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Application Note

Rapid Molecular Profiling of a Bladder and Lung Cancer Human Plasma Cohort using MetaboQuan-R

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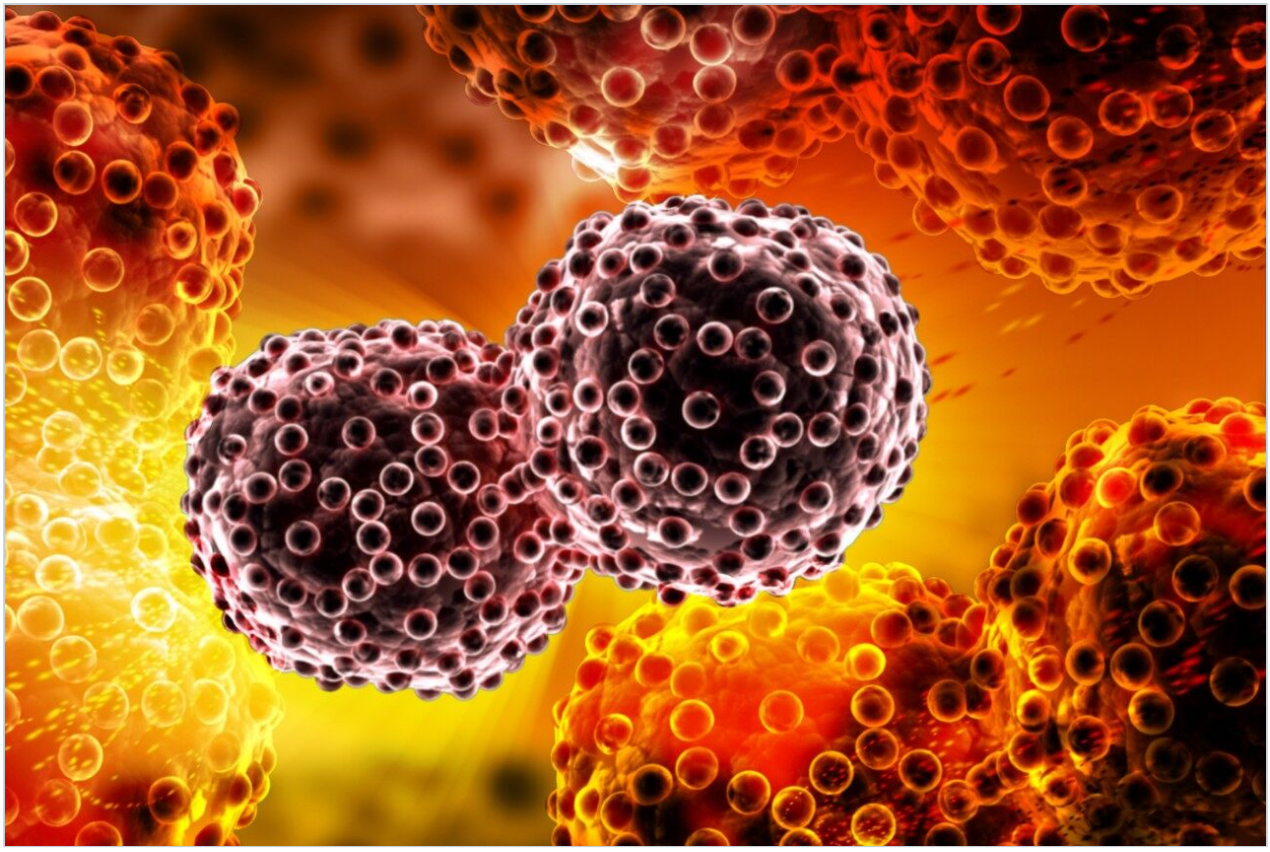

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Abstract

In this application note, we present a research study comparing plasma samples from bladder and lung cancer patients with healthy controls using MetaboQuan-R, a high-throughput targeted OMICS platform. We demonstrate three of the available methods to highlight some of the putitive markers associated with these cancer subtypes.

