

# Validation and application of a unique reference database for metabolomics – The Quantitative Metabolomics Database (QMDB)

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## Abstract

**Introduction:** Quantitative metabolomics reference data is scarce. Reliable reference data is important for validating metabolic biomarker signatures and to facilitate the evaluation of epidemiological cohort studies. The Quantitative Metabolomics Database (QMDB) provides quantitative ethylenediaminetetraacetic acid (EDTA) plasma metabolite concentration ranges for healthy human adults derived from analyses using the biocrates standardized MxP® Quant 500 kit. The samples were obtained from >1,000 healthy adults of all ages, using controlled epidemiological study data provided by several collaborating scientific institutes and universities.

**Aim:** This application note describes the QMDB and discusses its suitability as a reference in studies without a healthy control group. It also demonstrates how the QMDB can be used to validate the metabolomic results for a given control group as being within typical range.

**Methods:** The attributes of QMDB subjects were analyzed with descriptive statistics. Using principal component analysis (PCA), the metabolomes of the QMDB subjects were compared to those of several other healthy control groups. Metabolomics-based sample quality markers were assessed. The metabolite concentration ranges in the QMDB were statistically

compared to those from other databases or publications to assess their fitness for purpose. Finally, the QMDB data was put to the test in a case study.

**Results:** In the principal component analysis (PCA), the QMDB displayed high similarity to the other healthy control groups assessed. Only a small proportion of the QMDB mean concentrations (3-7%) were markedly different from those in other datasets. No outliers were detected. None of the QMDB samples had critical sample quality issues.

The concentration ranges observed in a study control group were successfully validated using the QMDB reference ranges. In a case study of patients with inflammatory bowel disease (IBD) without a control group, using the QMDB ranges as reference values resulted in the identification of significant differences previously described in a similar context.

**Conclusion:** The QMDB is suitable for use as a quantitative metabolomic reference database providing representative concentration ranges for the healthy population and customized subpopulations. The development of the QMDB thus represents an important step toward standardization in metabolomics.

# 1 Introduction

## 1.1 Background

Metabolomics profiling tools are used for the simultaneous quantification of metabolites representing the final or intermediate products of upstream biological processes. Studying metabolism contributes to the understanding of cellular processes and the influence of external factors (1).

While genomics reveals the probability of individuals developing certain physical traits and diseases, metabolomics provides a real-time snapshot of their actual current phenotype, influenced by genetics, lifestyle, and the environment, including pathogens, diet, and medication. Countless publications have shown that these factors can significantly alter metabolite concentrations (2, 3).

The metabolomes of body fluids have generated particular interest because these samples are readily available. Due to the accurate information it provides about a patient's physiological state, this data has the potential to be used for clinical diagnosis (4). Metabolomics is already commonly used for selected diagnostic purposes in clinical practice, such as newborn screening for genetic diseases (5), but its broader uptake in diagnostics has been hampered by the limited comparability and reproducibility of results produced at different sites with different cohorts. Fortunately, work has been done to help alleviate these pain points in recent years, and metabolomics is now widely viewed as a potential driver of cutting-edge developments such as precision medicine (6).

### Cutting-edge metabolomics technology

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) platforms are the

most widely used for metabolomics studies and often provide better sensitivity and metabolite coverage compared to other techniques. Until the early 2000s, researchers had to opt for either untargeted metabolomics, measuring the relative concentration changes of many metabolites, or targeted metabolomics, for the absolute quantification of a small, predefined number of metabolites. Since then, biocrates life sciences has developed several metabolomic kits for mass spectrometry that enable researchers to determine the absolute concentrations of a broad range of metabolites. Several interlaboratory ring trials using plasma and serum samples have proven that these kits enable robust and reproducible metabolite quantification, and yield comparable results when measured at different laboratories and with different instruments (7–9).

### Reference ranges for metabolite concentrations

This measurement approach opens up the possibility of establishing a new norm in obtaining reliable and reproducible metabolite concentrations. Clearly defined metabolite concentration ranges for healthy individuals could be used as reference values, a concept that has gained universal acceptance as one of the most powerful ways in which laboratory medicine can contribute to the clinical decision-making process (10).

Consequently, a number of publications have attempted to identify typical human plasma metabolite concentrations of this kind (11–14). However, the published mean concentration values for biocrates kit metabolites are heavily dependent on the characteristics of the respective study participants and are only of limited use to

other researchers. The Human Metabolome Database (HMDB; [www.hmdb.ca](http://www.hmdb.ca)) brings together mean metabolite concentrations and concentration ranges from diverse publications of varying quality, obtained with various methods. The resulting ranges thus lack consistency, and it is difficult for users to decide which of the listed ranges best applies to their study, if any. The jMorp database ([jmorp.megabank.tohoku.ac.jp](http://jmorp.megabank.tohoku.ac.jp)) developed by researchers from Tohoku University provides metabolite concentration ranges from a large group of

well-characterized samples quantified in their research laboratory, but this resource is limited to Japanese participants and does not offer customized sample selection.

There was therefore no way for the metabolomics community to access metabolite reference ranges from healthy participants in quality-controlled studies that were customizable and easy to use. biocrates developed the Quantitative Metabolomics Database (QMDB) in response to this need.

## 1.2 What is the QMDB?

The QMDB provides a collection of metabolite concentration ranges from healthy human individuals whose EDTA plasma samples have been measured using the MxP® Quant 500 or AbsoluteIDQ® p180 kits. Version 1.0 of the database contains samples from 1,082 adult humans of various ages obtained in collaboration with several scientific institutes and universities. In this context, healthy is defined as meaning that the participants underwent medical screening and were assessed as having no current or chronic physical or mental illness. Users can view the typical concentration range for any kit metabolite. Concentrations of individual samples are not provided.

