

## **SUPPLEMENTARY MATERIAL**

### **Profiling of circulating immune factors and host-derived extracellular vesicles in response to bacterial or malaria acute fever**

Natalia Tiberti<sup>1\*</sup>, Giulia Bertoli<sup>1</sup>, Cristina Mazzi<sup>2</sup>, Elisabetta Vezzelli<sup>1</sup>, Serena Zanzoni<sup>3</sup>, Francesca Alladio<sup>1</sup>, Zeno Bisoffi<sup>1</sup>, Francesca Tamarozzi<sup>1</sup>, Chiara Piubelli<sup>1</sup>, Dora Buonfrate<sup>1</sup>, Federico Giovanni Gobbi<sup>1,4</sup>

<sup>1</sup> Department of Infectious, Tropical Diseases and Microbiology, IRCCS Sacro Cuore Don Calabria Hospital, Negrar di Valpolicella, Italy

<sup>2</sup> Clinical Research Unit, IRCCS Sacro Cuore Don Calabria Hospital, Negrar di Valpolicella, Verona, Italy

<sup>3</sup> Centro Piattaforme Tecnologiche, University of Verona, Verona, Italy

<sup>4</sup> Department of Clinical and Experimental Sciences, University of Brescia, Brescia, Italy

**Supplementary Table S1. Detailed description of co-morbidities across the studied population of bacterial and malaria AFI.**

|                                                 | <b>Bacterial (n=22)</b> | <b>Malaria (n=25)</b> |
|-------------------------------------------------|-------------------------|-----------------------|
| <b>Cardiovascular diseases</b>                  | 3 (13.6%)               | 14 (56%)              |
| <b>Metabolic disorders</b>                      | 0                       | 4 (16%)               |
| <b>Oncological diseases</b>                     | 6 (27.3%)               | 0                     |
| <b>Autoimmune diseases</b>                      | 4 (18.2%)               | 1 (4%)                |
| <b>Gastrointestinal diseases</b>                | 7 (31.8%)               | 1 (4%)                |
| <b>Gastrointestinal parasites and helminths</b> | 3 (13.6%)               | 3 (12%)               |
| <b>HIV</b>                                      | 2 (9.1%)                | 6 (24%)               |
| <b>Respiratory</b>                              | 2 (9.1%)                | 0                     |
| <b>Bacterial pneumonia</b>                      | 0                       | 1 (4%)                |
| <b>Influenza B virus</b>                        | 1 (9.1%)                | 0                     |
| <b>Epstein Barr Virus</b>                       | 1 (9.1%)                | 0                     |

**Supplementary Table S2. Demographic and main clinical characteristics of the control cohort with other infections.**

|                                                  | <b>TB (n=38)</b>   | <b>Par (n=9)<sup>a</sup></b> |
|--------------------------------------------------|--------------------|------------------------------|
| <b>Age (years), median [range]</b>               | 28 [18 - 67]       | 21 [19 - 35]                 |
| <b>Sex (F), n (%)</b>                            | 13 (34.2%)         | 2 (22.2%)                    |
| <b>Max body temperature (°C), median [range]</b> | 37.4 [36.4 - 40.9] | 36.4 [36 - 36.8]             |
| <b>LMIC, n (%)</b>                               | 38 (100%)          | 8 (88.9%)                    |
| <b>Continent of origin</b>                       |                    |                              |
| <i>Africa</i>                                    | 29 (76.3%)         | 8 (88.9%)                    |
| <i>Asia</i>                                      | 8 (21.1%)          | 0                            |
| <i>Europe</i>                                    | 0                  | 1 (11.1%)                    |
| <i>Latin America</i>                             | 1 (2.6%)           | 0                            |
| <b>Co-morbidities, yes (%)</b>                   | 13 (34.2%)         | 1 (11.1%)                    |
| <b>HIV, yes (%)</b>                              | 2 (5.3%)           | 0                            |
| <b>Delay<sup>b</sup>, number of days</b>         |                    |                              |
| 0                                                | 24 (63.2%)         | 9 (100%)                     |
| 1                                                | 10 (26.3%)         | 0                            |
| 2                                                | 4 (10.5%)          | 0                            |

<sup>a</sup> Gastrointestinal parasites and helminths: *S. mansoni* (n=3); *S. haematobium* (n=3), Hookworm; *G. duodenalis*; *E. vermicularis*

<sup>b</sup> Delay between patient's fever peak and collection of the serum sample analysed in the present study  
LMIC: lower middle income countries; na, not applicable

