COMPREHENSIVE DISCOVERY LIPIDOMIC WORKFLOW WHICH UTILIZES A NOVEL, MULTI-REFLECTING TOF WITH INTEGRATED INFORMATICS, PROVIDING HIGHLY CONFIDENT LIPID CHARACTERIZATION AND QUANTIFICATION

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INTRODUCTION

Despite developments in analytical technology the detection and identification of lipids remains a significant challenge. To streamline the process of data acquisition and analysis we have developed a workflow which combines the attributes of the Xevo™ MRT Mass Spectrometer (Figure 1), with data analysis powered by the Mass Analytica™ Lipostar2 software. The Xevo MRT is a novel next generation QTof- combining multi-reflecting technology with a ESI source. This instrument utilise technology and learning from the SELECT SERIES™ MRT. It is applicable for a wide range of applications with a specific focus on metabolomics and lipidomics.

Lipids play a crucial role in various cellular functions, such as survival, proliferation, and death. They are involved in storing chemical energy, cellular signaling, constructing cell membranes, and facilitating cell-to-cell interactions in tissues. These processes are closely linked to the development of cancer, including transformation, progression, and metastasis. Colorectal cancer (CRC) (Figure 2), also known as bowel, colon and rectal cancer is the 2nd leading cause of cancer death worldwide and 3rd most common cancer in the US [1]. The benefits of the new workflow approach are demonstrated using the lipidomics data acquired in DIA mode derived from the analysis of plasma samples from colorectal cancer (CRC) and healthy control plasma. We highlight the key features and benefits of the Xevo MRT coupled to UPLC™ combined with Lipostar2 software. The resulting data showed dysregulation of the lipid metabolism pathways based on CRC as well as cancer type.

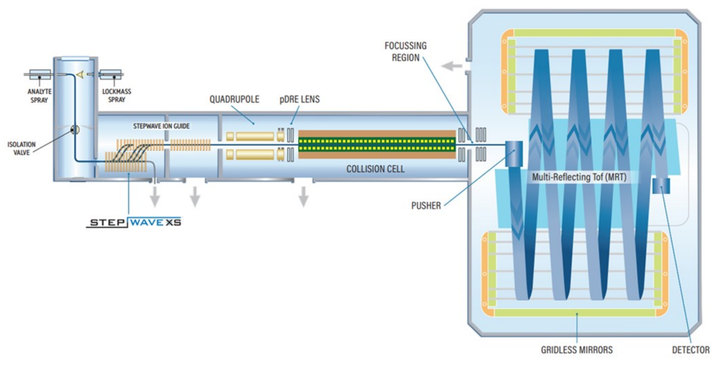


Figure 1. Waters Xevo MRT Mass Spectrometer schematic showing the new gas cell and transfer optics, the gridless TOF design and the new detector

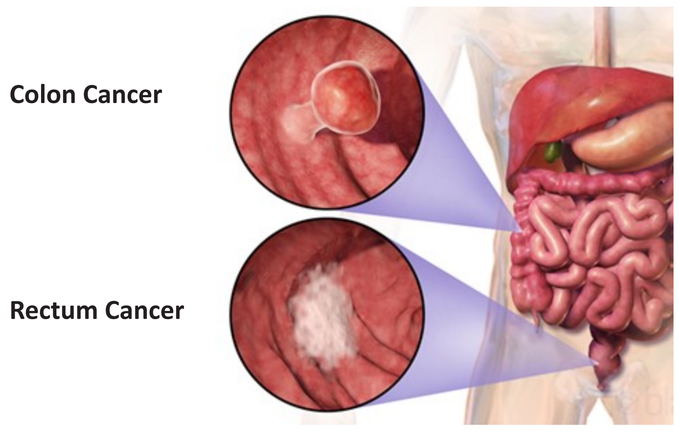


Figure 2. Location and examples of colorectal cancer tumors ie. colon and rectum cancer

XEVO MRT KEY FEATURES

- **New gas cell & transfer optic design enables simultaneous high sensitivity & resolution**
- **Gridless TOF design enables a highly folded 4m flight path in compact 0.65m x 0.4m analyzer allowing for resolution up to 100k (FWHM)**
- **Long life detector ensures stable response over long time periods and large ion currents enabling the analysis of big sample cohorts**
- **Acquisition system samples and processes data at an effective sampling rate of 60Gb/s enabling the analysis of complex matrices**

DATA TRANSFER

The Xevo MRT MS is operated using waters_connect software platform. Data can be transferred using to Lipostar2 using the application program interface or as mzML files for added flexibility (Figure 3).