### Filter options for customized reference ranges

One of the QMDB's unique advantages is its filter options menu, which enables users to define subgroups of the healthy population to be used to calculate customized reference ranges. Users can set a desired range for age and body mass index (BMI), and filter by sex and/or race/ethnicity. They can also restrict the sample selection based on fasting status at the time of collection as well as lifestyle factors including fitness, alcohol consumption, and smoking status. The display options allow

users to restrict the metabolites or metabolite classes displayed, making it easy for them to focus on their metabolites of interest. The selected options can then be saved in the menu ready for their next visit, and users can switch between their saved subpopulations with a single click.

### Concentration range table and descriptive statistics

After selecting the matrix of interest (currently only human EDTA plasma) and optional filters, the concentration range for each metabolite is calculated and displayed in a table. The concentration range table can be sorted individually and provides descriptive statistics for values including the mean, median, minimum, maximum, first and third quartiles and standard deviation. The number of samples, the percentage of samples above the limit of detection (LOD), and the typical LOD threshold are also provided for each metabolite. Users have the option to display the identifiers that link the metabolites to the corresponding entry in the HMDB database. The table can be exported as a text, CSV, or Excel file, and includes a summary of the selected filters for future reference.

## Data visualization and comparative statistics

The QMDB comes with an Excel template for visualizations and statistics that makes it easy for users to exploit the export file to draw boxplots showing concentration distributions. This can be downloaded from the online user manual. The Excel template also allows for direct side-by-side comparison of QMDB-derived concentration ranges with users' own data, or to compare different subsets exported from the QMDB.

The database also gives users the option to generate the mean and standard deviation from logarithmically transformed ( $\log_2$ ) concentration values. The log transformation establishes the normal distribution of the concentration data, which is required for parametric statistical tests. The Excel template also contains a worksheet that facilitates statistical comparison of means based on mean concentration, standard deviation, and number of samples (15, 16). This enables users to perform basic comparative

statistics and to identify any significant concentration differences.

## An ever-growing resource

The QMDB data will be supplemented with further eligible samples from other studies on a regular basis, making the reference ranges even more accurate over time and ensuring that the concentration ranges remain reliable even when working with granular filters. However, users will also have the option of reverting to an older version of the database if they want to repeat a reference range calculation conducted at an earlier stage.

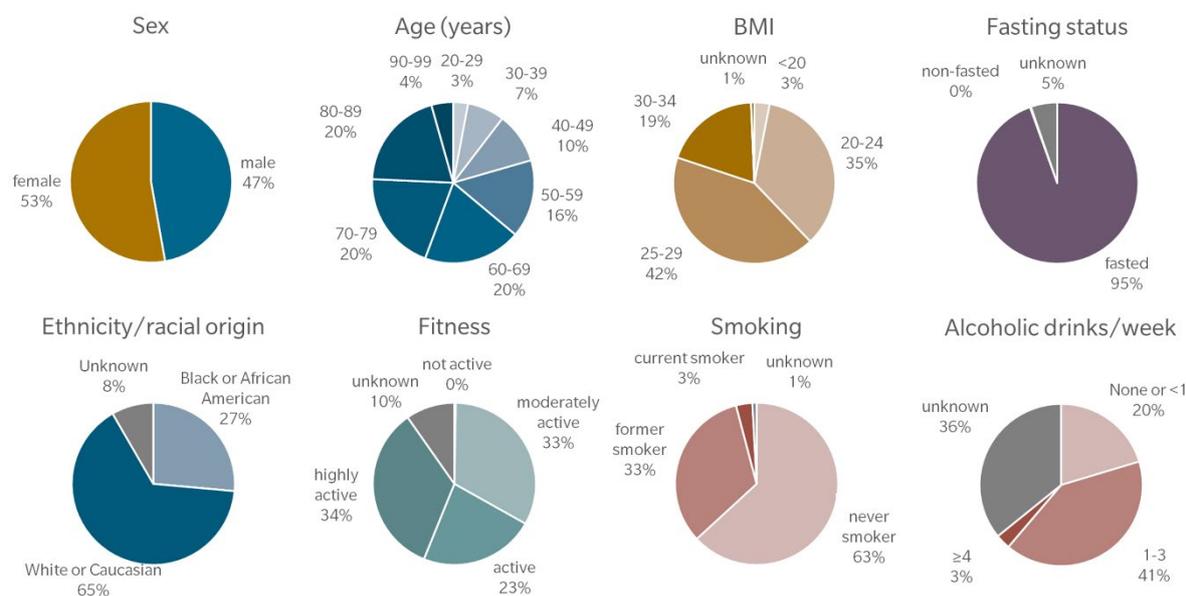
Anyone using a biocrates kit to measure values for healthy human subjects is encouraged to provide their data for the reference database and thus contribute to improving its quality. Contributors will receive a special discount on QMDB access and can use its features to filter for their own data, making it easier to compare to the total reference database.

## 2 Sample selection and demographics of the QMDB

To establish the QMDB as a reference database with a sufficiently large number of well-characterized samples from healthy participants, biocrates collaborated with the National Institutes of Health (NIH) and various universities, who contributed data from epidemiological studies to the QMDB.

Several thousand EDTA plasma samples from several studies were measured using the biocrates standardized MxP® Quant 500 kit at different centers. Since some of the contributing institutions required confidentiality to be maintained, we are unable to disclose how many samples from which studies contributed to the QMDB.

For Version 1.0 of the QMDB, 1,082 participants were selected, for whom the majority of the following data was available: sex, age, BMI, fasting status, race/ethnicity, fitness, smoking status, and number of alcoholic drinks per week. A considerable proportion of these samples were contributed by the NIH and were acquired only from participants that met the "IDEAL" standard established by NIH scientists (17). The demographics of the participants included in the QMDB are shown in Figure 1.



**Figure 1: Breakdown of demographic characteristics.** In Version 1.0 of the QMDB, the healthy human participants from whom the EDTA plasma samples were derived ( $n = 1,082$ ) are categorized by sex, age, BMI, fasting status, race/ethnicity, fitness, smoking status, and number of alcoholic drinks per week.