**Supplementary Table S3. Distribution of the concentration of all assessed markers in bacterial and malaria AFI.**

|                                              | Bacterial |         |                  | Malaria |         |                  | Bacterial/Malaria |                      |                      |
|----------------------------------------------|-----------|---------|------------------|---------|---------|------------------|-------------------|----------------------|----------------------|
|                                              | n         | Median  | Range            | n       | Median  | Range            | FC                | p-value <sup>a</sup> | q-value <sup>b</sup> |
| <b>Haematology</b>                           |           |         |                  |         |         |                  |                   |                      |                      |
| <b>WBC (cell x 10<sup>9</sup>/L)</b>         | 52        | 9.36    | 2.06 - 16.06     | 73      | 5.00    | 2.30 - 8.80      | <b>1.87</b>       | <0.001               | <b>&lt;0.001</b>     |
| RBC (cell x 10 <sup>12</sup> /L)             | 52        | 4.29    | 3.86 - 4.60      | 73      | 4.50    | 4.10 - 4.83      | 0.95              | 0.030                | 0.054                |
| Lymphocytes (cell x 10 <sup>9</sup> /L)      | 51        | 1.19    | 0.24 - 2.83      | 65      | 1.20    | 0.20 - 2.83      | 0.99              | 0.900                | >0.9                 |
| Monocytes (cell x 10 <sup>9</sup> /L)        | 51        | 0.48    | 0.10 - 1.14      | 65      | 0.50    | 0.10 - 1.20      | 0.96              | 0.900                | >0.9                 |
| <b>Neutrophils (cell x 10<sup>9</sup>/L)</b> | 51        | 7.10    | 0.83 - 13.73     | 65      | 3.09    | 0.60 - 6.90      | <b>2.30</b>       | <0.001               | <b>&lt;0.001</b>     |
| <b>Eosinophils (cell x 10<sup>9</sup>/L)</b> | 51        | 0.08    | 0.00 - 1.01      | 65      | 0.02    | 0.00 - 0.20      | <b>4.00</b>       | <0.001               | <b>&lt;0.001</b>     |
| Basophils (cell x 10 <sup>9</sup> /L)        | 51        | 0.02    | 0.00 - 0.10      | 65      | 0.01    | 0.00 - 0.10      | <b>2.00</b>       | 0.031                | 0.054                |
| <b>Platelets (cell x 10<sup>9</sup>/L)</b>   | 52        | 230.50  | 42.00 - 698.00   | 73      | 80.00   | 15.00 - 219.00   | <b>2.88</b>       | <0.001               | <b>&lt;0.001</b>     |
| Haemoglobin (g/dL)                           | 52        | 12.45   | 8.00 - 15.70     | 73      | 12.20   | 8.40 - 16.30     | 1.02              | >0.9                 | >0.9                 |
| <b>Acute phase reactants</b>                 |           |         |                  |         |         |                  |                   |                      |                      |
| <b>Fibrinogen (g/L)</b>                      | 48        | 6.25    | 3.39 - 11.40     | 63      | 4.18    | 2.68 - 7.60      | <b>1.50</b>       | <0.001               | <b>&lt;0.001</b>     |
| <b>ESR (mm/H)</b>                            | 51        | 62.00   | 13.00 - 120.00   | 58      | 47.00   | 15.00 - 103.00   | <b>1.32</b>       | 0.021                | <b>0.039</b>         |
| <b>PCT (pg/mL)</b>                           | 52        | 157.72  | 14.17 - 19870.01 | 73      | 768.64  | 34.86 - 19870.01 | <b>0.21</b>       | <0.001               | <b>&lt;0.001</b>     |
| <b>LDH (U/L)</b>                             | 51        | 196.00  | 96.00 - 1120.00  | 63      | 304.00  | 168.00 - 629.00  | <b>0.64</b>       | <0.001               | <b>&lt;0.001</b>     |
| CRP (mg/L)                                   | 49        | 130.56  | 1.00 - 342.31    | 73      | 158.26  | 4.98 - 376.87    | 0.82              | 0.500                | 0.600                |
| <b>Circulating immune factors</b>            |           |         |                  |         |         |                  |                   |                      |                      |
| <b>MMP-9 (ng/mL)</b>                         | 52        | 436.14  | 72.07 - 1488.59  | 73      | 285.73  | 56.18 - 968.70   | <b>1.53</b>       | <0.001               | <b>&lt;0.001</b>     |
| <b>MMP-2 (ng/mL)</b>                         | 52        | 154.99  | 98.21 - 283.84   | 73      | 128.48  | 81.72 - 309.90   | <b>1.21</b>       | <0.001               | <b>&lt;0.001</b>     |
| <b>VCAM-1 (ng/mL)</b>                        | 52        | 1424.44 | 586.65 - 6420.30 | 73      | 1803.42 | 429.85 - 6656.88 | <b>0.79</b>       | 0.017                | <b>0.033</b>         |
| <b>IL-4 (pg/mL)</b>                          | 52        | 23.44   | 6.14 - 135.64    | 73      | 42.44   | 6.14 - 149.51    | <b>0.55</b>       | <0.001               | <b>0.002</b>         |
| <b>IFN<math>\gamma</math> (pg/mL)</b>        | 52        | 18.36   | 0.29 - 368.86    | 73      | 38.22   | 0.29 - 2968.07   | <b>0.48</b>       | <0.001               | <b>&lt;0.001</b>     |
| <b>TNF<math>\alpha</math> (pg/mL)</b>        | 52        | 16.32   | 1.08 - 84.34     | 73      | 34.76   | 1.62 - 228.09    | <b>0.47</b>       | <0.001               | <b>&lt;0.001</b>     |
| <b>CXCL10 (pg/mL)</b>                        | 52        | 58.00   | 7.94 - 4291.50   | 73      | 140.28  | 10.99 - 3576.25  | <b>0.41</b>       | <0.001               | <b>&lt;0.001</b>     |

