MALDI-nanochip based Screening of Exosomal Biomarkers: Application to Cancer Diagnostics
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1. Overview
We previously demonstrated we could rapidly distinguish fluorouracil resistant cancer sample groups based on protein profiling of extracellular vesicles using a linear benchtop MALDI TOF instrument [1]. The aim of this follow up work is to identify the proteins that are differentially expressed in the different sample groups in order to better understand the disease processes and to support the rapid screening approach developed previously.

Here we present the results from this study using a high performance reflection MS/MALDI-TOF platform (Fig. 1) for the comparative proteomic profiling of circulating extracellular vesicles (EV) extracted from plasma samples of patients with colorectal cancer, postoperative colorectal cancer patients, IBD patients and a healthy control group in view of liquid biopsy applications (as a potential application for liquid biopsy oncological diagnosis).

2. Introduction
Exosomes are small cell-derived vesicles (50-150 nm) which are increasingly recognised as a promising source of circulating biomarkers for non-invasive diagnostics from bodily fluids (liquid biopsy). MALDIMS profiling of exosomal proteins was demonstrated as being capable to detect cancer-cell specific molecular signatures which can be used to differentiate between cancer types and stages as well as different grades of chemoresistance of cancer cells [1, 2]. This distinguishes MALDIMS as promising tool for application in liquid biopsy based cancer diagnostics.

Here the identification of the disease-related exosomal biomarkers represents a challenging task. Here we present a MALDI-nanochip platform in combination with bioinformatics data analysis for the comparative profiling and detection of exosomal proteins as potential cancer biomarkers.

3. Methods
Blood samples were prepared according to standard methods and exosomes were isolated using sequential (ultra)centrifugation. Proteins were solvent-extracted, dried under vacuum and stored at -80°C before analysis. Proteins were directly analysed and subsequently subjected to tryptic digestion after application to the MALDI-nanochip (Tetris) (Fig. 1). On-chip digests were dried, washed and covered with 0.5 μL CHCA in ACN:2:5:7:TFA = 70:30 (v/v). Alternatively, samples were digested in solution, desalted using C18-ZipTips and applied to FlexMass-DS targets (Shimadzu). For protein profiling the AXIMA-Performance (Shimadzu) instrument was used. Protein identification was performed using the MALDI-TOF MALDI-TOF/TOF spectrometer (Shimadzu) with Mascot protein database search (Swiss/Uniprot). Statistical analysis was performed using Clover MS Data Analysis (Clover Biosoft) and eMSTAT (Shimadzu) software.

4. Results
40 plasma samples from patients with colon cancer (CRC) preop (operative), inflammatory bowel disease (IBD) and healthy controls were used for evaluation. First, protein extracts were analysed by MALDI-MS in the range of m/z 2000-20000 which has previously been shown to contain most informative peaks of exosomes [2]. A comparison of the mass spectra showed distinct differences particularly in the range above m/z 8000 after exosome isolation. PLS-DA of the whole dataset recorded on the MALDI-nanochip showed a good clustering and separation of the samples belonging to the four study groups (Fig. 2).

Next, tryptic digests of the individual samples were subjected to peptide mass fingerprinting (PMF) in order to identify the discriminatory peptide peaks between the study groups based on multivariate data analysis (Fig. 3) and MS/MS analysis

5. Discussion
The work presents a MALDI-nanochip based protein profiling and identification workflow for the analysis of exosomal proteins as potential clinical biomarkers. Using bioinformatics, data analysis of mass spectral features, samples from patients with colon cancer, IBD and healthy subjects could be clearly separated. The peak intensities were found to vary greatly depending on the method of sample cleanup. By using the Tetris slide we were able to remove some of the more abundant proteins which are usually detected using ZipTip cleanup and discover otherwise undetected discriminating peaks. This looks promising to establish a high-throughput screening platform for clinical purposes (e.g. cancer diagnostics) in the future. We now aim to develop this method further to support patient group differentiation based on putative marker peaks with confident protein identifications.

6. References

Figure 1 – Overview of the sample preparation and analysis workflow.

Figure 2 – Representative protein MALDI mass spectra of selected patient samples of the four study groups (A-D) recorded before and after protease digestion. PLS-DA plots of all patients of the study groups recorded using (iii) standard target slide and (iv) MALDI-nanochip sample processing.

Figure 3 – Comparison of MS spectra illustrating several peaks where the signal intensity has been markedly enhanced when using Tetris slide sample washing (red) versus Zip-Tip™ sample cleaning (blue).

was then performed on the discriminating peaks to identify the digested proteins using Mascot. From in-solution digests of more abundant plasma proteins (e.g. alpha-1-antitrypsin, immunoglobulin heavy alpha 1, haptoglobin, fibrinogen gamma chain, etc.) were identified in selected samples of the study groups. These proteins were also found in the MALDI-nanochip processed samples but they showed no differentiation between the study groups. In contrast, several

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