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SHORT COMMUNICATION

Metabolomics by UHPLC–MS: benefits provided by complementary use of Q-TOF and QQQ for pathway profiling

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Abstract

Introduction Non-targeted metabolic profiling using high-resolution mass spectrometry (HRMS) is a standard approach for pathway identification despite technical limitations.

Objectives To assess the performance of combining targeted quadrupole (QQQ) analysis with HRMS for in-depth pathway profiling.

Methods Serum of exercising patients with type 1 diabetes (T1D) was profiled using targeted and non-targeted assays.

Results Non-targeted analysis yielded a broad unbiased metabolic profile, targeted analysis increased coverage of purine metabolism (twofold) and TCA cycle (three metabolites).

Conclusion Our screening strategy combined the benefits of the unbiased full-scan HRMS acquisition with the deeper insight into specific pathways by large-scale QQQ analysis.

Keywords Targeted · Non-targeted · Pathways · Metabolism

1 Introduction

The new generation of high-resolution mass spectrometers (HRMS) delivering unbiased full-scan information with high mass accuracy (< 3–10 ppm) has promoted non-targeted metabolic profiling by liquid chromatography coupled to mass spectrometry (LC–MS) to a gold standard for identifying regulated pathways in biological samples. This status has been gained as no prior knowledge of the regulated metabolites is required. Non-targeted metabolic profiling with HRMS has also demonstrated excellent robustness and hundreds of samples can be profiled if appropriate internal standards or quality control samples are used (Lewis et al. 2016). However, non-targeted LC–MS metabolomics suffers from major limitations: an ease of detector saturation, limited linear range and a complex integration pipeline influenced among other parameters by the peak picking algorithms (Myers et al. 2017). In contrast, the integration of the chromatographic ion signals measured on a triple quadrupole (QQQ) in a selected reaction monitoring (SRM) mode is relatively straightforward and measuring up to hundreds of metabolites in a single run is nowadays possible (Cai et al. 2015; Yuan et al. 2012). This performance is usually achieved by restricting the acquisition time of the metabolite to its elution window and by using the increased polarity switch capacity of QQQ.

Until recently, a major drawback for the implementation of such large-scale SRM assays on QQQ was the need to optimize the fragmentation conditions for each metabolite separately (e.g., *m/z* of the fragments, collision energy, transfer voltage), a process which often required metabolite standards and manual effort. The recent introduction of METLIN-MRM (Domingo-Almenara et al. 2018), a database containing the transitions of more than 15000 molecules, the numerous published protocols in literature and the

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introduction of data-independent acquisition (DIA) of MS/MS spectra currently allows for an accelerated development of SRM methods without the need of metabolic standards (Chen et al. 2013; Zha et al. 2018). Targeted acquisition on QQQ has also shown improved sensitivity, reproducibility and lower dependency to signal normalization compared to non-targeted acquisition on HRMS (Chen et al. 2013; Shao et al. 2015). Hence, large-scale targeted assay could become an interesting addition to non-targeted analysis for in-depth characterisation of multiple metabolic pathways.

In this study, we will demonstrate that the combination of full-scan non-targeted Q-TOF and targeted QQQ analysis improves the characterisation of metabolic profiles, and thus, the understanding of biochemical processes in pathways during metabolomics studies. As a proof of concept, we profiled serum samples from a previously described crossover study with T1D patients in which energy metabolic pathways were modulated by physical exercise (Bally et al. 2017). T1D is an important metabolic disease affecting many young and active people and is known for its complex interplay of metabolic processes during exercise (Dube et al. 2013).

2 Materials and methods

2.1 Study design and sample preparation

For the evaluation of the LC–MS methods, we used the serum of 12 male patients with T1D undergoing 90 min of isoenergetic exercise with (intermittent high-intensity, IHE) and without (continuous moderate intensity, CONT) interspersed sprints as previously described (Bally et al. 2017). Serum was collected before (0 min), during (80 min) and after (210 min) exercise. Metabolites were extracted by protein precipitation with organic solvent. Details of the extraction protocol are provided in the supplementary information. To evaluate the linear range, we additionally pooled the serum from healthy volunteers and analysed in triplicates a dilution series of the extracted serum (undiluted, 1:3, 1:9, 1:27, 1:81 and 1:243 dilution).