|                                      |    |        |                |    |        |                |             |        |                  |
|--------------------------------------|----|--------|----------------|----|--------|----------------|-------------|--------|------------------|
| IL-6 (pg/mL)                         | 52 | 16.10  | 0.06 - 124.10  | 73 | 12.32  | 0.06 - 814.68  | 1.31        | 0.200  | 0.300            |
| ICAM-1 (ng/mL)                       | 52 | 507.98 | 4.46 - 1253.78 | 73 | 508.57 | 1.40 - 1880.67 | 1.00        | 0.600  | 0.700            |
| NGAL (ng/mL)                         | 19 | 117.42 | 43.26 - 306.60 | 52 | 117.70 | 44.88 - 306.60 | 1.00        | 0.900  | >0.9             |
| TRAIL (pg/mL)                        | 52 | 32.05  | 1.93 - 142.58  | 73 | 39.72  | 4.68 - 159.04  | 0.81        | 0.062  | 0.100            |
| IL-8 (pg/mL)                         | 52 | 18.42  | 0.57 - 119.33  | 73 | 22.90  | 0.57 - 477.93  | 0.80        | 0.300  | 0.400            |
| OPN (ng/mL)                          | 52 | 33.66  | 5.63 - 126.93  | 73 | 42.06  | 7.22 - 350.47  | 0.80        | 0.045  | 0.074            |
| <b>EVs - group1 (normalised MFI)</b> |    |        |                |    |        |                |             |        |                  |
| CD63 <sup>+</sup>                    | 39 | 2.12   | 1.54 - 2.58    | 45 | 1.97   | 1.37 - 2.44    | 1.08        | 0.066  | 0.100            |
| <b>CD62P<sup>+</sup></b>             | 39 | 1.30   | 0.20 - 2.35    | 45 | 1.02   | 0.22 - 2.72    | <b>1.27</b> | 0.010  | <b>0.022</b>     |
| CD41b <sup>+</sup>                   | 39 | 1.32   | 0.38 - 2.64    | 45 | 1.03   | 0.28 - 3.18    | 1.28        | 0.100  | 0.140            |
| <b>CD81<sup>+</sup></b>              | 39 | 0.34   | 0.11 - 1.02    | 45 | 0.56   | 0.28 - 1.15    | <b>0.61</b> | <0.001 | <b>&lt;0.001</b> |
| <b>CD9<sup>+</sup></b>               | 39 | 0.46   | 0.25 - 0.79    | 45 | 0.40   | 0.12 - 1.22    | <b>1.15</b> | 0.011  | <b>0.023</b>     |
| <b>CD29<sup>+</sup></b>              | 39 | 0.37   | 0.14 - 0.66    | 45 | 0.27   | 0.13 - 0.69    | <b>1.37</b> | 0.011  | <b>0.023</b>     |
| <b>HLA-DR/DP/DQ<sup>+</sup></b>      | 39 | 0.13   | 0.03 - 0.88    | 45 | 0.43   | 0.13 - 1.47    | <b>0.30</b> | <0.001 | <b>&lt;0.001</b> |
| CD42a <sup>+</sup>                   | 39 | 0.30   | 0.10 - 0.83    | 45 | 0.28   | 0.09 - 0.86    | 1.07        | 0.400  | 0.500            |
| <b>CD8<sup>+</sup></b>               | 39 | 0.06   | 0.00 - 0.27    | 45 | 0.12   | 0.02 - 0.39    | <b>0.50</b> | 0.002  | <b>0.005</b>     |
| HLA-ABC <sup>+</sup>                 | 39 | 0.07   | 0.00 - 0.22    | 45 | 0.11   | 0.00 - 0.47    | 0.64        | 0.057  | 0.091            |
| <b>EVs - group2 (normalised MFI)</b> |    |        |                |    |        |                |             |        |                  |
| CD44 <sup>+</sup>                    | 39 | 0.04   | 0.00 - 0.30    | 45 | 0.05   | 0.01 - 0.12    | 0.80        | 0.400  | 0.500            |
| CD49e <sup>+</sup>                   | 39 | 0.04   | 0.01 - 0.09    | 45 | 0.03   | 0.00 - 0.13    | 1.33        | 0.083  | 0.120            |
| <b>CD24<sup>+</sup></b>              | 39 | 0.04   | 0.01 - 0.10    | 45 | 0.02   | 0.00 - 0.18    | <b>2.00</b> | <0.001 | <b>&lt;0.001</b> |
| <b>CD105<sup>+</sup></b>             | 39 | 0.03   | 0.00 - 0.12    | 45 | 0.05   | 0.02 - 0.11    | <b>0.60</b> | <0.001 | <b>0.002</b>     |
| <b>CD146<sup>+</sup></b>             | 39 | 0.01   | 0.00 - 0.11    | 45 | 0.03   | 0.00 - 0.31    | <b>0.33</b> | <0.001 | <b>&lt;0.001</b> |
| <b>CD40<sup>+</sup></b>              | 39 | 0.03   | 0.00 - 0.06    | 45 | 0.02   | 0.00 - 0.05    | <b>1.50</b> | 0.001  | <b>0.003</b>     |
| <b>CD31<sup>+</sup></b>              | 39 | 0.03   | 0.00 - 0.05    | 45 | 0.02   | 0.00 - 0.06    | <b>1.50</b> | 0.003  | <b>0.006</b>     |
| ROR1 <sup>+</sup>                    | 39 | 0.01   | 0.00 - 0.09    | 45 | 0.01   | 0.00 - 0.08    | 1.00        | 0.200  | 0.200            |
| CD14 <sup>+</sup>                    | 39 | 0.02   | 0.00 - 0.04    | 45 | 0.01   | 0.00 - 0.04    | 2.00        | 0.076  | 0.110            |