## Sample storage

As part of the studies contributing to the QMDB, the EDTA plasma samples were collected in accordance with guidelines for biomarker studies (18). As per the protocols, blood samples were immediately stored at 4°C and centrifuged within 4 hours from collection. After centrifugation, the plasma was immediately aliquoted and frozen at -80°C. The samples underwent no more than two freeze-thaw cycles prior to metabolomic quantification. The sample storage time before analysis was available for 63% of the samples and ranged between 0.9 and 14.1 years, with an average of 7.2 years.

## General observations

The demographics of the QMDB data were not expected to reflect the general population, since only samples from healthy participants were included and the individuals enrolled in the studies were primarily older white adults.

The distribution of metabolite concentrations may thus be biased due to imbalances in the age, BMI, race/ethnicity, and lifestyle parameter categories. While this may skew the calculated mean and median concentrations, the total concentration ranges (minimum and maximum concentrations) in the database should not be markedly affected.

## Sex

The QMDB currently contains slightly more samples from women than from men. As sex is one of the main confounders in biological studies, users are encouraged to study effects not only in both sexes together, but also in men and women separately. This can easily be done using the filter options to generate female and male sets of reference ranges.

## Age

The average age of participants was 64.9 years. Since no participants <20 years of age were included, this is much higher than

in the general population (41.8 years in developed countries in 2020) (19). For the oldest age category (90–99 years), it was difficult to identify participants with no medical conditions. The decision was therefore taken to include participants who were healthy but had previously undergone a surgical procedure such as hip replacement or hernia repair and had made a full recovery at the time of sample collection. Controlled hypertension was also omitted from the exclusion criteria.

### **BMI**

The threshold for excluding participants from the QMDB was set to BMI  $\geq 35$ , which is the definition of severe obesity according to the World Health Organization (WHO) (20) and is frequently used as a threshold for morbid obesity in modern medicine (21, 22). The mean BMI of the QMDB participants was 26.5, which is considered overweight in the adult population, but appears to be accepted as healthy in individuals over 60 years of age (23).

### **Fasting status**

Most of the samples currently included in the QMDB were obtained from fasting individuals, since this was a requirement of the sample collection protocols used in the studies. Fasting status was unknown for only 5% of the samples. Venous blood samples were collected in the morning after an overnight fast. Participants were not allowed to smoke, engage in physical activity, or take medication before the blood sample was collected.

### **Race and ethnicity**

The majority (65%) of the QMDB participants were White and of Caucasian

descent. A minority was of Hispanic/Latino origin. Black or African American participants represented 27% of those providing samples to the QMDB. There are plans to expand the races and ethnicities represented in the QMDB in the future.

### **Fitness**

Fitness was assessed with questionnaires regarding the performance, frequency, or duration of various high-intensity activities. Based on this, the participants were categorized as “not active”, “moderately active”, “active”, or “highly active”. The categorization based on high-intensity activities correlates with overall physical activity, which was also estimated from the questionnaire. It should be noted that even participants in the highest category were not typically athletes, but simply more active than average. It is also important to note that for samples from other studies added to the QMDB in the future, fitness may not have been assessed in the exact same way. The fitness categories should therefore be viewed as approximate labels.

### **Smoking status and alcohol consumption**

The smoking status and drinking behavior of participants is also included in the QMDB. As with physical activity, this information was self-reported, so it is possible that participants under- or overestimated their smoking status and alcohol consumption. We note that only 3% of the participants were current smokers, which is likely connected to the fact that smoking increases the risk of a whole host of diseases, and thus only a fraction of smokers was healthy enough to be eligible for the database.

## 3 Validation of the QMDB data

### 3.1 Aim

The QMDB was developed with the aim of defining metabolite concentration ranges for healthy individuals and making these available as reference ranges for studies measured with quantitative metabolomics, in particular those using biocrates technology, independently of the sample collection site and measuring laboratory. Researchers are naturally hesitant to rely on external reference values in scientific studies since experience has shown that external controls are usually less suitable

than controls obtained in the same study. This is especially true for metabolomics, since the harmonization of metabolite measurements obtained by different laboratories has historically been a challenge.

The validation work therefore focused on assessing whether the QMDB metabolite concentration ranges were comparable to the concentration ranges for independent healthy control groups from other studies.

### 3.2 Methods

#### External datasets

The QMDB data was compared to six other datasets comprising plasma metabolite concentration values for healthy control cohorts. To maximize the number of metabolites common to the QMDB and each dataset, the search for these datasets was limited to studies with metabolomics quantification using the MxP<sup>®</sup> Quant 500 or AbsoluteIDQ<sup>®</sup> p180 kits, as the entire set of AbsoluteIDQ<sup>®</sup> p180 metabolites is covered by the MxP<sup>®</sup> Quant 500 panel.

Dataset 1 (Q500) originated from a ring trial in which the performance of the MxP<sup>®</sup> Quant 500 kit was tested in different laboratories with different instruments using the same samples (manuscript in preparation). These EDTA plasma samples were commercially obtained and from healthy volunteers: three male and three female participants. The study also included measurements of SRM 1950, a standard reference plasma for metabolomics research developed by the National Institute of Standards and Technology (NIST), in collaboration with the NIH, to support technological developments in metabolomics research (24). These samples were measured in triplicate.

Dataset 2 (Neuro) originated from a study of age-related neurological disorders, comprising a control group of 68 healthy adults whose EDTA plasma samples were measured at biocrates laboratories using the AbsoluteIDQ<sup>®</sup> p180 kit. No metadata – such as sex, age, BMI, or lifestyle parameters – was available for these samples. Quality control identified 4 samples with lipemia and 9 samples with low sample quality; these were removed prior to statistical analysis, leaving a total of 55 samples.

Dataset 3 (Nutr1) originated from the Karlsruhe Metabolism and Nutrition (KarMeN) study. A subset of values produced using the AbsoluteIDQ<sup>®</sup> p180 kit was taken from a publication (25) describing 252 EDTA plasma samples from healthy adults: 150 men and 102 women, non-smoking, predominantly White, with a mean age of 45.9 years and a mean BMI of 23.9, considered to be healthy based on anthropometric, clinical, and functional assessments.

