A Dual UHPLC-HRMS-Based Fecal Metabolomics and Lipidomics Analysis and Automated Data Processing Pipeline for Comprehensive Gut Phenotyping

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ABSTRACT:
In recent years, feces has surfaced as the matrix of choice for investigating the gut microbiome-health axis because of its non-invasive sampling and the unique reflection it offers of an individual's lifestyle. In cohort studies where the number of samples required is large, but availability is scarce, a clear need exists for high-throughput analyses. Such analyses should combine a wide physicochemical range of molecules with a minimal amount of sample and resources, and downstream data processing workflows that are as automated and time efficient as possible. We present a dual fecal extraction and UHPLC-HR-Q-Orbitrap-MS-based workflow that enables widely targeted and untargeted metabolome and lipidome analysis. A total of 836 in-house standards were analyzed, of which 360 metabolites and 132 lipids were consequently detected in feces. Their targeted profiling was validated successfully with respect to repeatability (78% CV<20%), reproducibility (82% CV<20%) and linearity (81% R^2>0.9), while also enabling holistic untargeted fingerprinting (15 319 features, CV<30%). To automate targeted processing, we optimized an R-based targeted peak extraction (TaPEx) algorithm relying on a database comprising retention time and mass-to-charge ratio (360 metabolites and 132 lipids), with batch-specific quality control curation. The latter was benchmarked towards vendor-specific targeted and untargeted software and our IPO/XCMS-based untargeted pipeline in Lifelines Deep cohort samples (n = 97). TaPEx clearly outperformed the untargeted approaches (81.3 *vs.* 56.7-66.0% compounds detected). Finally, our novel dual fecal metabolomics-lipidomics-TaPEx method was successfully applied to Flemish Gut Flora Project cohort (n = 292) samples, leading to a sample-to-result time reduction of 60%.

Metabolomics is a holistic top-down analytical approach that maps the whole of small (<2 500 Da) molecules present in any biological matrix, as such offering an accurate reflection of an individual's physiological state. Metabolomics is highly useful for studying disease pathology, discovering clinical biomarkers, and developing new therapeutic strategies[1,2]. Because the human gut microbiome plays a vital role in human health, but 16S rDNA sequencing characterization lacks quantitative functional annotation, feces has been popularized as a metabolomics matrix in recent years[3]. Fecal metabolomics provides a complementary functional readout of the gut microbial metabolism, as well as its interaction with the host and environmental factors including dietary and drug intake[4].

Currently, analysis of fecal metabolites and lipids using ultra-high performance liquid chromatography (UHPLC) hyphenated to high-resolution mass spectrometry (HRMS) requires at least two separate strategies for both sample preparation and analysis[5–7]. The typical workflow for both metabolomics and lipidomics involves multiple time-consuming steps including freeze-drying, weighing and homogenization of the sample, extraction of the to-be-measured molecules from the sample with optimized solvent mixtures and purification, followed by chromatographic separation and detection[8]. Due to the increased interest in the implementation of fecal metabolomics for the analysis of large batches of samples (n>100) in light of e.g. longitudinal cohort or exposomics studies where sample availability is scarce[9], there is a clear need for comprehensive high-throughput analyses combining an as large as possible physicochemical range and physiological scope of molecules, using a minimal amount of sample material and consumables.

Besides requiring wide-scale, yet also high-throughput metabolomics sample analysis, there is also a distinct need for less time-consuming data processing and analysis workflows,

wherein automatization of large-scale metabolite profiling is envisioned. The (semi)-automated high-throughput metabolite profiling of metabolites and lipids may be accomplished by two approaches: untargeted peak search[10–12] and targeted peak extraction[13]. In untargeted peak search, a feature table is obtained through preprocessing algorithms, most importantly automated peak picking, and then a search is performed based on compound data such as retention time (RT) and mass-to-charge ratio (*m/z*), to annotate a selection of target compounds[10–12]. In targeted peak extraction, the extracted ion chromatogram (EIC) of the known compound is constructed directly from the data file[13,14] using the compound-specific information (RT, *m/z*, etc.).

The main challenge in targeted processing is that it requires vast expertise to curate the chromatographic peaks, is labor-intensive and thus very time-consuming. Furthermore, it relies heavily on proprietary, vendor-specific software (e.g. Thermo's XCalibur™). These proprietary tools are often easy to use, offering an extensive graphical user interface (GUI), but limit reproducibility and transparency between labs or even within the same lab[15]. Although open-source alternatives for targeted processing exist, e.g., Skyline: a software package for quantitative metabolomics[16], the work remains highly repetitive and moreover still requires a high level of training.

Due to the high sensitivity and broad coverage of untargeted LC-MS-based metabolomics and lipidomics, a vast amount of high-dimensional data is generated, with the detection of thousands of metabolites per sample[17–19]. Manual processing of this immense number of compounds is deemed impossible, and as such several proprietary and open-source software tools have been developed to automate data (pre)processing, including e.g., Compound Discoverer™ (Thermo Fisher Scientific, San Jose, CA, USA), XCMS[11], OpenMS[20] and MZmine[21]. Two important challenges in untargeted preprocessing of metabolomics data are parameter optimization and the detection of false positive peaks[22–24]. The first challenge entails that proprietary and open-source parameter settings for preprocessing are frequently difficult to interpret and suboptimal parameter settings can lead to biased results[25–27]. Thus, parameter optimization is required for the different steps in data preprocessing (extraction of ion traces, peak detection, adjusting RTs, grouping peaks into features, …), which all involve different algorithms (e.g. centWave[10] and OBI-Warp[28]). Therefore, to facilitate automatic parameter optimization, several tools have been developed, like e.g., Isotopologue Parameter Optimization (IPO)[26], Autotuner[25] and Paramounter[27]. The hence-obtained optimized parameters generally outperform default settings but can be topped by expert-chosen parameter settings[29]. The second challenge in untargeted metabolomics preprocessing is the retrieval of false positive peaks, which add unwanted noise and might end up as falsely discriminant features in downstream comparative analysis[24]. Different strategies are being employed to counter this. A first strategy attempts to improve peak picking performance by tweaking the centWave algorithm, such as ADAP and EIC+[24,30]. A second strategy involves filtering poor-quality peaks by means of tools like MetaClean, Peakonly, and NeatMS, which use deep learning and machine learning classification to classify peaks as real, high-quality peaks or as noise[31–33]. Alternative tools also rely on filtering, e.g. the Comprehensive Peak Characterization (CPC)[18] algorithm, which characterizes and filters the peaks after XCMS processing to reduce data complexity and increase data quality.