|                    |    |      |             |    |      |             |      |       |       |
|--------------------|----|------|-------------|----|------|-------------|------|-------|-------|
| CD133 <sup>+</sup> | 39 | 0.01 | 0.00 - 0.44 | 45 | 0.01 | 0.00 - 0.05 | 1.00 | 0.300 | 0.300 |
|--------------------|----|------|-------------|----|------|-------------|------|-------|-------|

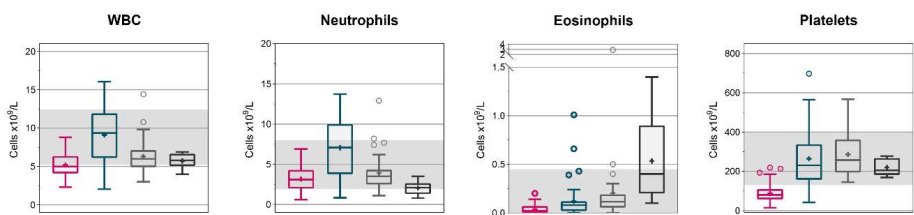
<sup>a</sup>Wilcoxon rank sum test; Wilcoxon rank sum exact test

<sup>b</sup>False discovery rate correction for multiple testing

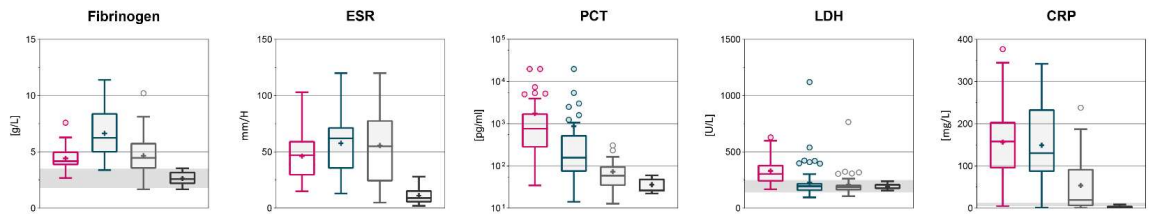
Definitions - WBC: white blood cells; RBC: red blood cells; SR: erythrocyte sedimentation rate; PCT: procalcitonin; LDH: lactate dehydrogenase; CRP: C reactive protein; OPN: osteopontin; PCT: procalcitonin; MFI: mean fluorescence intensity; FC: fold change.