Dataset 4 (French) was taken from a published study conducted by the Institut Servier of 800 healthy French volunteers aged between 18 and 86, with an even distribution by sex, not on any medication

and considered to be healthy on the basis of their medical history, clinical examination and standard laboratory tests (12). The EDTA plasma samples were measured with the AbsoluteIDQ® p180 kit.

Dataset 5 (Nutr2) comprised data from the A-DIET Confirm study conducted in Ireland. This included 186 healthy men and women, aged between 18 and 60 years, with a BMI in the range of 18.5–30 and not consuming any supplements or prescribed medication. The lithium-heparin plasma samples for these participants were measured using the AbsoluteIDQ® p180 kit (14).

Dataset 6 (Japan) was downloaded in January 2022 from the jMorp database, which contains metabolome and proteome data for plasma samples obtained from healthy Japanese volunteers from the Tohoku Medical Megabank Cohort Study (26). The concentration ranges obtained from measuring up to 7,079 samples (34% male, 66% female) with the MxP® Quant 500 kit were downloaded and used for analysis.

### Assessing sample quality

Twenty years' experience in metabolomics enables biocrates to identify metabolic markers of reduced sample quality. The quality of plasma samples may be reduced at an early stage, for instance if blood samples are left at room temperature for several hours after the blood draw before being processed to plasma by centrifugation, or if the plasma is not frozen at -80°C or below within an acceptable time after centrifugation (27, 28). Sample quality is also reduced by repeated thawing and freezing, for example due to repeated aliquoting or shipping samples to different laboratories without enough dry ice. The identity of these markers, which consist of metabolite concentrations as well as their sums and ratios, are not disclosed here since this is unpublished internal knowledge.

For Datasets 1-3, the metabolite concentrations from the individual sample measurements were available, enabling sample quality analysis. We assessed the metabolite concentrations and metabolite ratios known to be altered if samples are not processed in time or undergo several freeze-thaw cycles and removed low-quality samples prior to data analysis.

### Statistical analysis

All of the datasets were compared to the QMDB metabolite concentration ranges. First, only the metabolites present in both the external dataset and the QMDB were considered in the comparison. The metabolite PC ae 38:1 was excluded from further analysis since its concentrations were consistently markedly higher in the AbsoluteIDQ® p180 datasets than in the MxP® Quant 500 datasets. Side-by-side boxplots were then created using the Excel template for visualizations and statistics linked to from the QMDB user manual. The resulting *p* values were adjusted for multiple testing using the established Benjamini & Hochberg method (29).

The following three criteria were used to identify metabolites with markedly different concentration ranges when comparing the external datasets with the QMDB:

- At least a 1.5-fold difference between the mean concentrations.
- Statistical significance after correcting for multiple testing ( $q < 0.01$ ).
- No overlapping of the standard deviations for the mean concentrations.

We set a comparatively strict significance threshold to allow for the fact that even small fold changes easily become statistically significant due to the large sample size. We expected the QMDB metabolite concentration ranges to fit the ranges for the external healthy control datasets relatively well. An acceptance

threshold was thus defined to allow a maximum of 10% of the metabolite concentration ranges assessed to be markedly different between the QMDB compared to any of the external datasets. Percentages were used instead of the metabolite count to accommodate the fact that two of the control datasets were derived using the MxP® Quant 500 kit and four using the AbsoluteIDQ® p180 kit, which covers considerably fewer metabolites.

An outlier analysis was conducted for the mean concentration values from the various datasets by calculating Tukey’s fences (30), using conventional Tukey’s fencing ( $k = 1.5$ ). This method works best with nine or more data points but can be applied to datasets with as few as five data points, although the number of outliers may be slightly overestimated in such cases (31).

This analysis was conducted for 146 metabolites for which mean concentration values were present in the QMDB dataset and four or more of the external datasets. While this method is very good at detecting outliers when most of the mean concentrations are in a similar range,

extreme values are not recognized as outliers if there is high variance between the values in the different datasets. To identify mean concentrations that were very different from the means in the other datasets but were not identified as outliers using the Tukey’s fences method, for all 163 metabolites for which values were present in at least three datasets, the mean concentration in each dataset was compared to the consensus concentration across the datasets, calculated as the median of the mean concentrations. A threefold difference was set as the threshold for extreme values.

Based on the individual sample measurements available for Datasets 1-3, a principal component analysis (PCA) was also performed, which provided an informative overview of compatibility with the QMDB. As a PCA cannot handle values that are missing because they were < LOD, the datasets were cleaned, removing all metabolites for which less than 80% of the values were above the LOD. Missing values in the remaining metabolites were imputed between LOD and LOD/2 using a logspline imputation method (32).

### 3.3 Results and discussion

#### Sample quality

**Table 1: Percentage of samples with high quality.** Based on metabolologically assessed quality markers, sample quality was determined for four datasets (QMDB, Q500, Neuro, and Nutr1).