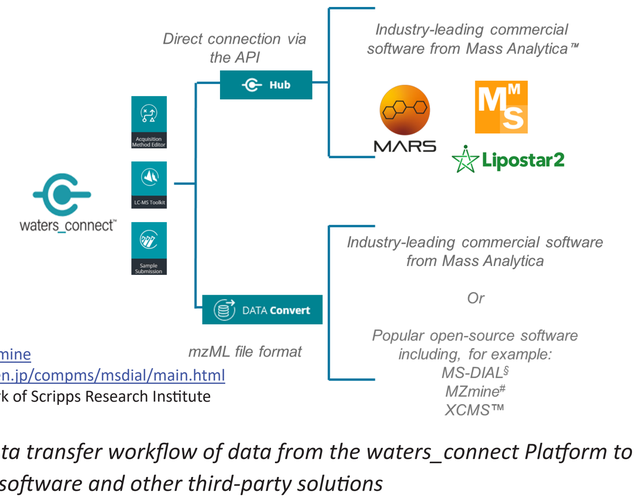
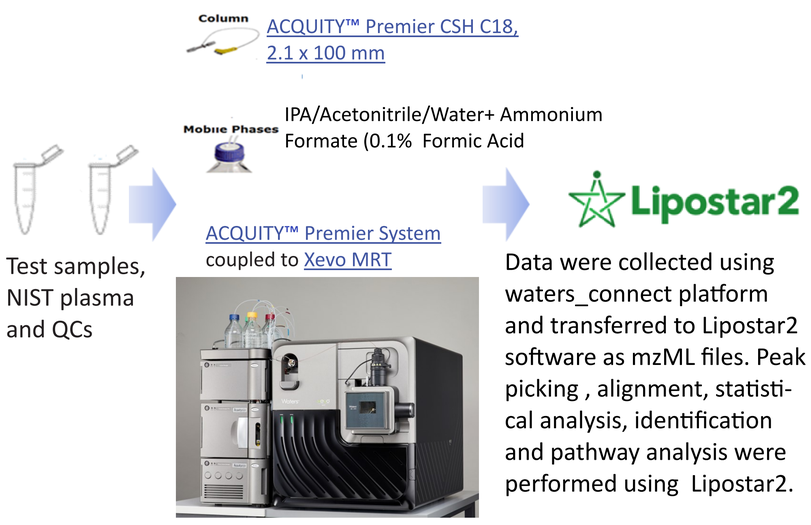


Figure 3. The data transfer workflow of data from the waters_connect Platform to Mass Analytica software and other third-party solutions

METHODS

- **High throughput Reversed-Phase Lipid profiling method for large samples sets gradient and MS conditions (12 min run time)** [2]
- **Mobile phases:**
  - A: 600:400 (Acetonitrile: Water) 10mM Ammonium Formate, 0.1% Formic Acid
  - B: 900:100 (IPA: Acetonitrile) 10mM Ammonium Formate, 0.1% Formic Acid
- **ACQUITY Premier UPLC I-Class and ACQUITY Premier UPLC CSH C18 (2.1 x 100 mm, 1.7 µm**
- **Xevo MRT | Multi Reflecting Time of Flight Mass Spectrometer | Waters**
- **MS^E data acquisition** [3,4] **Continuum**
- **10Hz scan speed**
- Human Plasma Pilot study samples (Innovative Research, Novi, MI, USA
  - 6 Health Patients
  - 6 CRC Patients (4 Colon and 2 Rectum)
- Test samples and QCs prepared in duplicate using a simple protein precipitation method described by Sarafin *et al*., 2014 [5], where 25µL plasma were transferred to Eppendoff tubes followed by 125 µL IPA of 200 -fold dilution EquiSPLASH LIPIDOMIX™ (Avanti, Birmingham, AI USA)
- Samples injected in triplicate in a randomized order (NIST SRM plasma (Sigma Aldrich, Poole, UK and Study pool QC samples injected every 6 sample injections)



CONCLUSION

- **Combination of reversed–phase UHPLC, high-resolution mass spectrometry and an intelligent workflow driven software allows for the rapid lipidomic analysis of biological samples**
- **Robust performance at speed in a compact footprint**
- **Excellent mass accuracy for improved identification confidence**
- **Lipostar2 software provides a streamline, flexible solution for handling waters connect LC-MS data sets (DDA or DIA)**
- **Extensive statistical analysis and pathway tools using Lipostar2 software to enable biomarker discovery and novel biological interpretation**