In this work, the consecutive extraction and dual analysis of both the metabolome and lipidome (Fig. 1A) were optimized and a total of 836 in-house metabolite and lipid standards encompassing a wide physicochemical variety (Fig. 1B, Table S1) were screened. Of these, 360 metabolites and 132 lipids were consistently detected in feces and their targeted profiling was successfully validated with respect to repeatability, reproducibility and linearity, while also enabling holistic untargeted fingerprinting in parallel. To automate the targeted processing of said 360 fecal metabolites and 132 lipids, and as such maximally harvest the underlying molecular signature of the human gut metabolome, we finetuned our in-house R-based untargeted peak picking pipeline (IPO/XCMS-based) (Fig. 1C) and developed and optimized an R-based targeted peak extraction approach (TaPEx) (Fig. 1D). The latter utilized method-specific metabolome and lipidome database searches with entries (RT and *m/z*) re-iterated per batch, employing quality control samples run throughout the batch. TaPEx was subsequently benchmarked towards manual vendor-specific software-based targeted data analysis (XCalibur™), IPO/XCMS[34] and vendor software-based untargeted peak picking (Compound Discoverer™) on our validation QC samples (n = 39) and a subset of the Lifelines Deep (LLD) cohort (n = 97). Finally, our novel dual fecal metabolomics-lipidomics analytical methodology, followed by automated TaPEx targeted profiling and IPO/XCMS-based untargeted fingerprinting were successfully applied to the Flemish Gut Flora Project (FGFP) Long cohort (n = 292), resulting in a total sample-to-result time reduction of 60% (Fig. 1E).

MATERIALS AND METHODS

Analytical Standards and Reagents. Analytical standards (n = 836) (Table S1, Fig. 1B), internal standards (ISTDs) for metabolomics (n = 6; alanine-d3, dopamine-d4, tyrosine-d2, phenylalanine-d2, deoxycholic acid-d4 and indole-3-acetic acid-d5) and ISTDs for lipidomics (n = 2; palmitic acid-d31 and 1,2-dimyristoyl-d54-sn-glycero-3-phosphocholine (14:0 PC-d54)) were purchased from Sigma-Aldrich (St-Louis, Missouri, USA), ICN Biochemicals Inc. (Ohio, USA), TLC Pharmchem (Vaughan, Ontario, Canada) and Gianni Degreve Cambrigde Isotope Laboratories Inc. (Tewksbury, Massachusetts, USA). Solvents (methanol, ethanol, acetonitrile, dimethyl sulfoxide and IPA) were acquired from Fisher Scientific UK (Loughborough, UK) and VWR International (Merck, Darmstadt, Germany).

Biological Samples. For optimization and validation purposes, fecal samples from 4 healthy donors (2 females, and 2 males, average age = 29 ± 6 years), hereafter called QC samples (n = 4), from our prospective UGent – LCA biobank (BR-184) were used.

For benchmarking of the data processing pipeline, selected samples (n = 97, average age = 46 ± 11 year) of the LLD cohort were used. The LLD cohort (n = 1500) is a sub-cohort of the large prospective Lifelines cohort study[35]. Lifelines is a multidisciplinary prospective population-based cohort study examining the health and health-related behaviors of 167 729 persons living in the North of the Netherlands in a unique three-