**Supplementary Figure S1.** Tuckey box-plot showing the distribution of cells (a), acute phase reactants (b), circulating immune factor (c) and EVs (d) across the 4 groups investigated in the present study. Only markers significant for the comparison bacterial AFI vs. malaria AFI are reported. Grey areas represent reference ranges for healthy individuals for WBC, neutrophils, eosinophils, platelets, fibrinogen, LDH and CRP. ESR reference values were <34 mm/H for males and <38 mm/H for females up to 65 years old; or <37 mm/H for males and <45 mm/H for females above 65 years old.

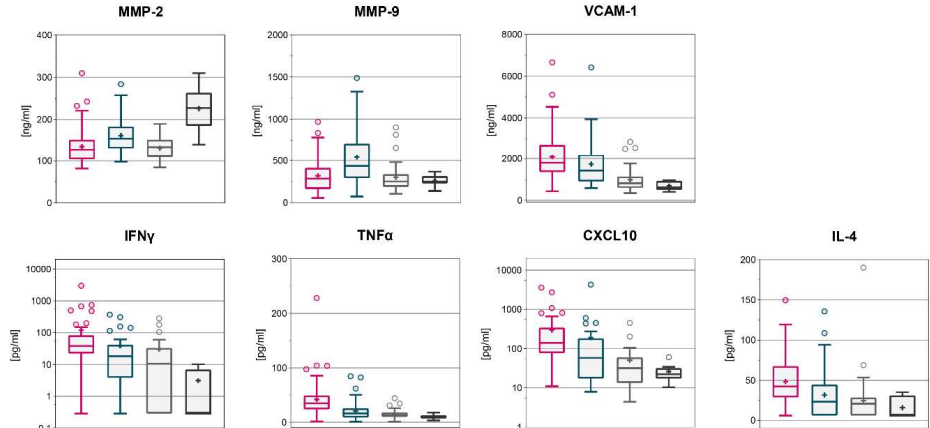
**a. Cells**



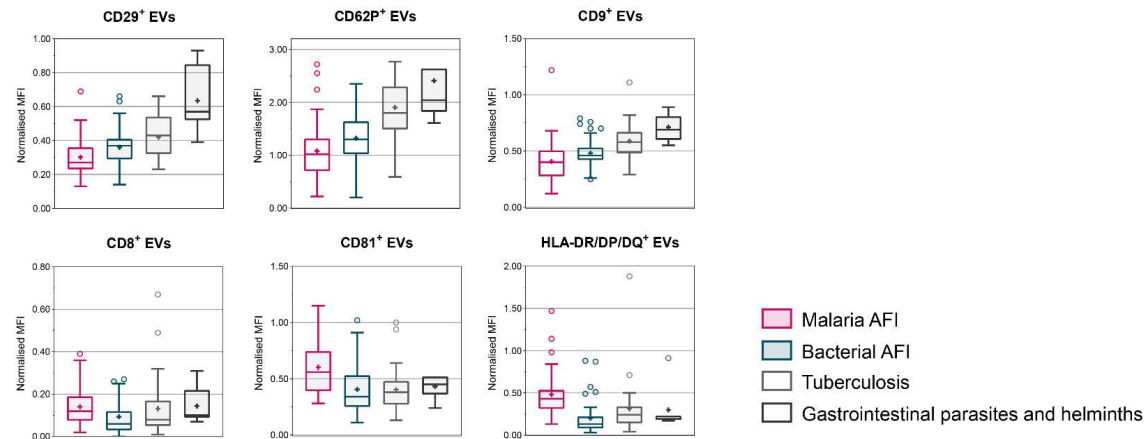
**b. Acute phase reactants**



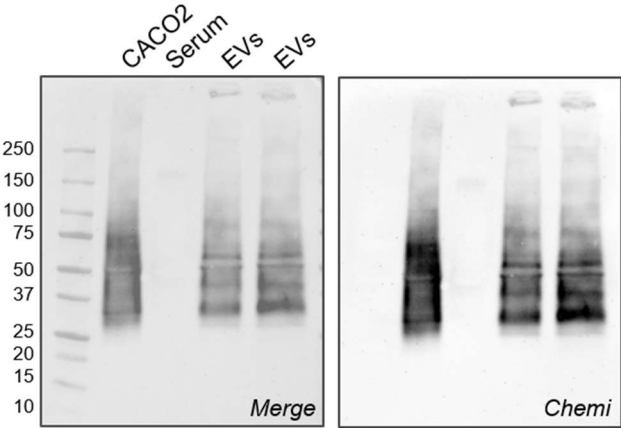