Benefits

- Simple, high-throughput workflow allowing the testing and semi-quantitative analysis of different compound classes with no method development
- A single, versatile LC-MS platform used for multiple compound classes (proteins, free fatty acids, triglycerides, amino acids, acylcarnitines, bile acids, etc.)
- Rapid analysis measuring markers of interest for large cohort, statistically driven studies

Introduction

Cancer is one of the most pressing global health concerns, responsible for almost 10 million deaths worldwide in 2018 alone.[1] A highly complex disease, cancer exists in many forms and emerges in a host of different bodily compartments. A combination of genetic and lifestyle factors, such as smoking, are known to increase the probability of cancer emerging, but the mechanisms of pathogenesis remain largely unknown. To improve prevention, diagnosis, and treatment, significant research and resources are being invested to better understand and characterize the complex biology underlying the emergence, propagation, and spread of cancer. One prominent outcome of the research to date is the appreciation that cancer is not a single disease and that a single type of cancer can take a very different course depending on the person affected. With an eye towards personalized medicine, more efficient early diagnosis, and increased surveillance, many research studies are relying on large study cohorts to generate statistically meaningful results. In order to empower these research studies, high throughput assays are required to process the large number of samples.

Cancers are characterized in part by an uncontrolled growth of cells, a phenomenon that is often associated with altered expression of proteins, lipids, and metabolites.[2,3] These diverse classes of biomolecules have

been shown to play a key role in cancer growth by providing energy to sustain rapid growth and higher mitotic rate. Recently, free fatty acid oxidations via carnitines and other derivatives have also been highlighted as another potential source of energy to fuel cancer cells.[4]

In this application note, we present a research study comparing plasma samples from bladder and lung cancer patients with healthy controls using MetaboQuan-R, a high-throughput targeted OMICS platform. This unique LC-MS platform allows for the rapid screening and semi-quantitative analysis of a variety of different compound classes (amino acids, bile acids, acylcarnitines, proteins, free fatty acids, triglycerides, etc). We demonstrate three of the available methods to highlight some of the putitive markers associated with these cancer subtypes. The three methods utilized include a protein method, which employs the Biognosys PlasmaDive Kit, and an amino acid methodology, which employs an acylcarnitine methodology. These last two classes of molecules were selected since they are known key energy sources in cancer biology.[2,3,4]

Experimental

All samples were prepared and run following the application notes included in each of the MetaboQuan-R method packages.[5] LC and MS methods were generated using the Quanpedia MetaboQuan-R databases included in each method package.

Sample preparation

Human plasma (Innovative Research, MI) consisting of controls (n=6) and patients diagnosed with lung (n=6) and bladder cancer (n=6) were used for the study.

Proteins

Plasma samples were reduced, alkylated, and tryptically digested overnight. Prior to LC-MS analysis, samples were spiked with the Biognosys PlasmaDive kit (Biognosys AG, Schlieren, Switzerland).

Acylcarnitines

Plasma was spiked with C10 decanoyl carnitine d4 at 312.5 ng/mL (Cambridge Isotope Laboratories, Cambridge, MA, USA) prior to protein precipitation using methanol.

Amino acids

Plasma samples were spiked with Valine d8 at 10 µg/mL (Sigma, Poole, UK) and proteins precipitated using sulfosalicylic (10%). The amino acids were then derivatized using the Waters AccQTag Kit (p/n: 186004535).

LC conditions

System:	ACQUITY UPLC I-Class
Column(s):	CORTECS T3 2.7 µm, 2.1 × 30 mm
Column temp.:	60 °C
Mobile phase A:	0.01% formic acid with 0.2 mM ammonium formate
Mobile phase B:	(B) 50% Isopropanol in acetonitrile with 0.01% formic acid and 0.2 mM ammonium formate
Injection volumes:	Peptides (6 µL); acylcarnitines (0.5 µL); amino acids (1 µL)
Flow rates: Peptides (0.15 mL/min), acylcarnitines and amino acids (1.3 mL/min)	
Gradient conditions:	Peptides – After an initial 2.5 min hold at 1% mobile phase B, tryptic peptides were eluted and separated with a gradient of 1%–45% mobile phase B over 2.9 min followed by a 2.5 min column wash at 85% mobile phase B. Acylcarnitines – After an initial 0.1 min hold at 2% mobile phase B, acylcarnitines were eluted and separated with a gradient

	of 2%–98% mobile phase B over 0.7 min followed by a 0.9 min column wash at 98% mobile phase B Amino acids – Derivatized amino acids were eluted and separated with a gradient of 1%–8% mobile phase B over 2.4 min followed by a 0.9 min column wash at 98% mobile phase B.