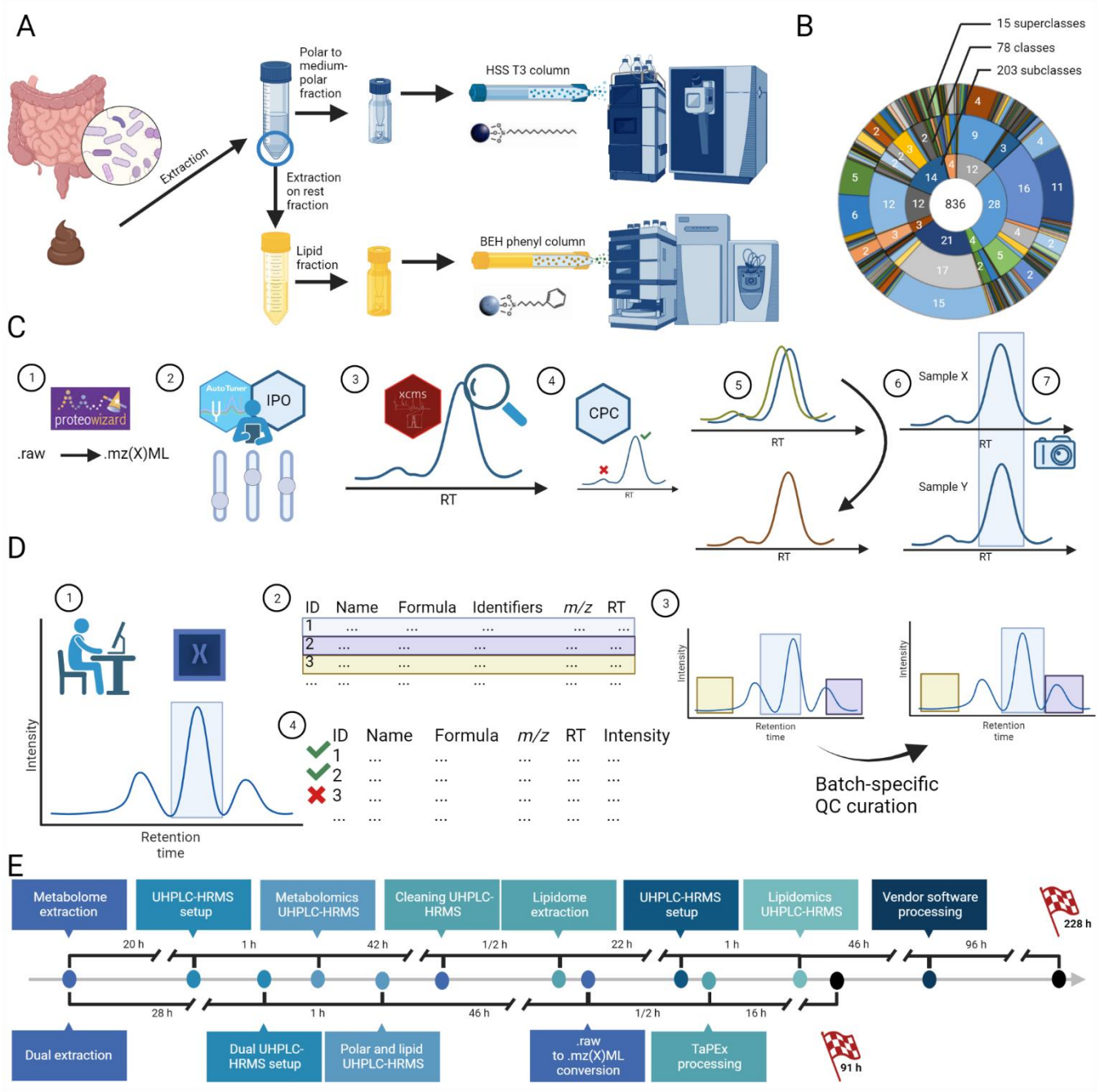

Figure 1. Visualization of the analytical and data analysis workflows used in this work. **A**. Dual extraction workflow. After sample preparation, metabolome extraction was performed, after which lipid extraction was performed on the rest fraction. Both fractions were subsequently analyzed in parallel on two state-of-the-art UHPLC-HRMS systems. **B**. Ontology of the LIMET in-house analytical standard library comprising a total of 836 analytical standards. Percentages are shown for the most abundant superclasses, classes and subclasses. **C.** Untargeted processing workflow. First, samples were converted from the vendor-specific to an open-source format (1). Next, parameters for XCMS processing were optimized using Autotuner, IPO and analyst knowledge (2). Peak picking was performed using the XCMS centWave algorithm (3) after which the CPC algorithm was used to assess peak quality. Afterwards, the XCMS OBI-Warp algorithm corrects retention time differences between the samples and aligns the peaks (5). Finally, the peaks are grouped into features using the XCMS density algorithm, and isotopes and adducts are annotated by CAMERA (6, 7). **D.** Overview of the TaPEx workflow. First, using vendor-specific software, a benchmark list of targeted compounds defined by their m/z and RT was constructed by experienced analysts (1, 2). Next, the chromatograms for the compounds in the database were extracted by TaPEx in a new sample set. To account for the shift in RT between batches of samples, the RT of the compounds was automatically curated based on batch-specific QC samples (3). Finally, an intensity table and diagnostic plots were returned, so the user could assess which compounds are reliably detected in the samples (4). **E.** Timeline of the different steps and their estimated time span to detect 360 metabolites and 132 lipids in 97 samples (and 20 QCs) in the classic, sequential and manual approach *vs.* our proposed parallel, semi-automated workflow.

generation design. It employs a broad range of investigative procedures in assessing the biomedical, socio-demographic, behavioral physical, and psychological factors which contribute to the health and disease of the general population, with a special focus on multi-morbidity and complex genetics from the North of the Netherlands[36]. All LLD cohort participants signed an informed consent form before sample collection. Individuals from the Flanders region of Belgium were recruited into the FGFP cohort through public announcements in print and social media through the FGFP website (www.vib.be/darmflora), from January 2013 onwards. Volunteers provided informed consent by mail and FGFP procedures were approved by the medical ethics committee of the University of Brussels/Brussels University Hospital (approval 143201215505, 5/12/2012). A declaration concerning the FGFP privacy policy was submitted to the Belgian Commission for the Protection of Privacy. FGFP samples and data were collected as described in Falony et al.[37] In short, stool samples were collected between June 2013 and April 2016 by mail. Sampling kits were sent to volunteers' home addresses and upon collection, samples were stored at -18 °C locally, cooled during delivery, and again stored at -18 °C upon arrival at a collection point until long-term storage was possible at -80 °C at the research facility. All samples were freeze-dried, ground, and sieved upon receipt at LIMET (within 48 h) and stored at -80 °C.

Optimization of Dual Fecal Metabolomics and Lipidomics Extraction. The metabolome extraction was optimized using an experimental setup in JMP 16 (SAS Institute Inc, Cary, USA), in which the effect of 3 parameters was screened with a full factorial design in line with our earlier fecal metabolomics work in a targeted and untargeted fashion[6]. A variable amount (50, 100 or 200 mg) of lyophilized homogenized feces was mixed with a proportionate amount (1, 2 or 4 ml) of ultrapure water (UPW). Then, 10 µl of a mixture of six ISTDs was added, after which a mixture of ice-cold methanol and UPW or a mixture of acetonitrile and UPW in a ratio of 25:75, 50:50, and 75:25 was added proportionally (250, 500 or 1000 µl) to the fecal slurry. Power analysis (JMP 16, SAS Institute Inc, Cary, USA) showed the highest intercept (p = 0.99) for the design with 2 replicas and 3 center points, resulting in 60 experimental runs. The effect of each factor was statistically evaluated based on the untargeted metabolome coverage (i.e., total number of detected components), as well as the summarized peak area of a representative selection of 161 metabolites based on our previous work[5,6] (marked with an * in Table S1).

For the fecal lipidome extraction, the residual fraction of the metabolome extraction was used as the starting point. Next, the obtained lipidome extracts were compared to extracts generated using our recently optimized single fecal lipidomics protocol[7]. This was statistically evaluated based on the untargeted lipidome coverage, as well as the summarized peak area of the 138 analytical standards included in our lipidomics target panel[3,7] (marked with an § in Table S1).