RESULTS

The validated supervised models of Controls vs CRC samples with and without QC samples as well as stratification based on CRC type, colon, and rectum are shown in Figure 4. The QCs are shown in red, they tightly cluster in the center of the model demonstrating good reproducibility over the analysis (Figure 4). When the QC samples (including NIST Plasma) were removed clear separation of the healthy controls and CRC is observed. Colon cancer and rectum cancer samples could also be separated when the QCs are removed since PLS-DA seeks to find the maximum separation between groups. The resulting Multivariate Analysis (MVA) statistical models were interrogated using a various tools such as Loading Plot, S plot and VIP scores (Figure 5). The Anova T tests is used to determine whether there is a statistical difference between averages of two groups. Cer(36:1) adducts are highlighted as being significantly different between healthy controls and CRC samples. A description of the Lipostar2 identification workflow is discussed in detail by Goracci *et al*., 2017 [6]. Briefly Lipostar2 uses a rule-based approach to generate theoretical fragments for lipid structures and simple traffic light system show lipid identification confidence. Figure 6 shows an example identified ceramide with low mass error and good fragmentation thus is assigned a green color in Lipostar2. Identifications with low fragmentation scores are assigned an amber color while those that are unidentified are red.

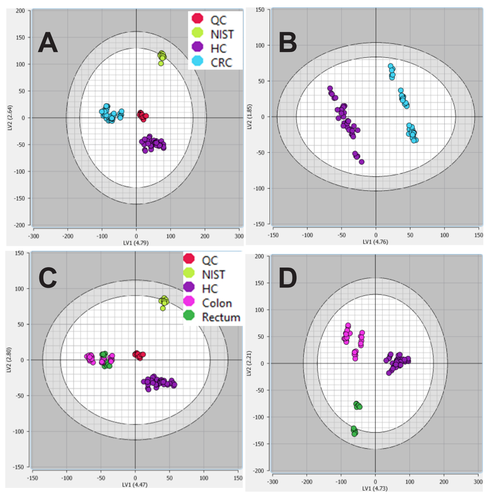


Figure 4. PLS-DA models of Controls vs CRC patients including NIST and study reference QCs (A) and with QCs removed from model (B). PLS-DA models of Controls vs Colon vs Rectum patients including NIST and study reference QCs (C) and with QCs removed from model (D)

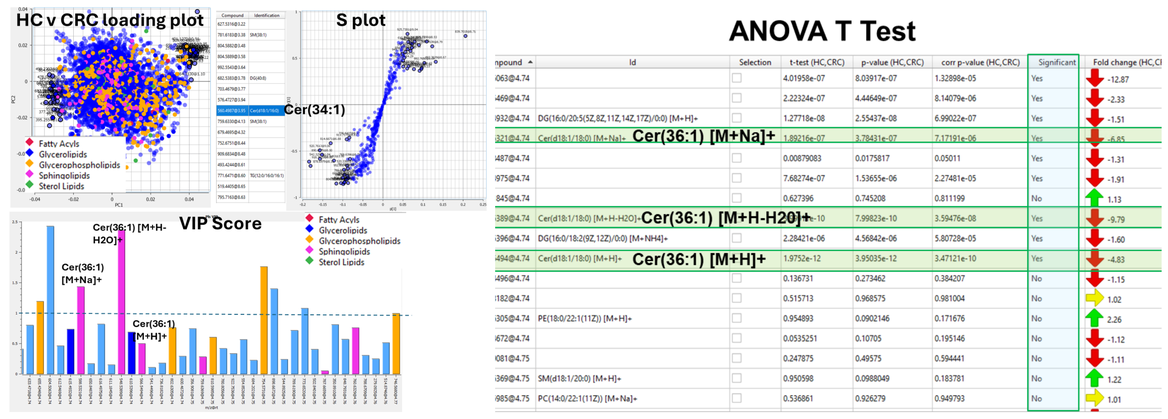


Figure 5. Various tools such as the loading plot, S Plot and VIP scores were used to interrogate the most relevant features driving clustering of sample cohort groups. Identified features can be coloured based on lipid class. The ANOVA T-Test were used to determine which lipids were significantly different between healthy controls and CRC samples.

DISCUSION

The box and whisker plots of ceramide and lysophoscholine lipids that have been reported to be involved with CRC dysregulation according to various peer reviewed publications [7, 8]as well as our findings from the MVA of samples are shown in Figure 7. Identified lipids are linked to the various pathways they have been associated with in peer reviewed literature in Lipostar2. Figure 8 shows a portion of the metabolic pathways of Prostate cancer and pancreatic cancer. These cancer-related pathway was used because no specific pathway related to CRC was available at this time. As an example, LPC is believed to decrease in concentration in Prostate cancer (highlighted in blue). The closest related known pathway for ceramides was Pancreatic cancer. As an example, Cer(34:1) is believed to increase in concentration in Pancreatic cancer (highlighted in red). Ceramide species are upregulated in CRC samples.

