Extracellular vesicles (EVs) are small vesicles produced by eukaryotic and prokaryotic cells, surrounded by a bilayer membrane and carrying many bioactive molecules. EVs are of great interest because of their important roles in cell communication, epigenetic regulation and possible application in disease diagnosis and therapeutic strategies. The isolation and separation of EVs usually involve complex and multi-step procedures that result in a heterogenous fraction of EVs of unconfirmed origin. The overlapping range of size, similar morphology and variable composition challenge precise separation of EV subpopulations. **The new analytical methods for isolation, separation and characterisation of EVs are essential to shed light on their structural diversity, physiological and pathological roles, and future diagnostic and therapeutic use.**

This project aims to explore the possibilities arising from the application of different modes of one- and two-dimensional high-performance liquid chromatography (HPLC) in EVs isolation and fractionation. Robust and efficient high-performance liquid chromatography techniques especially when directly coupled to mass spectrometry can accelerate basic research and quality control of drug formulations containing EVs.

Within the frame of the project it is planned to i) **develop a new HPLC method based on hydrophobic interaction chromatography (HIC) mode for effective isolation and separation of EV subpopulations; ii) develop two-dimensional HPLC methods involving two of three orthogonal separation modes based on hydrophobicity, size and ionic properties for comprehensive separation of EV fractions; iii) develop LC-Q-TOF method to study lipid composition of the EV fractions.** For the development of the method, human serum EVs and Cyanobacterial EVs will be used as biological samples. EV subpopulation fractions will be characterized with state-of-the-art techniques such as electron microscopy, western blot and nanoparticle tracking analysis by expertise collaborators.

**The main innovation of the project is** the introduction and evaluation of the applicability of a novel separation mechanism for EVs isolation and separation – HIC, that will **separate EVs based on their differences in membrane lipid composition, and thus their biogenesis – that is not currently possible with the conventional methods employed in the field of EV research.** The use of HIC is a new concept for separation of native EVs based on the differences in the hydrophobicity of their membrane. Conventionally, HIC is used for the separation, isolation and purification of proteins. Potential differences in lipid composition of EVs population may enable their fast and convenient separation and isolation. The ability of the HIC to separate EVs from other components of biological fluid and cell medium will be also assessed.

In the next stage of the project, it is planned to increase the resolving power of the 1D-LC HIC method, by implementing the offline 2D-LC technique, thus allowing for detailed insight into the heterogenicity of EVs populations, **not currently possible with the use of conventional methods.** Combinations of chromatographic modes will be tested to evaluate and compare the resolution of 1D and 2D modes for EVs subpopulation analysis.

The impact of chromatographic conditions on the resolution and selectivity for EVs analysis with the use of one- and two-dimension HPLC will be investigated and compared. The resolving power of the developed methods will be compared to the commonly used methods such as ultracentrifugation.

Additionally, The EV fractions obtained during one- and two-dimensional LC analyses will be analysed by high-performance liquid chromatography coupled to high-resolution mass spectrometry in a lipidomic manner to study the exact lipid composition of EV subpopulations. This will be achieved by the development of lipidomic methods allowing to obtain high lipidome coverage of EVs. Since the lipid composition of EVs and their biological function remains relatively unclear, it is of great importance to provide a tool allowing for lipidomic analysis of particular EV subpopulations instead of the total EVs

**It is expected that the tools developed in this project will actively contribute to getting deeper insights into EVs composition and biology, becoming well established and broadly applied methodologies for the isolation and separation of EVs subpopulations, and their further analysis.**