**Supplementary Table S1**. The detailed characteristics of the donkeys involved in the study

|  |  |  |
| --- | --- | --- |
| **N** | **Age (years)** | **BCS** |
| 1 | 2 | 4/5 |
| 2 | 4 | 4/5 |
| 3 | 3 | 3/5 |
| 4 | 12 | 3/5 |
| 5 | 19 | 3/5 |
| 6 | 2 | 4/5 |
| 7 | 4 | 4/5 |
| 8 | 13 | 3/5 |
| 9 | 3 | 3/5 |
| 10 | 12 | 3/5 |
| 11 | 5 | 3/5 |
| 12 | 19 | 3/5 |
| 13 | 2 | 4/5 |
| 14 | 4 | 4/5 |
| 15 | 13 | 3/5 |
| 16 | 19 | 3/5 |
| 17 | 3 | 3/5 |
| 18 | 19 | 3/5 |
| 19 | 2 | 4/5 |
| 20 | 19 | 3/5 |
| 21 | 4 | 4/5 |
| 22 | 19 | 3/5 |
| 23 | 5 | 3/5 |
| 24 | 19 | 3/5 |
| 25 | 5 | 3/5 |
| 26 | 19 | 3/5 |

**Materials**

The materials were used as received and are listed in the following table along with the corresponding suppliers:

|  |  |
| --- | --- |
| **Materials** | **Company** |
| n-Hexane 95% | TITOLCHIMICA, Pontecchio Polesine (Ro) Italy |
| Methyl alcohol HPLC | TITOLCHIMICA Pontecchio Polesine (Ro) Italy |
| Chloroform extra pure 99.5% | TITOLCHIMICA Pontecchio Polesine (Ro) Italy |
| PBS pH 7,4 RS | Carlo Erba, Milan ( Italy) |
| Polar Lipid Mixture (quantitative) | MATREYA LLC State College, PA, USA |
| non-Polar Lipid Mixture B (quantitative) | MATREYA LLC State College, PA,USA |
| Phosphatidylserine | MATREYA LLC State College, PA, USA |
| L-𝛼-Phosphatidylcholine | Merck, Darmstadt, Germany |
| ALUGRAM Xtra sheets 200x200mm | Carlo Erba, Milan Italy |
| Potassium hydroxide, pellets RPE - For analysis | Carlo Erba, Milan Italy |
| Sodium sulfate anhydrous RS - For anhydrification | Carlo Erba, Milan Italy |
| C14:0 – myristic acid methyl ester | Merck, Darmstadt Germany |
| C16:0 – palmitic acid methyl ester | Merck, Darmstadt Germany |
| C16:1 – palmitoleic acid methyl ester | Merck, Darmstadt Germany |
| C18:0 – stearic acid methyl ester | Supelco, Bellefonte, PA, USA |
| 9c, C18:1 – oleic acid methyl ester | Merck, Darmstadt, Germany |
| 11c, C18:1 – vaccenic acid methyl ester | Supelco, Bellefonte, PA ,USA |
| LA omega-6 – C18:2 – linoleic acid methyl ester | Merck, Darmstadt Germany |
| DGLA omega-6 – C20:3 dihomogammalinolenic acid methyl ester | Merck, Darmstadt Germany |
| ARA omega-6 – C20:4 – arachidonic acid methyl ester | Merck, Darmstadt Germany |
| EPA omega-3 – C20:5 – eicosapentaenoic acid methyl ester | Supelco, Bellefonte, PA, USA |
| DPA omega-6 – C22:5 – Docosapentenoic acid methyl ester | Merck, Darmstadt Germany |
| DHA omega-3 – C22:6 – docosahexaenoic acid methyl ester | Merck, Darmstadt Germany |
| Supelco 37 component FAME mix | Supelco, Bellefonte, PA, USA |

# GC analysis of FAME – Calibration procedure

For this study we chose to study a cluster of 12 fatty acids, which also corresponds to chromatographic peak areas >97%. This cluster consists of: 3 saturated fatty acids (SFA: myristic, palmitic and stearic acids); 3 monounsaturated fatty acids (MUFA, palmitoleic, oleic and cis-vaccenic acids); 4 polyunsaturated fatty acids omega-6 (PUFA, linoleic, dihomo-gamma linolenic, arachidonic, docosapentenoic acids); 2 polyunsaturated fatty acids omega-3 (PUFA, eicosapentaenoic and docosahexaenoic acids) as shown in Table 1 of the main text.