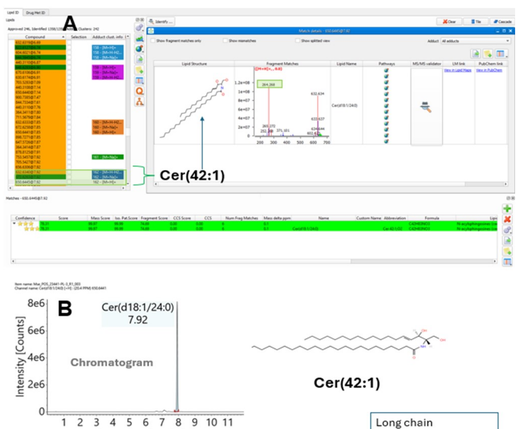


Figure 6.Example Cer(42:1) identification at 7.92 min using Lipostar2 (A) and in waters_connect (B). The long chain base fragment peak (264 m/z) is highlighted and shows a low mass error.

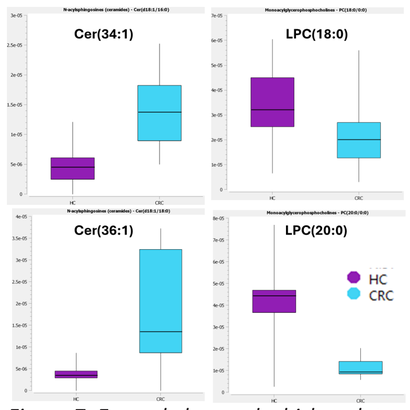


Figure 7. Example box and whisker plots of ceramides species and LPC species from the CRC sample cohort

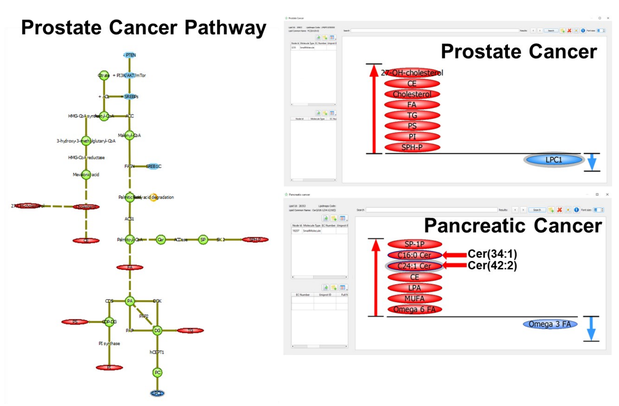


Figure 8. Metabolic pathway depicting "Prostate cancer and Pancreatic Cancer. The cancer pathways show LPC decrease in cancer patients while Ceramides increase in cancer patients

References

1. Morgan et al., 2023, Gut ;72:338-344. doi:10.1136/gutjnl-2022-327736
2. Isaac G *et al*., 2020 A Robust and Reproducible Reversed-Phase Lipid Profiling Method for Large Sample Sets 720006959, July 2020
3. Silva, J. C., Gorenstein, M. V., Li, G. Z., Vissers, J. P. C. & Geromanos, S. J. Absolute quantification of proteins by LCMSE: A virtue of parallel MS acquisition. Mol. Cell. Proteomics 5, 144–156 (2006)
4. Geromanos, S. J. et al. The detection, correlation, and comparison of peptide precursor and product ions from data independent LC-MS with data dependant LC-MS/MS. Proteomics 9, 1683–1695 (2009).
5. Sarafian et al., 2014, Objective Set of Criteria for Optimization of Sample Preparation Procedures for Ultra High Throughput Untargeted Blood Plasma Lipid profilingby UPLC-MS, Anal. Chem., DOI: 10.1021/ac500317c
6. Laura Goracci, Sara Tortorella, Paolo Tiberi, Roberto Maria Pellegrino, Alessandra Di Veroli, Aurora Valeri, and Gabriele Cruciani (2017) LipoStar, a Comprehensive Platform-Neutral Cheminformatics Tool for Lipidomics, Analytical Chemistry 2017 89 (11), 6257-6264, DOI: 10.1021/acs.analchem.7b01259
7. Alizadeh J, da Silva Rosa SC, Weng X, Jacobs J, Lorzadeh S, Ravandi A, Vitorino R, Pecic S, Zivkovic A, Stark H, Shojaei S, Ghavami S. Ceramides and ceramide synthases in cancer: Focus on apoptosis and autophagy. Eur J Cell Biol. 2023 Sep;102(3):151337. doi: 10.1016/j.ejcb.2023.151337. Epub 2023 Jun 26. PMID: 37392580.
8. Sheridan M, Ogretmen B. The Role of Ceramide Metabolism and Signaling in the Regulation of Mitophagy and Cancer Therapy. Cancers (Basel). 2021 May 19;13(10):2475. doi: 10.3390/cancers13102475. PMID: 34069611; PMCID: PMC8161379.