Final Dual Fecal Metabolomics and Lipidomics Extraction Protocol. To extract the fecal metabolome, 100 mg of feces was dispensed in 2 ml of UPW. After the addition of 12.5 µl of a 100 ng/µl mixture of six ISTDs, 0.5 ml of a mixture of ice-cold methanol and UPW (75:25, v/v) was added. The solution was thoroughly vortexed for 1 min, rotated for 10 min, and centrifuged for 10 min at 9000 rpm (room temperature) Next, the supernatant was collected and passed over a polyamide filter (25 mm diameter, 0.45 µm pore size, Macherey-Nagel, Düren, Germany). Depending on the size of the sample batch, a 1:3 (n>20) to 1:6 (n>100) dilution with UPW was additionally applied. Finally, 500 µL of the diluted extract was transferred to a glass LC vial.

To extract the fecal lipidome, the residual fraction of the metabolomics extraction was supplemented with 600 µl of methanol containing 0.01% (w/v) butyl-hydroxy toluene (BHT) to 100 +/- 0.50 mg and vortexed for 1 min. Next, 2.7 ml of methyl tert-butyl ether (MTBE) with 0.01% (w/v) BHT was added to the homogeneous mixture and vortexed for 30 s[7]. Subsequently, the sample was shaken for 20 min at 200 rpm at 20 °C in an incubator (New Brunswick Innova 42, Eppendorf). Thereafter, 1.5 ml of UPW with 2.5% trichloroacetic acid (w/v) was added, followed by centrifugation for 5 min at 3000 x g (20 °C). Then, 500 µl of the upper layer consisting of MTBE was collected and transferred to a Teflon tube (40 mL, VWR International, Darmstadt, Germany) and evaporated to dryness at 30 °C under a gentle stream of nitrogen. The residue was sequentially suspended in 125 µl of chloroform and 325 µl of methanol followed by 5 min of centrifugation at 3000 x g (20 °C). Finally, a 50-µl subfraction was transferred to an amber glass LC-vial and diluted 1:2 by the addition of 50 µl lipidomics ISTD mixture (concentrations between 1 and 100 ng/µl).

Optimization of Fecal Metabolomics HESI source and HRMS Parameters. A total of 10 parameters, i.e., ion spray voltage, sheath gas, aux gas and sweep gas flow, ion transfer tube and vaporizer temperature, S-lens Radio Frequency level, position source, mass resolution and injection time were further optimized using three replicas of fecal metabolome extracts per parameter. This was evaluated based on the untargeted coverage, as well as targeted processing of the same selection of 161 analytical standards used for the extraction optimization (marked with an * in Table S1).

Final Fecal Metabolomics and Lipidomics UHPLC-HRMS Analysis Protocols. For metabolomics, chromatographic separation was achieved using a Vanquish Flex UHPLC system (Thermo Fisher Scientific, San José, CA, USA), equipped with an Acquity HSS T3 column (150 x 2.1 mm, 1.8 µm) (Waters, Manchester, UK) kept at a constant temperature of 45 °C, as adapted from De Paepe et al.[5]. The binary solvent system consisted of UPW (A) and acetonitrile (B), both acidified with 0.1% formic acid. Using a flow rate of 400 $\mu L \cdot min^{-1}$, the following gradient was applied (solvent A, v/v): at 98% from 0-1.5 min, 98% to 75% from 1.5-7.0 min, 75% to 40% from 7.0-8.0 min, 40% to 5% from 8.0-12.0 min, at 5% from 12.0-14.0 min, 5% to 98% from 14.0-14.1 min, followed by a re-equilibration step of 4.0 min. Detection was performed using an Orbitrap Exploris™ 120 mass spectrometer (Thermo Fisher Scientific, San José, CA, USA), preceded by heated electrospray ionization (HESI-II source) in polarity switching mode (position: interL/M/1.5). Instrumental parameters included a sheath, auxiliary and sweep gas flow rate of 55, 25 and 3 arbitrary units (a.u.) respectively, heater and capillary temperature of 300 and 300 °C respectively, S-lens RF level of 50%, and a spray voltage of 2.9 kV for both positive and negative ionization mode. The *m/z* scan range was set from 53 to 800 Da, the automatic gain control target was 1 x 10^6 ions, the maximum injection time was 70 ms and the mass resolution was 120 000 Full Width at Half Maximum (FWHM) (1 Hz). The sample injection volume was 10 µL.

For the lipidomics analysis[7], chromatographic separation was achieved by means of a Dionex UltiMate 3000 XRS UHPLC system (Thermo Fisher Scientific, San José, CA, USA), equipped with an Acquity ethylene-bridged hybrid (BEH) phenyl column (150 × 2.1 mm, 1.7 µm) (Waters, Manchester, UK) kept at a constant temperature of 45 °C. A Hypersil Gold column (50 x 2.1 mm, 1.9 µm) (Thermo Fisher Scientific, San José, CA, USA) was installed between the LC pump and injector valve, with the purpose to delay any lipophilic compounds originating from the solvent system. Hereby, a binary solvent system consisting of UPW (solvent A) and methanol (solvent B), both acidified with ammonium acetate (3.5 mM), was used to establish a gradient elution program. The following proportions (v/v) of solvent B were used: 0−1 min at 75%, 1−2 min from 75% to 90%, 2−6 min from 90% to 98%, 6−15 min from 98% to 100%, and 15−17 min at 100%, followed by 3 min of equilibration at initial conditions. A constant flow rate of 300 µL.min^{-1} and a column oven temperature of 40 °C were set. A Q-Exactive mass spectrometer was employed (Thermo Fisher Scientific, San José, CA, USA) for detection, equipped with a HESI-II source that was operated in polarity switching mode (positioned in 0/B/1). The same instrumental mass spectrometric parameters were applied as described by Van Meulebroek et. al.[7]. An aliquot of 5 µl was injected into the chromatographic system.

Method Validation. In accordance with the guidelines of Naz et al., the method's analytical performance was thoroughly assessed in a targeted and untargeted fashion[38]. For the targeted analysis, we evaluated the consistent abundance (present in 30/30 QCs) of our 836 in-house analytical standards (Table S1, Fig. 1B) in the fecal QC samples (detected in 30/30 QCs) using our optimized dual extraction protocol. A total of 360 metabolites and 132 lipids could be detected successfully, both as an analytical standard and in our fecal QC samples (Table S2) and were further retained for targeted validation of our methods.