2.2 LC–MS analysis

Briefly, metabolic profiling of extracted serum was performed by a non-targeted and targeted approach using Q-TOF and QQQ mass spectrometers, respectively. The serum metabolites were separated by reversed-phase chromatography with water and methanol acidified with 0.1% (v/v) formic acid as mobile phases. The samples were analysed in the positive (+) and negative (−) electrospray ionization (ESI) mode. The scheduled QQQ method covered 114 metabolites involved in multiple core energy pathways (Table S1). The non-targeted Q-TOF method was slightly adapted from the previously published one (Rindlisbacher et al. 2018). Details of the LC–MS methods are provided in the supplementary information.

2.3 Data processing and analysis

The targeted evaluation of the data collected with the non-targeted Q-TOF and targeted QQQ approaches were processed with Skyline (MacCoss Lab, version 4.1.0) (MacLean et al. 2010). The raw data from the Q-TOF were noise reduced with the Waters Compression, Noise Reduction and Archival Tool software (noise reduction 5, FWHM 25000, version 1.10). For the non-targeted metabolic profiling, Progenesis QI (version 2.3, Nonlinear Dynamics, Newcastle, UK) was used for chromatographic alignment, peak picking and ion pattern deconvolution as previously described (Rindlisbacher et al. 2018). Metabolites solely detected by targeted QQQ approach were also searched in the raw non-targeted Q-TOF data using Skyline for masses of the most probable adducts ($[M+H]^+$, $[M+Na]^+$, $[M+H–H_2O]^+$, $[M+K]^+$, $[M–H]^-$, $[M–]^-$, $[M+HCOO]^-$, $[M–H_2O–H]^-$). Among them, xanthosine detected by Q-TOF was below the abundance-filtering threshold of ≥ 200 for each metabolic feature and was thus excluded from analysis. All non-targeted metabolic features were searched against the Human Metabolome Database (HMDB, version 4.0) (Wishart et al. 2018) based on exact mass (≤ 8 ppm) and against an in-house database containing retention times of authentic standards in addition to exact mass for identification. Features identified against our in-house database with a retention time deviation < 0.4 min were accepted as potential identity. Conformation of identity was evaluated using MS/MS spectra information from traveling wave ion-mobility mass spectrometry (TWIM-MS) or MS^E data. The level of confidence in identification (Sumner et al. 2007) was 1 for all mentioned metabolites (Table S2). Univariate statistical analysis was performed in Skyline or Progenesis QI using Student's *t* test and false discovery rate was estimated according to Benjamini Hochberg (threshold set at q ≤ 0.05).

3 Results and discussion

We first established a targeted LC–MS method for 114 metabolites involved in various core energy pathways and implemented a consolidated metabolite extraction procedure which minimized the amount of sample required for analysis (i.e., typically 50 µL serum). To evaluate the repeatability of our targeted QQQ and non-targeted Q-TOF methods, we analysed the repeated injections of the quality control (QC, n = 10) sample inter-spread during measurement of the 72 serum samples from patients with T1D on both devices. Overall, 33 and 17 metabolites were detected

with both approaches in ESI + and ESI− mode, respectively. The coefficient of variation (CV) of the peak area was low with median CV ≤ 6% for both approaches, indicating high intra-assay precision and reliable data acquisition with both MS platforms (Fig. 1a). Compared to the Q-TOF instrument, the QQQ was 2.2-fold and 1.5-fold more precise in repeated measurements of the QC sample in ESI + mode (median CV of 4.2% for Q-TOF and 1.9% for QQQ) and ESI− mode (median CV of 6% for Q-TOF and 4.1% for QQQ), respectively. The lower technical variability of the QQQ assay improves the detection of smaller differences between groups. Our findings are in line with previous work reporting improved reproducibility of QQQ compared to HRMS instruments (Chen et al. 2013; Shao et al. 2015).