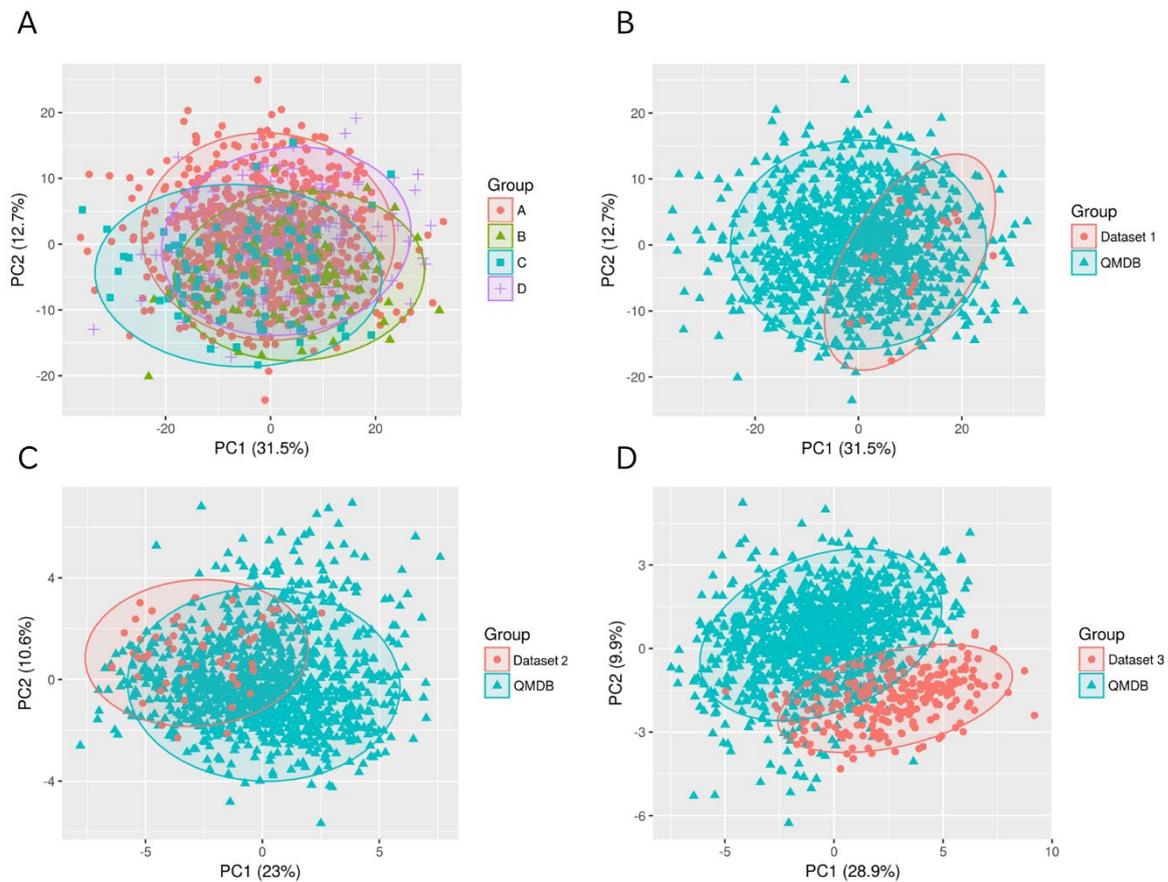
Dataset	High-quality sample score
QMDB	100%
Q500	100%
Neuro	87%
Nutr1	100%

Table 1 shows the percentage of high-quality samples in each dataset as assessed based on the quality markers. The sample

quality was very good (100%) in three datasets (QMDB, Q500, and Nutr1), while the Neuro dataset had the lowest score (87%), with nine samples identified as being low-quality. As noted in the section above, these were removed from the dataset before statistical analysis.

#### Principal component analysis

PCA was used to visualize and compare the QMDB data with each of the six external datasets, and to examine the homogeneity of the datasets that were compiled to create the QMDB. All concentrations were target-normalized to biocrates quality control level 2 (QC2) samples, as is required for all MxP® Quant 500 measurements.



**Figure 2: Comparisons of datasets using PCA:** (A) Comparison of the four QMDB subsets, and (B-D) comparison of the QMDB samples with the external datasets 1 (Q500), 2 (Neuro), and 3 (Nutr1).

The four subsets included in the QMDB are shown in Figure 2A. The high degree of overlap between the subsets demonstrates the comparability of the target-normalized MxP® Quant 500 results across the studies.

The results for the external validation of the QMDB differed between the three datasets. Like the QMDB, Dataset 1 (Q500) consisted of metabolite data acquired with the MxP® Quant 500 kit and target-normalized to biocrates QC2 samples. A high similarity of the sample metabolomes was consistently observed across both datasets (Figure 2B).

Dataset 2 (Neuro) was acquired with the AbsoluteIDQ® p180 kit and target-normalized to biocrates QC2 samples. The MxP® Quant 500 kit covers all of the metabolites included in the AbsoluteIDQ® p180 kit, and both kits yield absolute

concentrations. The results should therefore be compatible, and the two datasets did indeed show almost complete overlap (Figure 2C). Overall, the sample diversity of the QMDB was higher than that of Datasets 1 and 2 (Figure 1B-C), reflecting the greater diversity of participants. Dataset 3 (Nutr1) was also acquired with the AbsoluteIDQ® p180 kit but normalized to internal controls. Interestingly, the concentrations detected in this dataset were far less comparable to those of the QMDB (Figure 2D). The PCA loading plots (not shown) revealed differences in the concentration ranges between the two datasets for numerous metabolites, especially phosphatidylcholines (PCs). Although most of the differences were not large on a single metabolite basis, taken together they resulted in a clear separation of the

samples, since PCs are the largest metabolite class covered by the AbsoluteIDQ® p180 kit.

It seems likely that the different normalization strategy employed for Dataset 3 was the cause of the difference in the metabolite concentrations. As this was the only dataset for which a separation was seen, we are unable to determine the reason for this deviation with certainty. However, we would encourage QMDB users to employ target normalization to QC2 samples whenever possible, to guarantee optimal comparability with QMDB datasets.

We note that the QMDB sample distribution in the PCA and the variability explained by the first two principal components in Figures 2A and 2B were identical, indicating that the addition of the few extra samples from Dataset 1 did not impact the overall dataset variability. Datasets 2 and 3 included more samples, and only the QMDB metabolites present in these p180 datasets were included in the analysis, resulting in markedly different sample distribution and variability explained by the first two principal components in Figures 2C and 2D.