MS conditions

MS system:	Xevo TQ-S micro
Ionization mode:	ESI (+) at 2.0 kV
Ion source temp.:	150 °C
Cone gas flow rate:	50 L/hr
Desolvation temp.:	650 °C
Acquisition mode:	Multiple reaction monitoring (MRM)

Data processing

LC-MS data were processed using Skyline (Washington University, Seattle, USA). Additional data visualization and statistical analysis were performed using Metaboanalyst.[6]

Results and Discussion

MetaboQuan-R is a targeted OMICS solution that allows for the rapid testing, semi-quantitative analysis of multiple compound classes using a single LC-MS and informatics platform. All LC and MS methods were

generated using the MetaboQuan-R Quanpedia databases available at Waters.com/targetedomics and did not require any optimization. The overall workflow is described in Figure 1, where the total analysis time for all three assays was 1.5 days (including data processing and review).

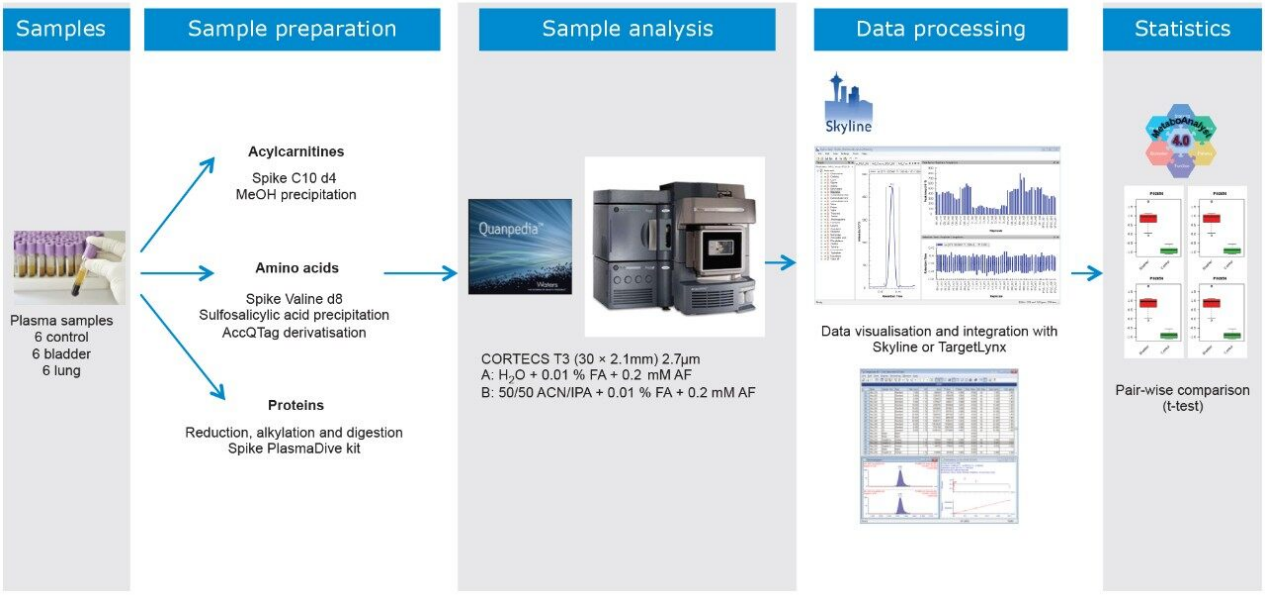

Figure 1. MetaboQuan-R workflow. Briefly, samples were prepared according to the compound class of interest. LC and MS methods were generated using the Quanpedia databases and data acquired using the ACQUITY UPLC-I Class System coupled to a Xevo TQ-S micro Triple Quadrupole Mass Spectrometer. Data were processed with Skyline or TargetLynx and statistical analysis performed.

Data were collected for 18 plasma samples (six controls, six bladder and six lung cancer), each sample being run in duplicate (proteins) or triplicate (acylcarnitines and amino acids). Quality controls (consisting of a pool constructed from all samples) were acquired every ninth injection. In total, 206 injections were performed and final results highlighting significant markers were obtained in 34 hours from injection points.

Altogether 128 components (80 proteins, 20 acylcarnitines, and 28 amino acids) were detected and quantified using Skyline, generating a coefficient of variation (CV) of less than 10% for the quality control (QCs) samples. Example chromatograms extracted from the QCs are shown in Figure 2.