In biological samples, metabolite concentrations vary strongly and can span multiple orders of magnitude. Exceedance of the linear range and detector saturation with high abundant metabolites is an often encountered obstacle with Q-TOF instruments operating in full scan mode. An improved linearity is usually reached with QQQ, which may exceed four to five orders of magnitude without detector saturation in contrast to two to three orders of magnitude for Q-TOF instruments (Vrhovsek et al. 2012). We evaluated the linear range of both mass spectrometers by analysing a dilution series made from pooled serum from healthy volunteers. Among the 114 metabolites analysed with the QQQ method, 83 metabolites were detected by both the targeted and non-targeted approaches. Peak areas of the commonly detected metabolites were extracted and the linearity of the dilution series was assessed by linear regression (Fig. 1b). The majority of the metabolites detected by the QQQ (61%) had an excellent linear correlation with $r^2 > 0.99$. In contrast, only 29% of the metabolites detected by the Q-TOF achieved similar linearity. Whereas 96% of the metabolites detected by the QQQ had an $r^2 > 0.95$, only 68% of the metabolites detected by the Q-TOF reached this linear correlation. Metabolites with poor linear correlation (i.e., $r^2 < 0.90$, 16%) were only measured on the Q-TOF. These results endorse previously published reports, which show improved linearity of QQQ compared to Q-TOF instruments (Chen et al. 2013; Holcapek et al. 2012). The wider linear range obtained with the QQQ was especially advantageous for detecting of very low abundant metabolites. These metabolites, which were overseen by full-scan analysis using Q-TOF, may reveal a deeper insight into specific metabolic pathways. Subsequently, a combination of full-scan non-targeted Q-TOF and targeted QQQ analysis as a new screening strategy for metabolomics data could be highly beneficial for in-depth generation of metabolic profiles.

To confirm this hypothesis, we further profiled serum samples of exercising patients with T1D using both approaches. After peak integration and filtration of the full scan Q-TOF data, 1220 features were detected in ESI + and 540 features in ESI− mode. The full-scan data revealed a comprehensive exercise-associated metabolic profile for patients with T1D and was used for discovery of discriminative features between the groups. Potential clinical implications were extensively discussed in our previous publication (Bally et al. 2017). A fraction of the features detected with Q-TOF was assessed by the targeted QQQ method: 71 and 33 metabolites in ESI + and ESI− mode, respectively. In total, 22 metabolites measured by the QQQ were significantly regulated between CONT and IHE during (80 min) and after (210 min) exercise and are documented together with the fold changes in Table S3. These commonly detected metabolites showed consistent fold changes with both methodological approaches, underlying the reproducibility of results generated with both the QQQ and Q-TOF approach.

In line with previously reported results, the most prominent changes were detected in β-oxidation and purine metabolism (Bally et al. 2017). The coverage of acylcarnitines derived from fatty acid oxidation was similar for both profiling approaches. Acetylcarnitine ($p = 0.003$ for targeted, $p = 0.004$ for non-targeted) was significantly upregulated in IHE compared to CONT in both MS-based approaches at 80 min. Hexanoylcarnitine ($p = 0.002$ for targeted, $p = 0.001$

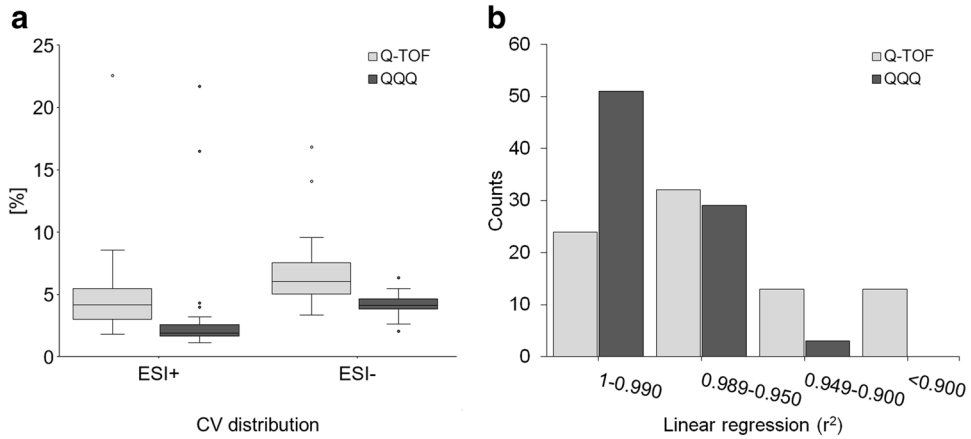

Fig. 1 Repeatability and linearity of response determined for the targeted QQQ and non-targeted Q-TOF methods for the commonly detected metabolites. **a** Coefficient of variation (CV) of the peak area calculated from metabolites in QC samples of the exercising patients with T1D (n = 10 injections). **b** r^2 distribution of linear regression analysis of the dilution series made from pooled serum. QQQ analysis is shown in dark grey, Q-TOF analysis in light grey