### Comparison of metabolite concentration ranges

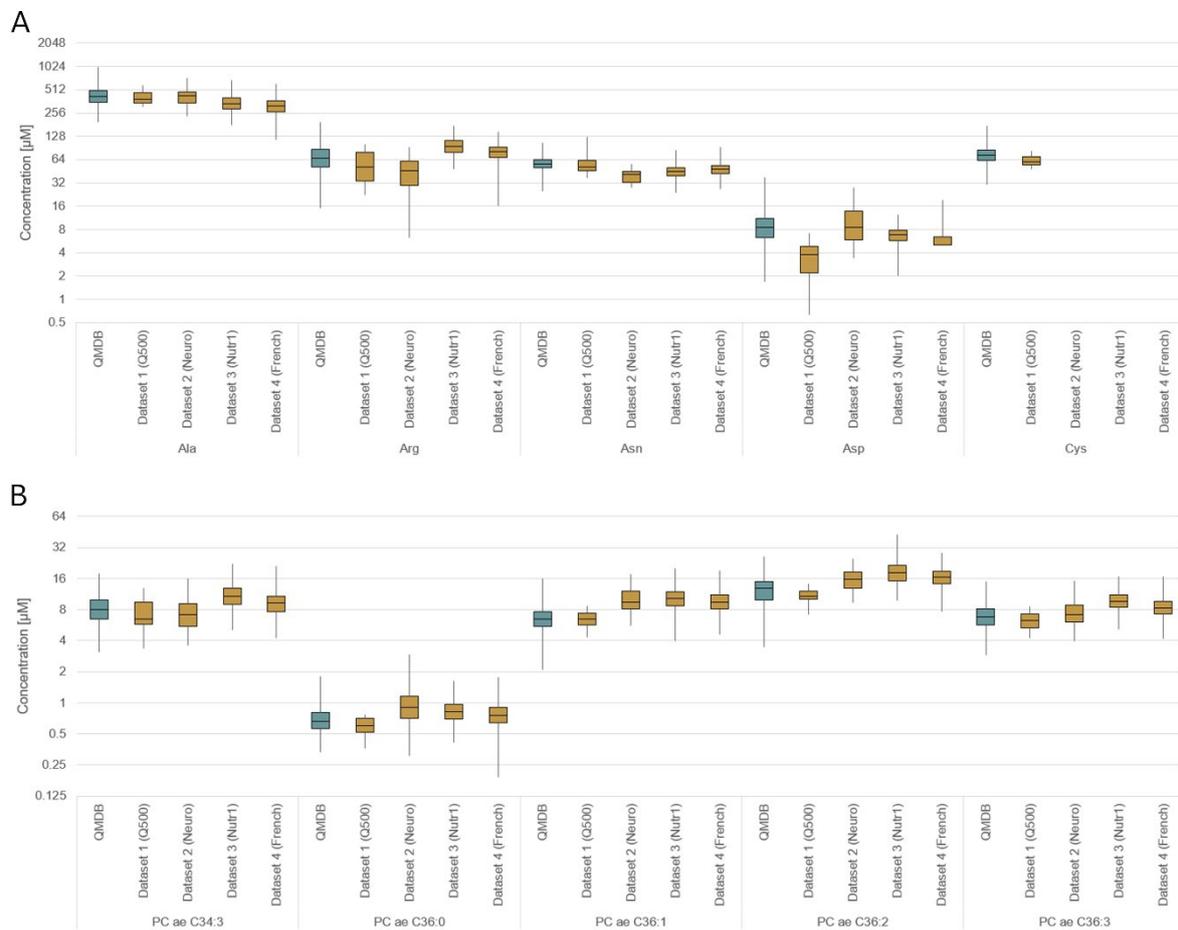
Using the QMDB Excel template for visualizations and statistics, we generated side-by-side boxplot comparisons of the metabolite concentration ranges from the QMDB and the six external control datasets. Representative examples for polar metabolites analyzed with LC-MS/MS and lipids analyzed with flow-injection analysis (FIA) MS/MS are shown in Figure 3.

Generally, the metabolite concentration ranges from the QMDB and the external datasets were highly comparable to one another. Five amino acids were used as example LC-MS/MS metabolites. These are quantified using a seven-point calibration

curve, resulting in quantitative concentrations with very low coefficients of variance (CV). Dataset 1 (Q500) displayed slightly lower aspartic acid (Asp) concentrations than the QMDB (Figure 3A), but this was not observed with Datasets 2-4. Similarly, Dataset 2 (Neuro) displayed a slightly lower median for asparagine (Asn) concentrations, and Dataset 3 (Nutr1) had a slightly higher median for arginine (Arg), which was not observed in the other datasets. The amino acid concentration ranges in Dataset 4 (French) corresponded to those in the QMDB. It should be noted that cysteine (Cys) concentrations were absent from Datasets 2-4 because cysteine is not covered by the AbsoluteIDQ® p180 kit.

Several acyl-alkyl-phosphatidylcholines (PC ae) were selected as examples of metabolites measured with FIA-MS/MS. These are quantified using a one-point calibration, resulting in quantitative concentrations that may have a slightly higher CV. In Dataset 1 (Q500), the PC concentrations were very similar to those in the QMDB (Figure 3B). In Dataset 2 (Neuro) and Dataset 4 (French), the median concentrations were sometimes slightly higher for some PCs, but still very similar overall. The PC concentration ranges for Dataset 3 (Nutr1) were higher than in the other datasets. This was consistent for most, but not all, PCs. Other metabolite classes did not show a similar shift. This shift toward higher PC concentrations is the most probable cause for the separation of Dataset 3 from the QMDB in the PCA.

As expected, the concentration ranges varied between metabolites. However, the boxplots shown in Figure 3 demonstrate that, although the QMDB comprises several datasets characterized by a broad range of ages, races/ethnicities, and other factors, this did not result in a larger variance in the concentration range of single metabolites compared to the smaller, less diverse external datasets.