**c. Circulating immune factors**



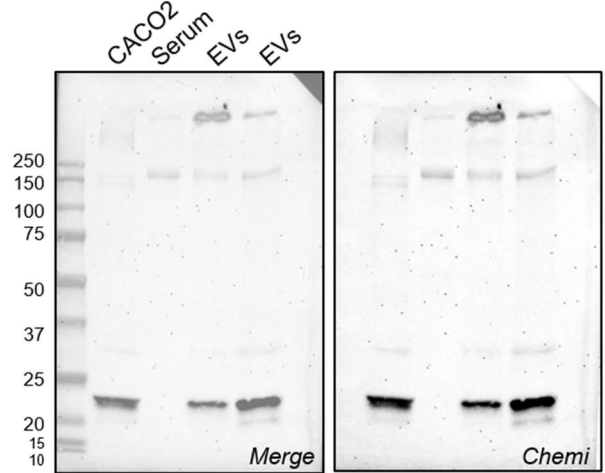
**d. Circulating EVs**



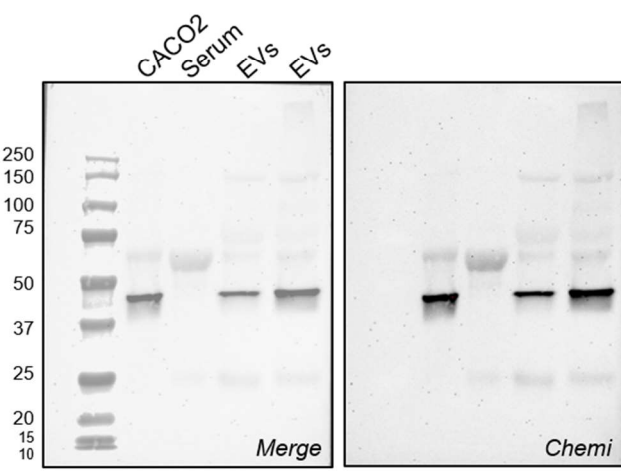
**Supplementary Figure S2.** Entire images of the western blot experiments presented in the main manuscript. For each protein target, both the merged colorimetric and chemiluminescent signals (merge) and only the chemiluminescent signal (chemi) are reported.



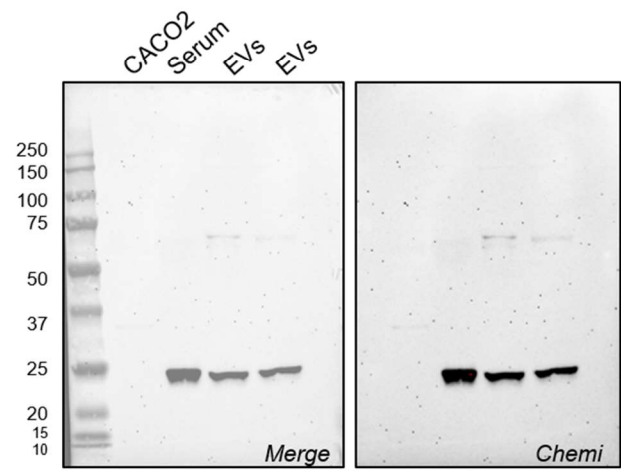
**CD63**, in 5% non-fat milk  
Anti-mouse-HRP, 1/1500  
Predicted size 30-60kDa  
Non-reducing conditions



**CD81**, in 5% non-fat milk  
Anti-mouse-HRP, 1/1500  
Predicted size 25kDa  
Non-reducing conditions



**Flotillin 1**, in 5% non-fat milk  
Anti-rabbit-HRP, 1/2000  
Predicted size 47kDa



**APO-AI**, in 5% non-fat milk  
Anti-rabbit-HRP, 1/2000  
Predicted size 27-31kDa