for non-targeted), octanoylcarnitine ($p = 0.001$ for targeted, $p < 0.001$ for non-targeted), decanoylcarnitine ($p = 0.001$ for targeted, $p < 0.001$ for non-targeted), dodecanoylcarnitine ($p = 0.002$ for targeted, $p = 0.001$ for non-targeted) were due to the increased energy consumption significantly downregulated in IHE at 80 min. Myristoylcarnitine/tetradecanoylcarnitine was detected by both approaches, but was solely discriminative between IHE and CONT at 80 min using the QQQ approach ($p = 0.04$ for targeted, $p = 0.07$ for non-targeted). For the purine metabolism profiled with the targeted QQQ approach, adenosine monophosphate (AMP, $p = 0.002$), inosine ($p = 0.01$), hypoxanthine ($p < 0.001$ in ESI− and ESI+), xanthosine ($p < 0.001$), xanthine ($p = 0.04$) and uric acid ($p = 0.002$ in ESI− and ESI+) levels were increased at 80 min during IHE compared to CONT (Fig. 2a). Furthermore, xanthosine ($p < 0.001$) and uric acid ($p = 0.001$ in ESI− and $p < 0.001$ in ESI+) remained elevated after exercise at 210 min in the IHE group, indicating increased purine turnover during IHE compared to CONT. The discriminative features from the purine metabolism isolated by the QQQ approach were partly confirmed by the Q-TOF analysis: hypoxanthine (t = 80 min, $p = 0.009$ in ESI− and $p = 0.002$ in ESI+), xanthine (t = 80 min, $p = 0.08$ in ESI− and $p = 0.02$ in ESI+) and uric acid (t = 80 min: $p = 0.009$ in ESI− and $p = 0.03$ in ESI+; t = 210 min: $p = 0.007$ in ESI− and $p = 0.04$ ESI+) were increased in IHE compared to CONT. Inosine was detected as not significantly changed at 80 min ($p = 0.09$ in ESI− and $p = 0.07$ in ESI+) with the Q-TOF approach, but showed increased abundance in IHE compared to CONT (Fig. S1a).

Interestingly, AMP, adenosine, adenine and xanthosine were exclusively detected by the QQQ, leading to a twofold increased purine pathway coverage compared to the non-targeted Q-TOF analysis. Furthermore, three metabolites of the TCA cycle were exclusively detected by the QQQ: malic acid, succinic acid, and alpha-ketoglutaric acid (Fig. 2b). These metabolites are present at low concentration in serum and rapid metabolisation is known to occur. Their detection by the QQQ reflects the enhanced sensitivity for certain specific metabolites by this assay. Malic acid ($p < 0.001$) and alpha-ketoglutaric acid ($p = 0.03$) were significantly upregulated and succinic acid was detected with non-significant ($p = 0.25$) higher abundance in IHE compared to CONT at 80 min (Fig. S1b). Our results confirm that the strength of the targeted QQQ approach lies in the increased coverage of low abundant metabolites involved in core energy pathways.

The present work has a methodological focus applying targeted QQQ analysis in conjunction with Q-TOF to improve and consolidate metabolic pathway coverage. Our findings with HRMS of differences in acylcarnitine profiles and purine metabolites were corroborated by complementary targeted approach albeit with a twofold greater coverage of purine metabolism. Of note, non-targeted findings were expanded by novel discriminative features of TCA metabolism, which would have remained unrecognised with the exclusive use of HRMS. Higher abundance of TCA candidates such as malic acid, alpha-ketoglutaric acid and succinic acid with intermittent high-intensity compared to moderate intensity exercise link the increased ATP turnover illustrated by higher purine metabolites and differences in acylcarnitines, thereby providing a more comprehensive insight into specific exercise-induced metabolic effects. These results are in line with previous metabolomics-based studies investigating the plasma