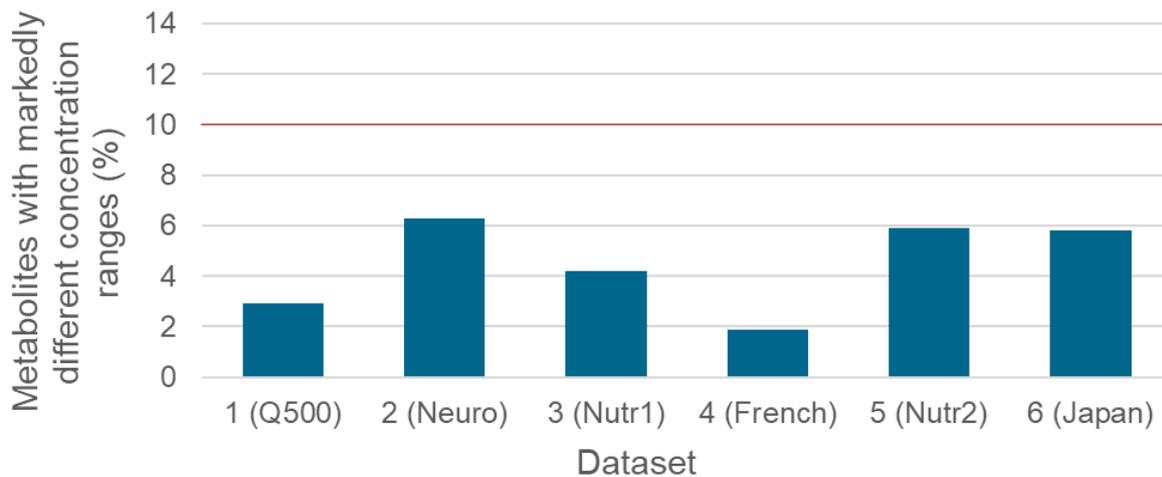


**Figure 3: Comparison of metabolite concentration ranges across the datasets:** (A) concentration ranges of the amino acids alanine (Ala), arginine (Arg), asparagine (Asn), and aspartic acid (Asp) analyzed with LC-MS/MS and (B) five PC ae analyzed with FIA-MS/MS in the QMDB and external control datasets 1-4.

For Datasets 5 and 6, only the medians and standard deviations were available, making the plots less informative and more difficult to read. They are therefore not shown here. The crucial question as to whether any of the differences in the concentration ranges were statistically significant was however assessed for all datasets.

The percentages of metabolites with a significantly and markedly different concentration range (as defined in the Methods section) across the six external control datasets and the QMDB are shown in Figure 4.

The proportion of markedly different concentration ranges was 6.3% or lower for all six datasets, meaning that the 10% threshold was not crossed. The QMDB thus met the validation criteria, indicating that its concentration ranges were sufficiently similar to those of the other control datasets. With a proportion of markedly and significantly different metabolite concentration ranges of around 2%, Dataset 4 (French) was the healthy control dataset with the most similar concentration ranges to the QMDB.



**Figure 4: Proportion of markedly different metabolite concentration ranges.** The percentage of metabolites with markedly different concentration ranges between the QMDB and external datasets 1-6 was assessed according to the defined criteria. An acceptance threshold was set at a maximum of 10% (red line).

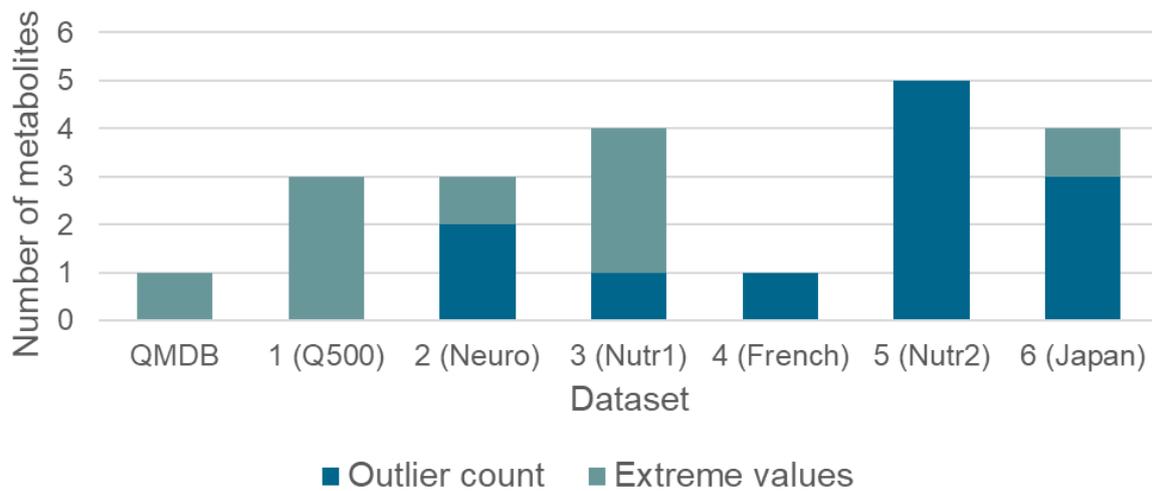
Very few significantly and markedly different metabolite concentration ranges were identified when comparing the QMDB with Datasets 1 and 4. Datasets 2, 5, and 6 had around 6% significantly and markedly different metabolite concentration ranges. Contrary to expectation, the percentage of different metabolites in Dataset 3 (Nutr1) was not found to be higher than the other datasets, even though its metabolome appeared to be quite different from the QMDB in the PCA. This discrepancy is due to differences in data handling. Dataset 3 differed from the QMDB mainly in the concentrations of PCs, which were higher, and fewer values were below the LOD compared to the QMDB. Values below the LOD were imputed for the PCA, leading to lower PC concentrations for the QMDB. For the comparison of means, only the measured values were considered and values below the LOD ignored. Consequently, there were fewer differences between the QMDB and Dataset 3.

### Detection of outliers and extreme values

The QMDB dataset and the six external control datasets were subjected to an

outlier analysis. The mean concentrations of each metabolite were compared across all seven datasets, and single metabolites in the dataset were flagged as outliers. All metabolites identified as outliers were excluded from the subsequent calculation of extreme values. The outliers identified among the 146 metabolites tested and the extreme values in each dataset for the 163 metabolites tested are displayed in Figure 5. In total, 121 metabolites were detectable across all seven datasets.