Instrumental, intra-, and inter-day assay variances were assessed in a targeted manner for each of the consistently detected metabolites and lipids and in an untargeted manner as a measure of precision. Instrumental precision was determined by repeatedly injecting (n = 10) a QC sample. For the intra-assay precision, multiple QC samples (n = 10) were extracted in parallel under identical experimental conditions, whereas inter-day assay precision (n = 30) included within-laboratory variations such as different analysts and different days[39].

Linearity was assessed for each of the consistently detected metabolites and lipids by considering a nine-point dilution series (1, 2, 5, 10, 20, 50, 100, 200, and 500 times) of a pooled QC (n = 4) with UPW. Usage of the peak area (100 000 au) as a decision criterium instead of the signal-to-noise ratio (S/N) was related to the properties of the HRMS, where S/N is often infinite. Linearity was also determined in an untargeted way. For each metabolite and lipid, linearity was evaluated based on the determination coefficient (R^2).

Data and File Conversion. Both metabolomics and lipidomics .raw files were acquired from the 39 QC validation samples (n = 4) and 97 LLD cohort samples. All .raw files were converted to the open-source .mzXML and .mzML formats using Proteowizard's MSConvert[40]. Each file was centroided and split into a positive and negative polarity open-source format file.

Manual Targeted Benchmark. To establish a ground truth for benchmarking, manual targeted data processing was carried out on all .raw full scan HRMS data files including metabolite identification and quantification using XCalibur™ 4.1 software (Thermo Fisher Scientific, San José, CA, USA) by at least two experienced analysts. Identification of a metabolite required congruence with the corresponding authentic analytical standards, i.e., the *m/z*-value of the molecular ion (mass deviation≤5 ppm), the C isotope pattern ($^{13}C/^{12}C$ isotope ratio, compliant with CD 2002/657/EC) and a retention time relative to that of the ISTD (max. deviation of 2.5%)[41] as such achieving the Tier 1 annotation level[42].

Untargeted Peak Picking Pipeline (IPO/XCMS). An untargeted feature table was generated using our in-house R (version 4.2.1) pipeline (Fig. 2C). Parameters for XCMS (version 3.18.0) preprocessing were optimized using IPO (version 1.21.0) and AutoTuner (version 1.3.0) and were further tuned based on expert knowledge and literature[29,43–46]. The parameters used can be consulted in Table S4. Prior to further preprocessing, empty spectra were filtered. Next, peak picking was performed with XCMS centWave and low-quality peaks were filtered using CPC. The CPC step was excluded during extraction and analytical method optimization. The remaining peaks were merged with neighboring peaks within each sample and RT alignment was performed using XCMS OBI-Warp. Then, the XCMS density algorithm was used to perform correspondence, in which detected peaks are matched between samples and groups them into features. Isotopes and adducts were identified using the CAMERA[47] R package (version 1.52.0).

Targeted Peak Extraction Pipeline (TaPEx). The most used data handling functions from XCMS and MSnbase[48], and code adapted from the MetEx package (https://github.com/zhengfj1994/MetEx, accessed on 19 August 2022) were wrapped into our database specific TaPEx pipeline (Fig. 2D). Based on an input table containing the targeted compounds defined by their *m/z* and RT, EICs were extracted from the data. Within these EICs, peaks were automatically recognized using the findPeaks algorithm to find local maxima (adapted from https://github.com/stas-g/findPeaks, accessed on 19 August 2022). A mass deviation of 5 and 10 ppm was allowed for metabolomics and lipidomics, respectively. For both metabolomics and lipidomics databases, an RT window of ± 0.3 min was applied. The output consisted of an intensity table containing the intensities of each compound in each sample and diagnostic plots that allow evaluation of the peaks. First, this method was applied to the validation QC samples. To ensure robustness in case of so-called RT drift (measured RT in samples deviating from RT in the database) caused by column wear, type of column used, etc. in larger cohorts such as LLD and FGFP, TaPEx was run first on three QC samples (one ran in the beginning, middle and at the end of the analysis batch). For these QC samples, the diagnostic plots were manually inspected to assess which peaks could be reliably detected by the algorithm (Fig. S1 and S2). Next, for the peaks that were reliably detected, the RTs to extract EICs in samples were automatically altered to the average RT of the peaks extracted in the previously analyzed QC samples (Fig. S3), resulting in a final intensity table of all components per sample.

Untargeted Peak Search. To search for known targeted compounds based on *m/z* and RT in the untargeted feature table obtained from our untargeted peak picking pipeline (IPO/XCMS)[34], the MetaboAnnotation[12] R package was used. This method was applied both on the validation QC samples and

samples from the LLD cohort data. To include a comparison with industry-standard software, the same methodology was applied on an untargeted feature table generated using Compound Discoverer™ 3.3 (Thermo Fisher Scientific, San José, CA, USA). The parameters of Compound Discoverer™ 3.3 (Table S5) were tuned to match those of IPO/XCMS as close as possible for a fair comparison in performance.

RESULTS AND DISCUSSION

Selection of Panel of Targeted Metabolites and Lipids. Our targeted panel of 836 metabolites (Table S1, Fig. 1B) for which analytical reference standards were acquired, was selected based on our initial list of relevant fecal metabolites from 2015[6] and 2018[5] and lipids from 2017[7]. The metabolites are characterized by a broad range of psychochemical properties and were considered relevant from a gastrointestinal perspective[49–53], while the selected fecal lipids covered all 8 LIPID-MAPS classes[54]. This list was further complemented with mostly metabolites and some lipids that were detected in the last 5 years in multiple fecal metabolomics and lipidomics studies[5,55–58], the HMDB[59] and recent work from Han et al. reporting on 833 gut microbial metabolites[3].

Optimization of Dual Metabolomics and Lipidomics Extraction. As a first step towards obtaining a dual extraction approach for both the fecal metabolome and lipidome, the extraction parameters that showed the highest impact based on prior stool metabolomics studies [6,49,50,60–64], i.e., starting weight of the fecal sample, type of solvent and solvent ratio were studied more in-depth using an experimental design (18 different combinations, 120 runs). The 4 combinations that generated the highest number of untargeted components (26 157 - 26 296), and highest peak areas and repeatabilities for the 161 target analytes (i.e., CV<20% for 123 - 135/161), were analyzed again in triplicate to corroborate the obtained results. A fecal sample mass of 100 mg, and methanol *vs.* UPW ratio of 75:25 (v/v) generated the highest number of untargeted components (26 296) with 15 966 of those untargeted features displaying a CV<20% and 135/161 targeted features displaying a CV<20%. This extraction protocol is relatively similar to earlier reported procedures for fecal metabolomics[6,64–66], the main differences pertain to the lower starting material mass (100 *vs.* >= 200 mg) and the different methanol *vs.* UPW ratio (75:25 *vs* 80:20).