# Taking into account the previously reported benchmark for membrane fatty acid profile [15] we proceeded with the quantitation of the fatty acids was carried out by calibration procedures, for which the following protocol has been followed:

# initially a *n*-hexane (HPLC grade, Titolchimica) 5mM solution of stearic acid methyl ester (2 mg in 1340 μL) was prepared and 1μl was directly injected to the Agilent 7890B GC system equipped with a flame ionization detector and a DB-23 (50%-Cyanopropyl)-methylpolysiloxane capillary column (60 m, 0.25 mm i.d., 0.25 μm film thickness). The following oven conditions were established to be kept for all the analyses: the initial temperature was 165 °C, held for 3 min, followed by an increase of 1 °C/min up to 195 °C, held for 40 min, followed by a second increase of 10 °C/min up to 240 °C, held for 10 min. The carrier gas was hydrogen, held at a constant pressure of 16.482 psi. The injections were repeated in triplicates.

# The second round of injections for calibration was then performed with 0.5 mM solution of the same fatty acid methyl ester (taking 100μL of the initial solution and diluting with 900μL of *n*-hexane), injecting 1 μL as previously described for triplicates.

# The same protocol was carried out using dilutions of 0.05mM, 0.005mM and 0.0005mM of stearic acid methyl ester.

# In all the injections a calibration curve was created using the software of the GC equipment (Agilent 8890B GC system). Using the concentration of 0.0005mM for methyl stearate, the corresponding peak area was detectable but not quantifiable, indicating this concentration as the limit of detection (LOD) of the specific GC system (<0.5nM). The same protocol has been followed for all the fatty acids of the cohort.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Reference FAMEs** | | | | |
| **SFA** | **C16:0** |  |  |  |
| **C18:0** |  |  |  |
| **MUFA** | **9c,C16:1** |  |  |  |
| **9c,C18:1** |  |  |  |
| **11c,C18:1** |  |  |  |
| **PUFA omega-3** | **EPA** |  |  |  |
| **DHA** |  |  |  |
| **PUFA omega-6** | **LA** |  |  |  |
| **DGLA** |  |  |  |
| **ARA** |  |  |  |

**Supplementary Figure S1**. Calibration curves of the 10 fatty acids chosen as representatives of the SFA, MUFA and PUFA families present in the erythrocyte membrane phospholipids.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Distribution FAME from Spermatozoa** | | | | | |
|  | | | | | |
| **SFA** | | | | | |
| **14:0** | | **16:0** | | **18:0** | |
|  | | | | | |
| **MUFA** | | | | | |
| **9c,C16:1** | | **9c,C18:1** | | **11c,C18:1** | |
|  | | | | | |
| **PUFA** | | | | | |
| PUFA omega-6 | | | | | |
| **LA** | **GLA** | | **ARA** | | **DPA** |
| PUFA omega-3 | | | | | |
| **EPA** | | | **DHA** | | |

**Supplementary Figure S2**. Distribution of the values in the population of healthy donkeys (n = 26) for each of the fatty acids using obtained from spermatozoa membranes (data are reported in Table 2 in the main text).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Individual Fatty Acids** | | | **Sum of families** |
| **SFA** | **C14:0** | **C16:0** | **C18:0** | **Total SFA** |
| **MUFA** | **9c,C16:1** | **9c,C18:1** | **11c,C18:1** | **Total MUFA** |
| **PUFA-ω3** | **EPA** | **DHA** |  | **PUFA omega-3** |
| **PUFA-ω6** | **LA** | **DGLA** | **ARA** | **PUFA omega-6** |
| **DPA** |  |  |

