**Comprehensive targeted LC-QTOF-MS metabolomics identifies novel metabolite changes associated with treatment of the rare bone disease Alkaptonuria**

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Metabolomics involves studying the entire complement of small molecule metabolites in a biological system and is increasingly recognised as a powerful phenotyping strategy to understand the pathophysiology underlying a broad range of disease. Data processing and identification of ‘unknowns’ is *the* bottleneck in metabolomics. To resolve this an approach has been evaluated for comprehensive targeted metabolomics employing three complementary LC-QTOF-MS methods and accurate-mass retention time (AMRT) databases generated in-house from 619 metabolite standards. The strategy was then applied to Alkaptonuria (AKU), a rare inborn error of tyrosine metabolism also known as ‘black bone disease’.

619 standards (mw:45-1354 Da) covering a broad range of primary metabolism, including carbohydrates, amino and organic acids and lipids, were analysed by three chromatographic methods (two reversed-phase, one normal-phase) coupled to an Agilent 6550 LC-QTOF-MS operated in positive and negative polarity. Data from the standards formed an in-house AMRT database for each method for identifying the structures of ‘unknown’ chemical entities detected from metabolic profiling of AKU urine. AMRT-based targeted feature extraction was performed on data of urine samples from 25 AKU patients (19-72 years) at baseline then at 3 (2mg every other day), 12 and 24 months (2mg daily) on nitisinone.

AMRT matching employed windows of ±10ppm (mass) and ±0.3 minutes (retention time). Combining data from the three methods enhanced coverage of the metabolome, achieving a total of 243 unique AMRT compound identifications that passed quality control filtering. In total, 24 positively-charged AMRT matches showed significant profile differences (FDR p<0.05 and fold change >2) from the pre-treatment sampling time point; 12 decreased abundance, 12 increased abundance. The altered metabolites included changes which are known following nitisinone treatment, including homogentisic acid (decreased) and tyrosine (increased), but also some previously unreported changes: tyramine, 3-methoxytyramine, 4-hydroxyphenylacetic acid, 4-hydroxybenzaldehyde and ethylmalonic acid increased; tryptophan, kynurenine, methyl-histidine, cAMP, xanthosine and paraxanthine decreased.

In conclusion, we have identified a number of novel metabolite changes in AKU urine following treatment with the promising drug nitisinone. The LC-QTOF-MS strategy will be an invaluable phenotyping tool for application to AKU and other rare bone diseases.