The residual metabolome fraction was used to perform a lipidomic extraction. Because our lipidomics extraction protocol was optimized relatively recently and displayed excellent results[7] both untargeted as well targeted (8 LIPID-MAPS classes), we did not re-optimize the solvent composition and other relevant extraction parameters, but merely compared our dual approach with the original protocol starting from crude feces. In comparison to the original single fecal lipidomics extraction[7], the dual approach generated 3 191 *vs.* 3 252 untargeted components, while 122 *vs.* 128/132 targeted lipids were detected. As such, the dual approach was considered sufficiently performant for implementation in large cohort studies. To the best of our knowledge, dual protocols have been reported for blood[67] and tissue[68], but is unique for feces.

Optimization of UHPLC-HRMS Metabolomics Analysis. The optimized dual extraction protocol was applied to a series of pooled QC samples. The obtained metabolomics extracts were further used to optimize the HESI source and HRMS parameters on the Exploris 120 mass spectrometer (Thermo Fisher Scientific, San José, CA, USA). Maximal untargeted feature counts (37 572) and number of consistently detected targeted features (140/161) were obtained with a sheath, auxiliary, and sweep gas flow rate of, respectively, 50, 25, and 3 a.u., a heater and capillary temperature of 300 °C and 300 °C, an S-lens RF level of 50 V and a spray voltage of ± 2.7 kV and deviated only minimally from those reported earlier for HESI-Orbitrap instruments in fecal metabolomics analysis [64,69,5].

Dual Metabolomics-Lipidomics Analytical Method Validation. We pursued the validation of our optimized dual metabolomics and lipidomics methodology using various performance characteristics (Table 1), which were assessed in a targeted and untargeted fashion. For the targeted metabolites and lipids consistently detected in the pooled QC samples (Tables S2 and S3) the instrumental variability, repeatability (intra-day variability), reproducibility (inter-day variability), and linearity were evaluated (Table 1, Tables S6 and S7). Targeted evaluation of the dilution series showed good linearity ($R^2 \geq 0.90$) for 304 out of 360 metabolites and 94 out of 132 lipids. Similar results for linearity were observed in previous studies for both fecal metabolomics and lipidomics, although the number of known compounds used for targeted validation was significantly higher in the current study (>15-fold)[6,7].

For targeted profiling, the FDA guidelines recommend a CV≤15%, except when operating close to the limit of detection (CV≤20%)[41]. For untargeted fingerprinting, a CV≤30% is generally recommended as acceptable precision[41]. Our novel dual metabolomics and lipidomics fecal extraction method established a high fecal metabolome coverage with a good level of repeatability and reproducibility. To the best of our knowledge, this is the first report presenting the repeatability and reproducibility of so many molecules, with such a broad physicochemical diversity (Fig. 1B) in feces [69,70].

Table 1. Summary of the UHPLC-HRMS validation of dual fecal metabolomics and lipidomics. For the targeted analysis, the number of analytes is reported. Regarding the untargeted analysis, the % refers to the % of components that match the specified criteria.

	Targeted		Untargeted	
	Metabolome	Lipidome	Metabolome	Lipidome
Linearity ($R^2 \geq 0.90$)	304/360	94/132	-	-
Precision				
Instrumental (CV<20%,	345/360	90132	-	-
CV<30%)	355/360	108/132	86%	49%
Intra-day (CV<20%,	344/360	57/132	-	-
CV<30%)	353/360	88/132	85%	45%
Inter-day (CV<20%,	336/360	49/132	-	-
CV<30%)	347/360	84/132	85%	45%

Fig. S4 shows our validation results for the 360 metabolites (A-C) and 132 lipids (D-E) per superclass. For the metabolites and lipids, respectively, eight out of twelve superclasses and six out of seven superclasses were presented as the remaining superclasses were only represented by one analyte. Notably, those compounds detected with a lower peak area (and thus abundance) generally also display higher CV-values, therefore being responsible for the outliers in Fig. S4. This was the case for i.a. cotinine, biliverdin, 4-methyl valeric acid, 2-methyl maleic acid, and adipoylcarnitine for which reproducibility CV-values

of 34.6%, 28.5%, 50.3%, 53.2%, and 51.2%, respectively, were obtained. Moreover, all metabolite superclasses presented relatively lower average CV values compared to the superclasses representing the lipidome.

Target Compound Database. For the method-specific metabolomics and lipidomics database, the detection of the aforementioned 360 targeted metabolites and 132 targeted lipids was confirmed in the validation QC samples using manual curation by an experienced analyst using Xcalibur™ 4.1, and as such the entry databases for our TaPEx pipeline were created (Tables S2 and S3).

Target Compound Detection. In the validation QC samples (n = 39), 298/360 metabolites and 98/132 lipids could be reliably detected by the TaPEx approach (see Table S8 and S9 for peak intensities). Using the untargeted feature list obtained when running our method-specific (metabolomics and lipidomics) optimized IPO/XCMS pipelines (Fig. 1C) 253/360 metabolites and 68/132 lipids were retrieved, respectively (peak areas in Table S10 and S11) while using the same approach with CD 3.3 generated 290/360 metabolites and 82/132 lipids (Fig. 2, peak areas in Table S12 and S13).

In the LLD cohort samples, TaPEx detected 304 metabolites and 96 lipids (intensities in Table S14 and S15), corresponding to 81.3% of all targeted compounds. To ensure reliable detection, TaPEx was applied on pooled QC samples (n = 20) that were run throughout the LLD cohort samples first (2 before and after and in between every 10 samples). Following visual inspection by an experienced analyst, our TaPEx algorithm automatically shifts the RT range to be used when applied to the samples based on the results of the batch-specific QCs (Fig 1. D). Using IPO/XCMS, respectively 264 metabolites and 61 lipids were found (areas in Table S16 and S17), CD 3.3 generated 218 metabolites and 61 lipids (Fig. 3, areas in Table S18 and S19) which corresponds to 66% and 56.7% of targeted compounds respectively.