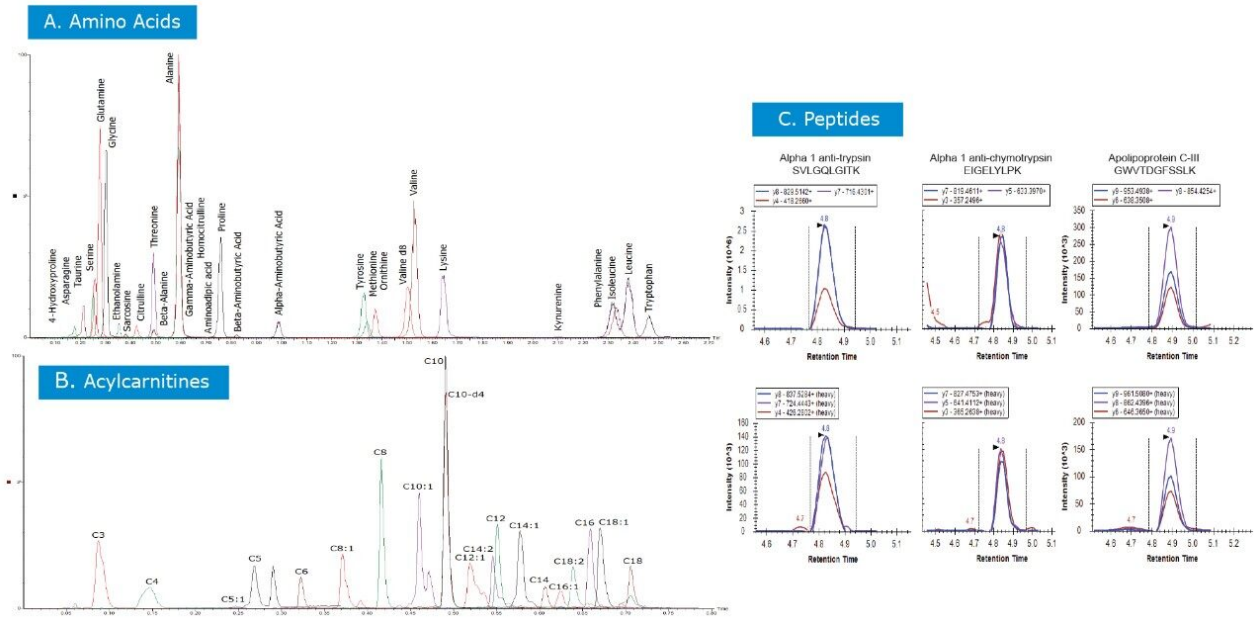

Figure 2. Example chromatograms were obtained from the QC samples for A) the amino acids MetaboQuan-R screening method and B) the acylcarnitines MetaboQuan-R screening method. Three example peptide chromatograms of the 100 monitored proteins with the MetaboQuan-R protein screening method are shown in C). The upper chromatograms show the three transitions for the native peptides and the lower chromatograms show the stable labelled (heavy) reference peptides.

High quantitative precision was demonstrated with low observed quantitative variance on the QC samples (Figure 3). Valine d8 (standard amino acid spiked in all samples for the amino acid screening) is used to illustrate the consistency of peak area (median CV of 5%) across the whole study (Figure 4).