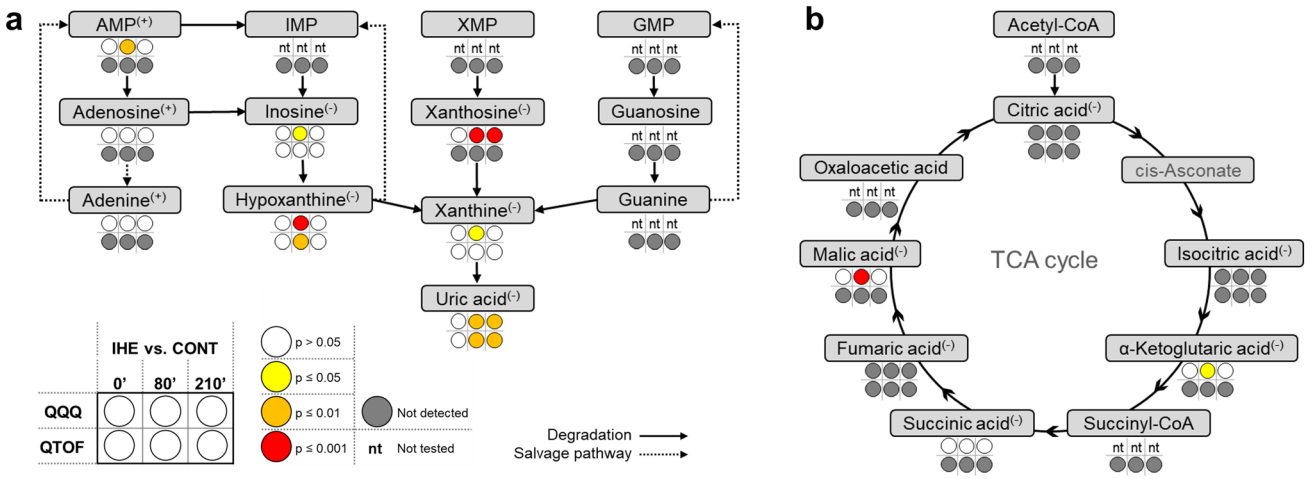

Fig. 2 Comparison of selected metabolites between IHE and CONT at each time point (0, 80 and 210 min) illustrated in simplified representations of the pathways. **a** Purine metabolism and **b** tricarboxylic acid (TCA) cycle using QQQ (upper line of circles) and Q-TOF (lower line of circles) methods. Metabolites were comprehensively upregulated in IHE compared to CONT. Superscript indices indicate measurement in positive or negative ESI mode. Yellow circles illustrate adjusted p-values ≤ 0.05; orange, p ≤ 0.01; red, p ≤ 0.001; grey, not detected; nt, not tested due to not incorporated in QQQ library (Color figure online)

signature of exercise in individuals with type 1 diabetes and healthy controls (Lewis et al. 2010; Brugnara et al. 2012).

The increased coverage of purine metabolism and TCA cycle emphasizes the strength of a combined analysis strategy: while the non-targeted Q-TOF approach could be used to discover unknown regulated pathways and metabolites, thereby expanding the understanding of biological processes, the targeted QQQ approach provides deeper insights into specific pathways. With our combined strategy, the gain in information was not at an expense of increased amount of sample due to the consolidated metabolite extraction method. The applied sample preparation proved suitable for HRMS full-scan profiling as well as targeted analysis with QQQ, and thus, will be of advantage for study cohorts with delicate collection of samples. Recent advances in databases containing multiple fragmentation transitions simplified and accelerated method development for targeted analysis and support a combined use of QQQ and Q-TOF analysis for metabolite and pathway profiling. In conclusion, our metabolomics screening strategy combines the benefit of the unbiased full-scan HRMS acquisition with the deeper insight into specific core energy pathways by large-scale QQQ analysis.

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Author contributions The following authors contributed to conception and design of the study: KF and CB; acquisition of data: KF and CB; data analysis: KF and CB. KF and CB were involved in drafting the article and all authors revised it critically for important intellectual content (KF, CRL, CS, GMF, LB, CB). All authors approved the final version of the article.

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Data availability The datasets generated and analysed during the current study are available from the corresponding author on request.

Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

Ethical approval The study was approved by the local Ethics Committee in Bern (KEK 001/14).

Informed consent Informed consent was obtained from all individual participants included in the study. Study-related activities were undertaken according to Good Clinical Practice guidelines.

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