Comparison of TaPEX to IPO/XCMS and CD 3.3. In the validation QC samples 80.5%, 65.2%, and 75.6% of the total number of metabolites and lipids were retrieved by TaPEx, CD 3.3- and IPO/XCMS, respectively (Fig. 2). None of the three methods managed to successfully retrieve all manually curated peaks. A total of 28, 17 and 3 components were uniquely retrieved by TaPEx, CD 3.3 and IPO/XCMS, respectively. It was expected that none of the approaches would succeed in retrieving all compounds from the manual benchmark. In manual curation, peaks that deviate from the expected peak shape and RT can still be integrated by the analyst. Furthermore, when using the default settings for peak detection, the XCalibur™ software applies gaussian smoothing to the data, which can cause few data points to present as high-quality chromatographic peaks. Whilst it has been demonstrated by Loziuk et al. that this smoothing does not impact the processing results[71], these peaks are less likely to be retained by the algorithms in XCMS and CD 3.3. The lower number of compounds detected in the CD 3.3- and IPO/XCMS-based approach was also expected because low-quality peaks were filtered at different points during both CD 3.3 and IPO/XCMS preprocessing (e.g., by the CPC algorithm) (Fig. 1C). TaPEx does not contain such (automatic) quality control and will thus include any data points within the given RT and *m/z* ranges. The discrepancy between CD 3.3 and IPO/XCMS shows that, despite our efforts to match XCMS parameters to those of CD 3.3, there are still (performance) differences in the algorithms for peak picking and quality filtering[24,46].

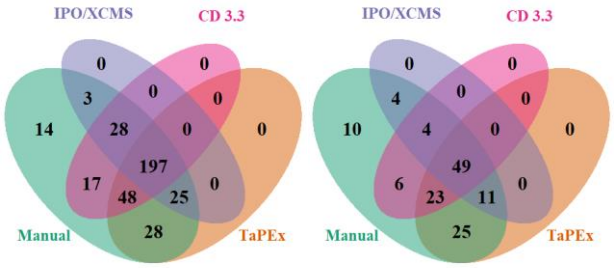

Figure 2. Venn diagram displaying the number of detected targeted compounds in the validation QC samples (n = 39) by the different approaches for metabolomics (left) and lipidomics (right).

The results displayed in Fig. 3 demonstrate that TaPEx was able to extract a larger number of metabolites and lipids compared to both CD 3.3- and IPO/XCMS-based approaches. Furthermore, it is notable that there was a greater overlap in compounds between TaPEx and IPO/XCMS, than between CD 3.3 and IPO/XCMS, reinforcing again that different peak picking algorithms gain variable results, as reported before[24,46].

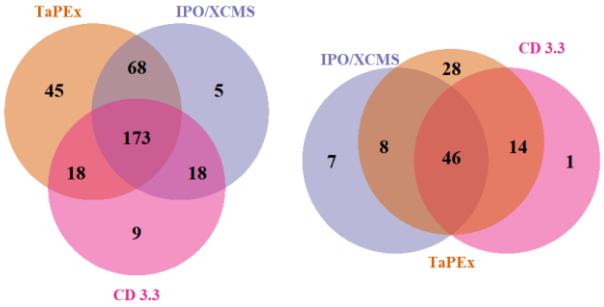

Figure 3. Venn diagram displaying the number of detected targeted compounds in the benchmark LLD sample data (n = 97) by the different approaches for metabolomics (left) and lipidomics (right).

Also, the number of compounds uniquely detected by TaPEx was greater than for both other approaches (Fig. 4). It is clear that TaPEx outperforms IPO/XCMS and CD3.3 in the detection of lipids, organic acids, nucleosides, organic nitrogen- and organoheterocyclic compounds. Furthermore, TaPEx successfully enabled the detection of larger compounds (*m/z* > 500), eluting at later RTs, while compounds uniquely detected by IPO/XCMS and CD3.3 were mainly metabolites characterized by lower *m/z*-values eluting at earlier RTs. Combined, the methods were able to retrieve all but 24 compounds from our targeted metabolome (n = 360) and lipidome (n = 132) fecal panel (95%). Compounds that could not be retrieved by any of the methods were mainly lipids and lipid-like molecules (Fig. S5). This was expected, as the results presented in Fig. 2 already displayed a lower retrieval rate for lipidomics compared to metabolomics. These results indicate that TaPEx is a promising leap forward in the automated detection of lipids and that future research should focus on improving automated peak-picking in lipidomics.

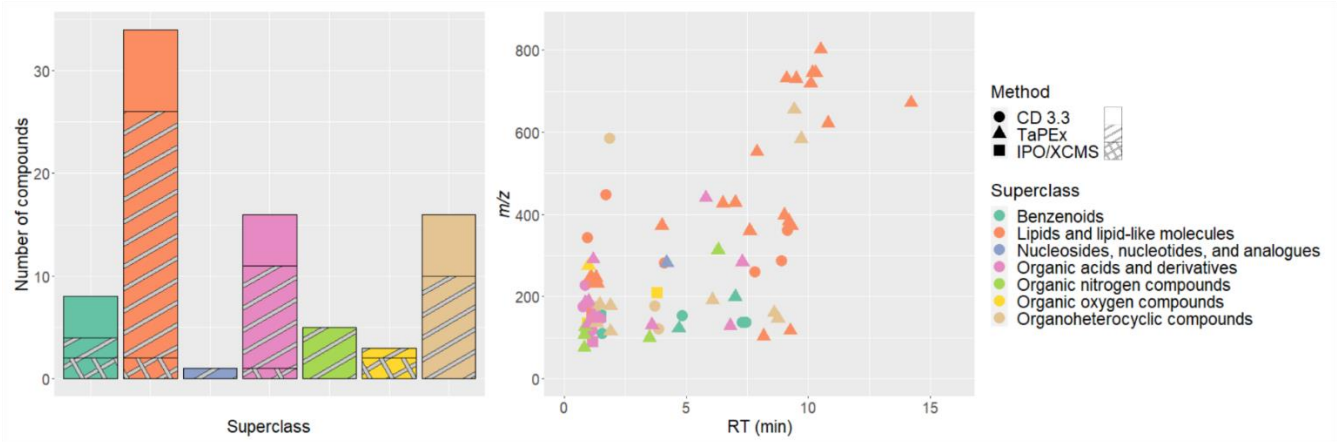

Figure 4. Visualization of the unique chemical space covered by the different methods used for targeted metabolome (n = 360) and lipidome (n = 132) peak detection. Left: bar plot presenting the number of compounds uniquely retrieved by each method, colored according to compound class. The pattern on the bars indicates the applied method. Right: Scatterplot of the uniquely retrieved compounds.

