MULTI-OMIC CHARACTERIZATION OF PLASMA FROM PATIENTS DIAGNOSED WITH BLADDER CANCER REVEALS MOLECULAR INSIGHT INTO IMMUNITY AND RECEPTOR SIGNALING PATHWAYS

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INTRODUCTION

Cancer is one of the most complex, life threatening diseases, existing in many forms which have unknown pathogenesis. A combination of genetic and lifestyle factors are known to contribute towards increasing the probability of encountering cancer. Lifestyle factors such as smoking are known to contribute towards bladder cancer, with the condition predicted to see >80,000 new cases diagnosed and >17,000 deaths in the USA for 2019.[1] The exact mechanism as to how these carcinomas develop during various stages is still not well understood. Here, we describe a multi-omic approach to reveal molecular factors that may be involved in these biomolecular processes. Combining lipidomic, metabolomic and proteomic approaches have helped to identify multi-factorial disease associated components and their related pathways.

METHODS

Sample preparation

Proteomics

Proteomic samples for all patients (n=12) were prepared by taking 20 µL undepleted plasma and reducing, alkylating and digesting overnight with trypsin (figure 1).

Metabolomics & Lipidomics

For metabolite extraction, proteins were removed from plasma and diluted with water, using cold acetonitrile. Lipids extractions were performed as previously described by Sarafin et al.[2] The resulting supernatant was collected for LC-MS analysis (figure 1). Pooled Quality Control samples (QCs) were created for both Metabolomic and Lipidomic experiments by combining aliquots of each sample prior to extraction.

LC-MS parameters

Peptides were chromatographically separated using an ACQUITY M-class UPLC, over a 30 min gradient of 1 to 40% acetonitrile/0.1% formic acid at 7 µL/min. Metabolite and lipid analysis used an ACQUITY I-class system, which employed HILIC (metabolites) and reversed-phase (lipids) separation. Small molecule metabolites were eluted over a 6.5 min gradient from 100 to 50% 5:95 water:acetonitrile (10 mM ammonium formate & formic acid) at 700 µL/min. Lipids were separated using a 20 min gradient from 3 to 40% isopropanol:methanol (10 mM ammonium formate) at 400 µL/min.

LC-MS data for all extracts was acquired using a Xevo G2-XS QToF mass spectrometer (Waters Corporation), operated in SONAR™ mode.[3,4]

Bioinformatics

Progenesis software (Waters Corporation) was used to process all data. Multivariate statistical analysis was performed using EZInfo (Umetrics, Umea). Pathway mapping was also conducted using MetaCore (Clarivate Analytics, London, UK).

1) Protein Statistics

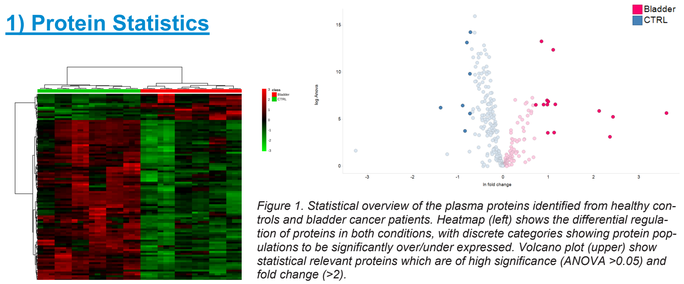


Figure 1. Statistical overview of the plasma proteins identified from healthy controls and bladder cancer patients. Heatmap (left) shows the differential regulation of proteins in both conditions, with discrete categories showing protein populations to be significantly over/under expressed. Volcano plot (upper) show statistical relevant proteins which are of high significance (ANOVA >0.05) and fold change (>2).

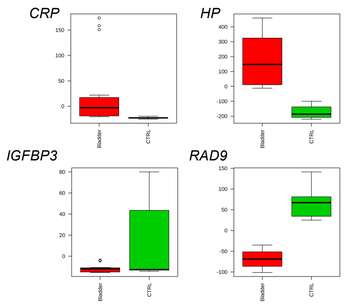


Figure 2. Example proteins resulting from statistical curation. Box-whisker plots show the expression changes at the group level. CRP for example has been reported previously as a cancer marker but lacks specificity for distinguishing cancer type(s). Other significant proteins include haptoglobin (HP), insulin-like growth factor binding protein 3 (IGFBP3) and cell cycle checkpoint control protein (RAD9).

3) Associating BMI with cancer subjects

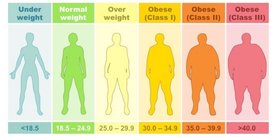
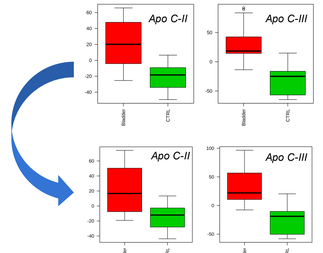
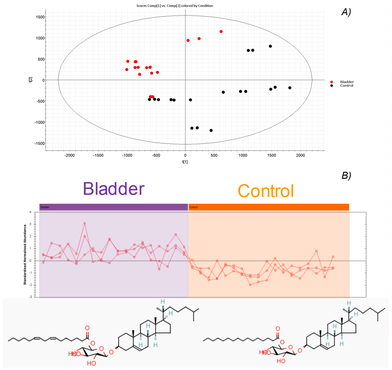