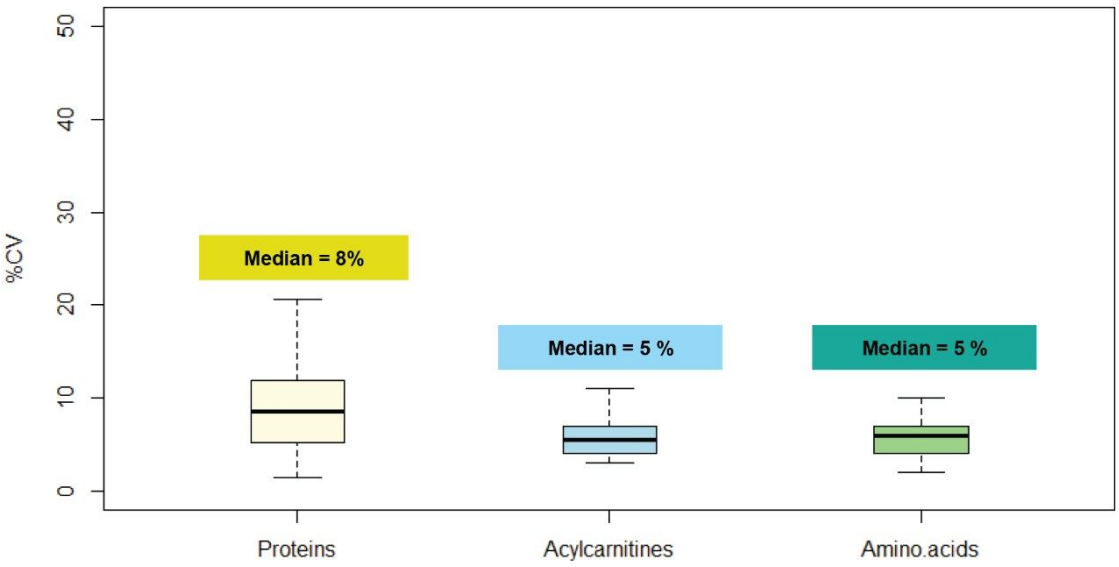

Figure 3. Box and whisker plot representing the distribution of the peak area CV across the quality control samples for the 128 detected and quantified components.

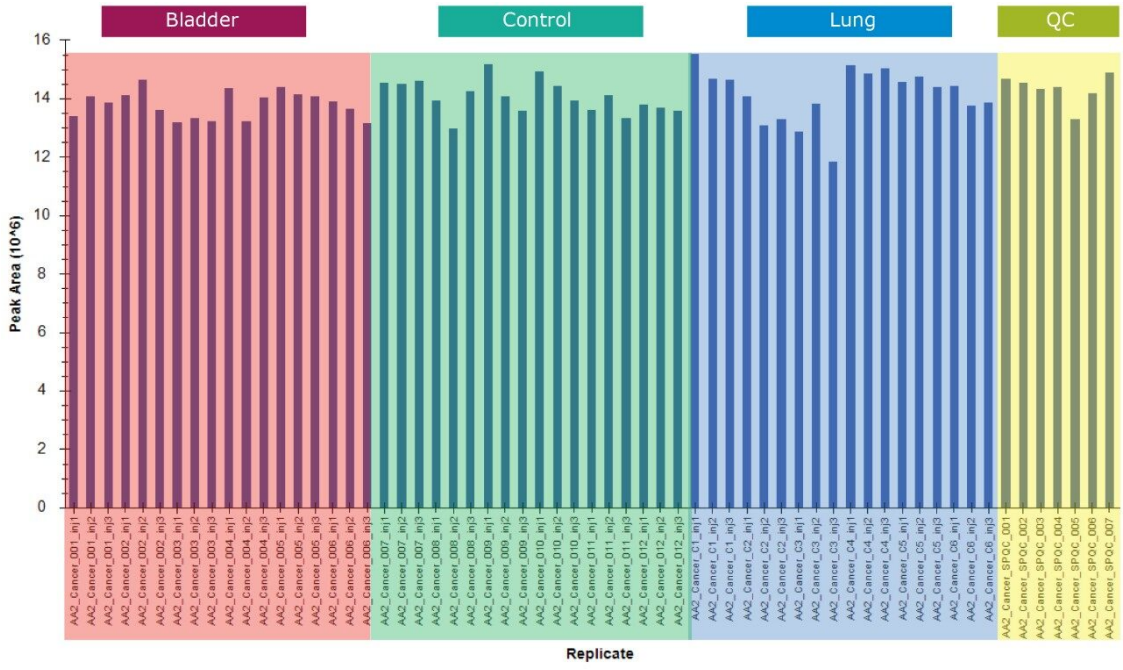

Figure 4. Peak area of the amino acid spiked standard (valine d8) extracted in Skyline from all sample analyses. The overall median CV was observed to be 5%.

Pair-wise comparisons (bladder vs. control and lung vs. control) using a t-test were performed on each

compound class. At the proteomic level, 10 proteins were identified as statistically differentiated in both the bladder and lung cohorts when compared with controls (Figure 5). Similarly, evaluation of the data corresponding to the acylcarnitines (Figure 6), and amino acids (Figure 7), show eight acylcarnitines for bladder cancer and nine for lung cancer were differentially expressed and seven amino acids were altered between the cancer cohorts and control group. Components that demonstrated the greatest differential expression from all three assays are provided in Table 1.