TaPEx Performance and Application to Larger Cohorts. Both results for the QC validation samples and the LLD cohort samples point towards TaPEx outperforming the untargeted approaches with respect to the number of targeted compounds generated successfully. TaPEx also outperforms the untargeted processing-based methods in terms of computing speed (~60 minutes *vs.* ~24 hours for 97 samples and 20 QCs on a machine equipped with an Intel® Core™ i9-9900 CPU) since it does not rely on generating an untargeted feature table first and lacks additional quality control algorithms. The lack of automatic quality control brings about the need to visually inspect which compounds are reliably detectable in QC samples first, rendering it a semi-automated approach. Still, TaPEx remains far less time-consuming compared to manual targeted processing or untargeted approaches (Fig. 1E). Indeed, TaPEx removes the need for manual processing of all compounds per sample by the analyst and is also suited for larger cohorts, as we then confirmed by its application on the FGFP cohort (n = 292). Following the analysis of 292 FGFP stool samples using our novel dual metabolomics-lipidomics analytical methodology, including manual prospection of the 58 QC samples run throughout both metabolomics and lipidomics batches, TaPEx was applied, and 351/360 metabolites and 103/132 lipids were reliably detectable in QC's and retrieved successfully in samples (intensities in Table S20 and S21). Interestingly, a larger number of targeted metabolites and lipids were detected in this cohort as compared to our validation QC and LLD cohort batch. We believe this is due the amount of different QC sample runs being evaluated. Since the analysis of the FGFP cohort was run over five days, multiple QC sample runs were evaluated to check if compounds were detected consistently over the different days. Since compound peak shape can differ between different QC sample runs, it is possible that some peaks were judged to be reliable because they were present in most of the runs. However, when the amount of evaluated QC runs is lower (as in the LLD cohort), the chance that these less consistent peaks are present in these runs and thus are judged unreliable is higher. Future development of our TaPEx approach includes elevated user-friendliness (e.g. GUI), implementation of automatic quality control prospection, parallelization of processing, and possibly publication as an open-source software package.

CONCLUSION

In 2015 and 2017, we optimized and successfully validated parallel extraction protocols and UHPLC-HRMS-based analysis methods for metabolomics[5,6] and lipidomics[7], respectively. To meet the current needs regarding fast, high-throughput metabolomics analysis, the metabolomics and lipidomics extraction protocols were optimized to establish a dual approach. The new combined (metabolomics and lipidomics) fecal extraction and analysis methods were validated in an untargeted and targeted fashion, the latter on 360 metabolites and 132 lipids. Validation demonstrated a large fecal metabolome and lipidome coverage with a high level of precision and good linearity for a wide variety of physicochemically diverse metabolites. Furthermore, we present the use of a novel targeted peak extraction approach, called TaPEx, as a robust and semi-automated method for targeted data processing, which relies on method-specific databases, and can also be expanded to other multi-small molecule analysis methods in adjacent fields. Our novel analytical and data analysis workflow encompasses a total time reduction from sample-to-result of up to ~60%. As such this work answers challenges in both the analytical and bioinformatic part of the field by presenting a validated and benchmarked, holistic and semi-automated workflow for gut phenotyping.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information (Supplementary Tables S1 – S21., Figures S1 – S5) is available free of charge on the ACS Publications website. All code can be found at https://github.com/UGent-LIMET/TaPEx.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Jasmien Van Arnhem (FWO GO12721N) and Lieselot Y. Hemeryck (1297623N) are supported by Research Foundation – Flanders. This work was supported by the Interuniversity Special Research Fund (iBOF) from Flanders (grant number BOFIBO2021001102). The Laboratory of Integrative Metabolomics research group is part of the Ghent University expertise center MSsmall. The Lifelines initiative has been made possible by subsidy from the Dutch Ministry of Health, Welfare and Sport, the Dutch Ministry of Economic Affairs, the University Medical Center Groningen (UMCG), Groningen University and the Provinces in the North of the Netherlands (Drenthe, Friesland, Groningen). The authors wish to acknowledge the services of the Lifelines Cohort Study, the contributing research centers delivering data to Lifelines, and all the study participants. FGFP procedures were approved by the medical ethics committee of the University of Brussels–Brussels University Hospital (approval 143201215505, 5/12/2012). A declaration concerning the FGFP privacy policy was submitted to the Belgian Commission for the Protection of Privacy. Written informed consent was obtained from all participants. The FGFP was funded with support of the Flemish government (IWT130359), the Research Fund–Flanders (FWO) Odysseus program (G.0924.09), the King Baudouin Foundation (2012-J80000-004), FP7 METACARDIS HEALTH-F4-2012-305312, VIB, the Rega Institute for Medical Research, and KU Leuven. Jingyuan Fu is supported by the Dutch Heart Foundation IN-CONTROL (CVON2018-27), the ERC Consolidator grant (grant agreement No. 101001678), NWO-VICI grant VI.C.202.022, and the Netherlands Organ-on-Chip Initiative, an NWO Gravitation project (024.003.001) funded by the Ministry of Education, Culture and Science of the government of The Netherlands. Alexandra Zhernakova is supported by the Dutch Heart Foundation IN-CONTROL (CVON2018-27), the ERC Starting Grant 715772, NWO-VIDI grant 016.178.056, and the NWO Gravitation grant Exposome-NL (024.004.017).

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