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Method development for quantification of DMET-proteins in the paediatric intestinal tract via LC-MS/MS using a QconCAT technique

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SUMMARY

Physiologically based pharmacokinetic (PBPK) modelling is a powerful alternative for paediatric clinical trials. Paediatric PBPK models require data on intestinal drug metabolising enzymes and transporter (DMET)-protein abundances, however only limited data is available. This is the first study to report *paediatric* duodenal DMET protein quantification using a QconCAT approach. Thirty-six paediatric intestinal biopsies have been obtained from which the total mucosal protein was extracted. Proteins were digested to peptides using FASP and peptide levels were quantified using LC-MS/MS. Preliminary results provide some insight on the effect of age on duodenal protein abundance.

INTRODUCTION

Drug development for the paediatric population is challenging, as clinical trials in this cohort often face ethical constraints (Batchelor and Marriott 2013). Modelling approaches, such as physiologically based pharmacokinetic (PBPK) modelling, can support paediatric drug development. PBPK software packages, such as the Simcyp simulator (Certara), combine physiological and anatomical data with *in vitro* information and mathematical models to accurately predict pharmacokinetic (PK) profiles *in vivo*. Reliable data on biophysical descriptors of the paediatric intestinal tract is crucial for accurate prediction of drug absorption, but fundamental data is minimal for this cohort (El-Khateeb, Vasilogianni et al. 2019). Intestinal drug metabolising enzymes and transporter proteins (collectively, DMET proteins) are important regulators of oral drug absorption (Harwood, Achour et al. 2015). Currently, limited data describes the ontogeny pattern for these proteins, based on less sensitive immunohistological examination or mRNA analysis, hence advanced characterisation using more sensitive methods are required. Peptide sequences that uniquely belong to a single protein (proteotypic) can be identified and quantified using LC-MS/MS. Heavy isotope labelled copies of proteotypic peptides can be employed for quantification. Previously, proteomic DMET protein quantification in adult liver and small intestine has been performed via a QconCAT (an artificial protein concatenating all proteotypic peptides of interest) approach spiked into the sample (Harwood, Achour et al. 2015, Couto, Al-Majdoub et al. 2020).

Previous reports quantified DMET-protein abundance in the adult small intestine, but only limited data for the paediatric cohort is available (Kiss, Mbasu et al. 2021). This study reports paediatric intestinal DMET protein abundance via a QconCAT approach. Target proteins include: ABC B1, C2, C3, C4, G2; SLC 15A1; CYP 2C19, 2C9, 2D6, 3A4; UGT 1A1, 1A4, 2B7, 2B17; CES2 amongst others.

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MATERIALS AND METHODS

Paediatric duodenal pinch biopsies were collected (± 17 mg) and stored at –80°C from patients undergoing endoscopy or enterostomy reversal as part of their clinical care at the Birmingham Children's Hospital. Informed consent was obtained from the carers of every participant (ethical approval: IRAS 251909). Protein extraction and digestion: frozen samples were manually cryopulverised using a mortar and stamper cooled with liquid N_2. The powder was resuspended in 100 µL radioimmunoprecipitation assay (RIPA) buffer with protease-inhibitor cocktail (cOmplete, Roche) and snap-frozen in liquid N_2. For batch processing, samples were thawed, incubated under gentle agitation (30 min at 4°C), sonicated and centrifuged at 9,000 *g* (5 min at 4°C). Protein concentration in the isolated supernatant was assessed using Pierce's Protein BCA Assay (Thermo Fisher) and adjusted to 1 µg protein/µL. A QconCAT containing proteotypic peptides for the target proteins (PaedCAT, PolyQuant) was spiked in. Proteins were reduced, alkylated and digested (Promega, Trypsin/Lys-C Mix, Mass Spec Grade) using a filter associated sample protocol (Amicon Ultra 0.5mL 3000, Merck) digestion. Peptide samples were stored at -70 °C until LC-MS/MS analysis. LC-MS/MS: peptide samples were analysed via LC-MS/MS (Shimadzu 8060NX), with a flow rate of 0.5 mL/min and a gradient of 3-95% acetonitrile over 10 min, returning to starting conditions, using an C18 HSS T3 column (Acquity Premier Peptide, 100 Å, 1.8 µm 2.1 x 100 mm, Waters). MRM and collision energy optimisation and protein quantification were performed in Skyline v21.2.0.

RESULTS AND DISCUSSION

Preliminary results are available for a 2- and 15-year-old participant for selected proteins, Table 1. These indicate DMET expression may be dependent on age. These values are compared to data previously reported in adults. In time, a full set of data from 36 duodenal samples will be included in our analysis which will provide a more detailed picture of the ontogeny of DMET proteins in the paediatric duodenum. Existing proteomic data on paediatric intestinal DMET abundance is available for the jejunum and ileum, where biopsies of ±150 mg were analysed (Kiss, Mbasu et al. 2021). The initial results here show that quantification of DMET proteins on significant smaller biopsies is possible as well. Current adult expression values via LC-MS/MS are based on jejunal or ileal biopsies, whereas in this study the paediatric biopsies are duodenal.

Table 1. *Preliminary protein expression levels (pmol/mg total mucosal protein) for selected DMET proteins in a 2 year old and 15 year old duodenal sample.*

Protein	2 year old	15 year old	Adult[a]
ABC B1	0.46	0.84	0.74 ± 0.22
CYP 2C9	158.54	75.24	1.52 ± 0.61
CYP 3A4	61.92	49.86	33.33 ± 5.70
UGT 2B7	3.82	8	1.84 ± 0.97

[a]: Jejunal adult mean expression values taken from (Couto, Al-Majdoub et al. 2020)

CONCLUSIONS

Initial results indicate duodenal DMET protein expression in paediatrics may vary with age. Further data on a larger sample size and more proteins are needed to determine the significance of this finding.

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