Markers	Bladder	Lung
P00488 Coagulation factor XII A chain	↓	
P00738 Haptoglobin	↑	↑
P01009 Alpha-1-antitrypsin	↑	
P01011 Alpha-1-antichymotrypsin	↑	↑
P02656 Apolipoprotein C-III	↑	↑
P02671 Fibrogen alpha chain	↑	↑
P02748 Complement component C9		↑
P02749 Apolipoprotein H		↑
P02750 Leucine-rich alpha 2-glycoprotein		↑
P02763 Alpha-1-acid glycoprotein 1	↑	↑
P19652 Alpha-1-acid glycoprotein 2	↑	↑
P68871 Hemoglobin subunit beta	↑	↑
Sarcosine	↑	↑
C8:1 Octenoyl carnitine	↓	

Table 1. List of the most statistically significant markers identified for lung and bladder cancer. The direction of protein expression is indicated by the arrows shown, up (over expressed), and down (under expressed).

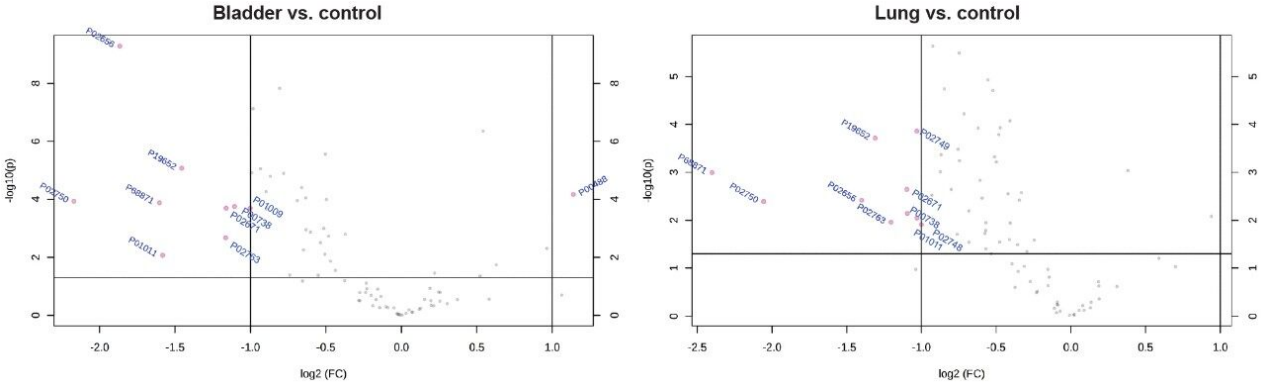

Figure 5. Volcano plot highlighting potential proteins of interest (pink dots) when comparing bladder vs. control and lung vs. control. In both scenarios, only features exhibiting ANOVA (p) values <0.05 and fold changes >2.00 were considered as significant. These significant proteins are listed in Table 1.