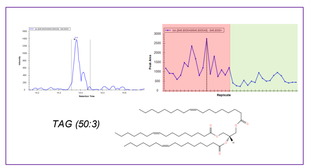
Figure 4. A large number of apolipoproteins (Apo) were also identified as being over expressed for bladder cancer patients. Application of the metadata to exclude patients with high BMI continued to show over expression of the Apo family for patients diagnosed with bladder cancer. Box-whisker plots opposite, represent a variety of Apo proteins which have been identified and therefore correlate with the increased levels of cholesterol and TAG which were recorded. Exclusion of individuals with high BMI, show these expression plots to have little change and therefore suggest these Apo proteins to be independent of BMI.

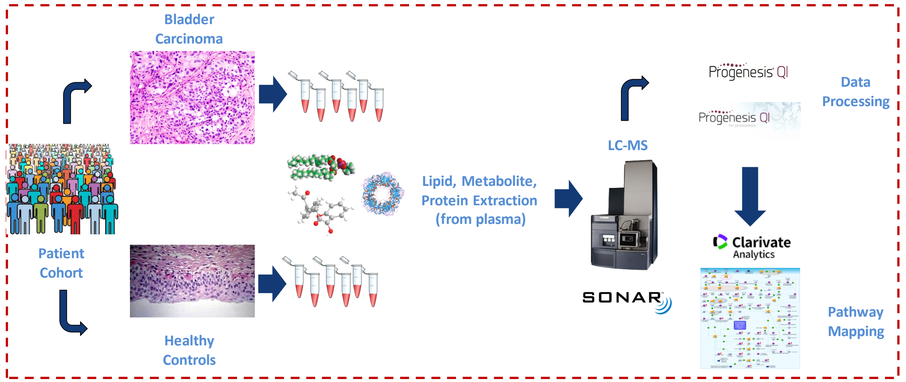
High BMI exclusion



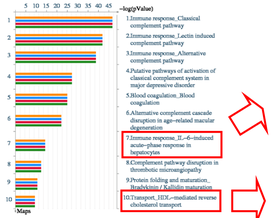
2) Lipid Statistics

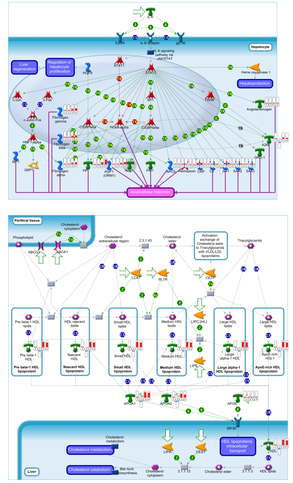






4) Pathway Analysis





IL-6 induced acute-phase response in hepatocytes

Transport HDL-mediated reverse cholesterol transport

ANALYSIS WORKFLOW

Figure 3. Unsupervised principal component analysis (PCA) reveals significant separation between healthy controls and bladder cancer patients (A). Cholesterols and in particular Glc-cholesterols appear to be over expressed for bladder cancer subjects when compared with controls. This is represented by the expression trend plot (B). Two example species identified following database searching are shown. Additional lipid species which show elevated levels in bladder cancer subjects are the triglycerides (TAG). An example is provided with TAG (50:3) being over expressed for a number of patients as shown with the expression trend plot (C). The shaded red area represents the bladder cancer cohort, whilst shaded green are the healthy controls.

CONCLUSION

- **Multi-omic analysis has been applied to a patient cohort consisting of both healthy controls and individuals with bladder carcinomas. Applying the analysis workflow using LC-MS, highlights differential groups of molecules which can be correlated with biological pathways.**
- **Differential analysis revealed specific protein groups to be expressed including apolipoproteins (APO) and protease inhibitors.**
- **Corresponding lipid analysis indicated that cholesterol derived lipid species are over expressed in bladder cancer subjects. Likewise, a similar trend was also observed for certain triglycerides.**
- **Accounting for patients with high BMI still highlighted the relevance of APO over expression with cholesterols/triglycerides, even with these patients discarded from the analysis.**
- **Mapping the statistical relevant molecules to biological pathways suggested changes related to the IL-6 induced acute-phase response and HDL transporter pathways.**

References

1. American Cancer Society - https://www.cancer.org/cancer/bladder-cancer/about/key-statistics.html
2. Sarafian et al., Objective Set of Criteria for Optimization of Sample Preparation Procedures for Ultra-High Throughput Untargeted Blood Plasma Lipid Profiling by Ultra Performance Liquid Chromatography-Mass Spectrometry, Anal. Chem., 2014; 86 (12), 5766-5774.
3. Gethings et al., Lipid profiling of complex biological mixtures by liquid chromatography/mass spectrometry using a novel scanning quadrupole data-independent acquisition strategy, Rapid Commun Mass Spectrom., 2017; 31 (19), 1599-1606.
4. Moseley et al., Scanning Quadrupole Data-Independent Acquisition. Part A: Qualitative and Quantitative Characterization, J Proteome Res., 2018; 17 (2), 770-779.

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