cells. IgG1 and IgG2b were used to correct for binding of antibodies to Fc receptors exposed on EVs.



Supplementary Figure 3. A titration curve with Innovin (a reagent that is prepared from purified recombinant human tissue factor combined with synthetic phospholipids) was generated to estimate the tissue factor coagulant activity present in human milk.