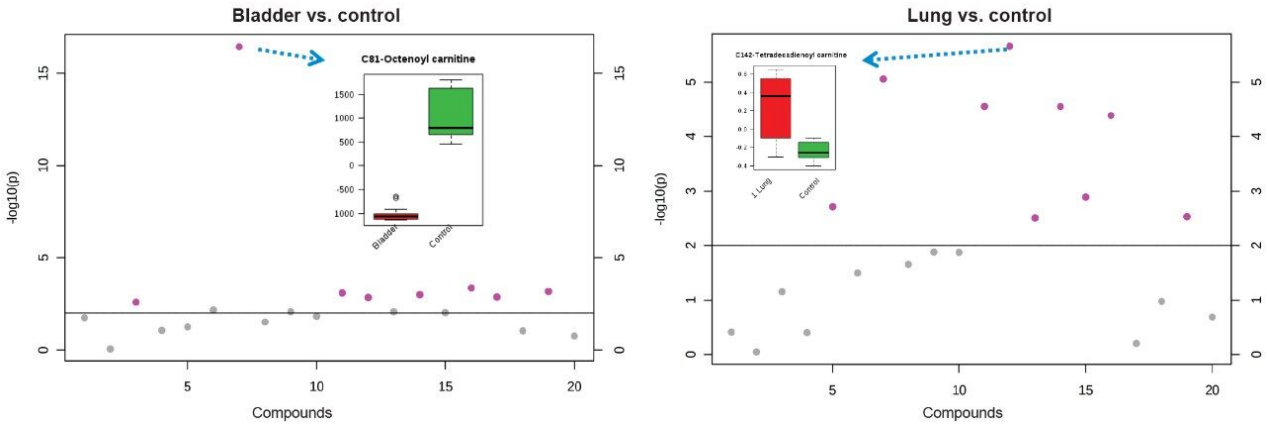

Figure 6. Pair-wise comparisons of the 20 acylcarnitines screened (t-test, FDR cut-off value adjusted to 0.01). The pink dots represent acylcarnitines that were detected as significantly different between the two conditions. C_8: 1-Octanoyl carnitine, for example, is shown to be under-expressed in a majority of bladder cancer subjects. The statistical differentiation of acylcarnitine markers for lung cancer also shows a number of significant markers (e.g. C14:2), but the degree of change was less stark than observed within the bladder cancer cohort.

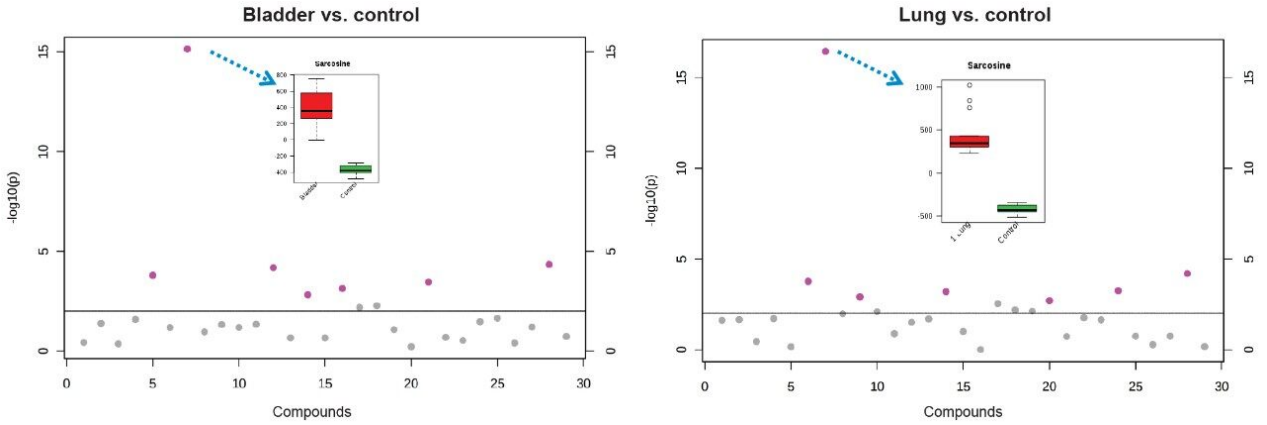

Figure 7. Pair-wise comparisons of the 28 amino acids (t-test, FDR cut-off value adjusted to 0.01). The pink dots represent amino acids that were significantly different between the two conditions. In particular, sarcosine was found to be significantly over expressed for both bladder and lung cancer subjects.

Although more statistical analysis and validation are required, some of those compounds identified here have been highlighted in previous cancer research studies. For instance, urine-based studies have also shown apolipoprotein C-III to be over-expressed in patients diagnosed with bladder cancer.[7] Likewise, sarcosine has also been demonstrated as being highly elevated in cases of pancreatic cancer,[8] while C_8:1 acylcarnitine was observed to decrease for bladder cancer subjects.[9]

Overall, the data demonstrates the capacity of the MetaboQuan-R platform to rapidly and efficiently identify potential and relevant markers of interest. Analyses can be implemented and executed without the need of extensive method development and results are generated in timelines necessary to support the processing of large sample sets.

Conclusion

- MetaboQuan-R was successfully applied to profile and compare a cohort consisting of healthy controls with bladder and lung cancer plasma-based samples.
- High precision was demonstrated with low observed variance on the quality control samples (CV<5%).
- Rapid LC-MS methods for multiple analyte classes were easily deployed.
- Markers of interest belonging to the different molecule classes (proteins, amino acids, and acylcarnitines)

were identified using the high throughput methodology. 11 markers were identified as the most statistically significant for bladder cancer, while 12 were highlighted for the lung cancer cohort.

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