**Supplementary Figure S3**. Spearman correlation with linear regression and parameters for healthy donkeys (n=26) using age and each fatty acid type and family obtained from spematozoa membranes (data are reported in Table 2 in the main text). Each member of the fatty acid family is given in a row, the last column being the sum of the corresponding fatty acid family.

|  |  |  |
| --- | --- | --- |
| **LIPID Indexes** | | |
| **Total PUFA** | **omega-6/omega-3 ratio** | **PUFA Balance** |
| **SFA/MUFA ratio** | **Unsaturation Index (UI)** | **Peroxidation Index (PI)** |

**Supplementary Figure S4**. Spearman correlation with linear regression and parameters for healthy donkeys (n=26) using age and lipid indexes obtained from erythrocyte membranes (data are reported in Table 2 in the main text). 1st row: total PUFA, omega-6/omega-3 and PUFA balance ratios; 2nd row: SFA/MUFA ratio, unsaturation and peroxidation indexes.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Individual Fatty Acids** | | | **Sum of families** |
| **SFA** | **C14:0** | **C16:0** | **C18:0** | **Total SFA** |
| **MUFA** | **9c,C16:1** | **9c,C18:1** | **11c,C18:1** | **Total MUFA** |
| **PUFA-ω3** | **EPA** | **DHA** |  | **PUFA omega-3** |
| **PUFA-ω6** | **LA** | **DGLA** | **ARA** | **PUFA omega-6** |
| **DPA** |  |  |

**Supplementary Figure S5**. Spearman correlation with linear regression and parameters for healthy donkeys (n=26) using motility (%) and each fatty acid type and family obtained from spematozoa membranes (data are reported in Table 2 in the main text). Each member of the fatty acid family is given in a row, the last column being the sum of the corresponding fatty acid family.

|  |  |  |
| --- | --- | --- |
| **LIPID Indexes** | | |
| **Total PUFA** | **omega-6/omega-3 ratio** | **PUFA Balance** |
| **SFA/MUFA ratio** | **Unsaturation Index (UI)** | **Peroxidation Index (PI)** |

**Supplementary Figure S6**. Spearman correlation with linear regression and parameters for healthy donkeys (n=26) using motility (%) and lipid indexes obtained from erythrocyte membranes (data are reported in Table 2 in the main text). 1st row: total PUFA, omega-6/omega-3 and PUFA balance ratios; 2nd row: SFA/MUFA ratio, unsaturation and peroxidation indexes.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Individual Fatty Acids** | | | **Sum of families** |
| **SFA** | **C14:0** | **C16:0** | **C18:0** | **Total SFA** |
| **MUFA** | **9c,C16:1** | **9c,C18:1** | **11c,C18:1** | **Total MUFA** |
| **PUFA-ω3** | **EPA** | **DHA** |  | **PUFA omega-3** |
| **PUFA-ω6** | **LA** | **DGLA** | **ARA** | **PUFA omega-6** |
| **DPA** |  |  |

**Supplementary Figure S7**. Spearman correlation with linear regression and parameters for healthy donkeys (n=26) using Low and High progressive motility (%) and each fatty acid type and family obtained from spematozoa membranes (data are reported in Table 2 in the main text). Each member of the fatty acid family is given in a row, the last column being the sum of the corresponding fatty acid family.

|  |  |  |
| --- | --- | --- |
| **LIPID Indexes** | | |
| **Total PUFA** | **omega-6/omega-3 ratio** | **PUFA Balance** |
| **SFA/MUFA ratio** | **Unsaturation Index (UI)** | **Peroxidation Index (PI)** |

**Supplementary Figure S8**. Spearman correlation with linear regression and parameters for healthy donkeys (n=26) using Low and High progressive motility (%) and lipid indexes obtained from erythrocyte membranes (data are reported in Table 2 in the main text). 1st row: total PUFA, omega-6/omega-3 and PUFA balance ratios; 2nd row: SFA/MUFA ratio, unsaturation and peroxidation indexes.