No outliers were detected among the mean concentrations of metabolites in the QMDB dataset. Only one was identified as an extreme: the mean concentration of the sphingomyelin SM 22:3 was just under one-third of the median mean concentration. Similarly, only one outlier was identified in Dataset 4 (French). The other datasets had three to five metabolites with mean concentrations markedly different from the other datasets. These results indicate that the QMDB and Dataset 4 are the two most representative datasets of all the healthy control datasets considered in this study. The below-average outlier count of the QMDB confirms its suitability as a reference database.



**Figure 5: Detection of outliers and extreme values.** Some mean concentrations of metabolites were detected as outliers (blue) and extreme values (green) in the QMDB and external datasets 1-6.

### 3.4 Evaluation

The results of this validation show that the QMDB metabolite concentration ranges are comparable to the concentration ranges of independent healthy control groups from different studies. All of the samples included had sufficiently high sample quality according to our quality markers. The PCA found that the metabolomes of the datasets used to compile the QMDB were very similar to one another. When the entire QMDB dataset was compared to another independent MxP® Quant 500 control dataset (Dataset 1), the metabolomes were also comparable according to the PCA.

The only suboptimal result was the separation between the QMDB samples and the samples from Dataset 3 based on the metabolome in the PCA. A shift in the metabolite concentration ranges for several lipids in Dataset 3 compared to the QMDB appears to have been responsible for this. In the statistical comparison of means, Dataset 3 did not stand out, but this was because values below the LOD were not considered. The differences may be connected to the fact that the metabolite concentrations in this dataset were not normalized to QC2 target values, as was the

case with the QMDB, Dataset 1, and Dataset 2. We conclude that if studies have not been normalized to QC target values, there is a risk of the ranges not fitting as closely. We therefore generally recommend using the QMDB metabolite concentration ranges as healthy control reference values only for studies that have been target-normalized.

The metabolite concentration ranges from the QMDB were comparable to the ranges calculated from Dataset 1 as well as four AbsoluteIDQ® p180 healthy control datasets (Datasets 2–5). This was evidenced by a low percentage of significantly and markedly different metabolite concentration ranges. The QMDB also displayed the lowest number of outliers and extreme values, when mean concentrations of metabolites across all datasets were compared to one another. These results indicate that the QMDB reference ranges are consistent with other healthy control datasets obtained with biocrates technology, and that the QMDB is therefore suitable for use as a quantitative metabolomic reference database for EDTA plasma samples from healthy adults.

## 4 Applications of the QMDB

### 4.1 Use cases for the QMDB

The QMDB provides typical metabolite concentration ranges for the healthy adult population that have been validated for use as reference values. The option for customized selection of subgroups from the healthy population also provides insights into metabolomic differences based on race/ethnicity, sex, and lifestyle factors. The QMDB data can be used in numerous ways:

- Users can utilize the QMDB reference ranges in their own studies to determine whether their control group is representative of the normal healthy population.
- In studies without healthy controls, for example comparing two treatments, users can compare the concentration ranges with the QMDB ranges to investigate which treatment results in the most normal metabolic values.
- If researchers discover healthy individuals in their study whose metabolome differs from the rest of the group, they can use the QMDB concentration ranges to determine whether these values are still within normal range, or if the individual is a true outlier. This can help exclude outliers, or participants who have not adhered to the study protocol, for example.
- For certain research questions comparing different subgroups solely within the healthy population, researchers may be able to skip collection of their own samples and data and use the QMDB concentration ranges for *in silico* studies.
- The exported values can be employed by data scientists, for instance using pattern recognition algorithms to identify novel metabolite signatures behind participant attributes.

### 4.2 Case study – Using the QMDB in studies without a healthy control group

#### Background

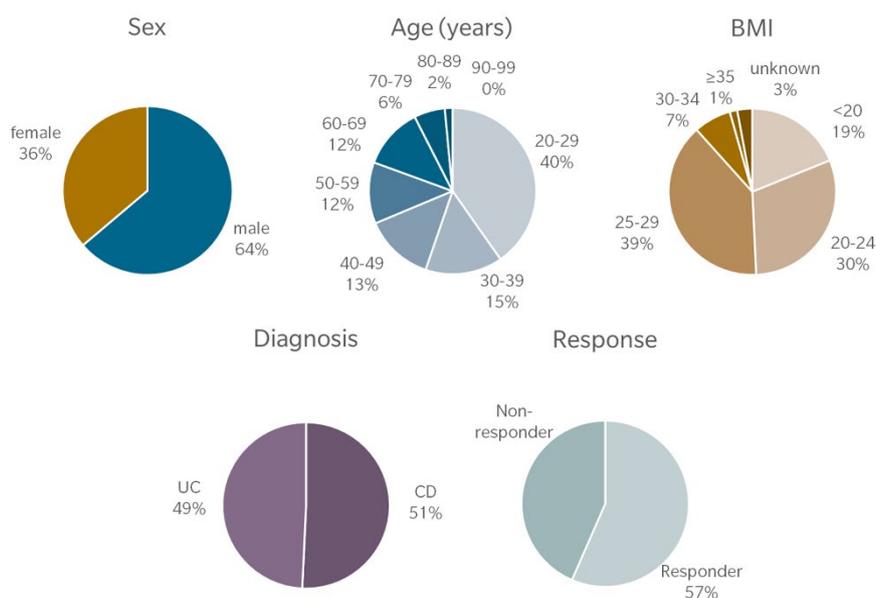
This case study demonstrates how the QMDB can be used in studies without a healthy control group. In this study, a dataset of 69 EDTA plasma samples from patients with inflammatory bowel disease (IBD) was provided by a collaboration partner. The dataset consisted of female and male patients diagnosed either with Crohn's disease (CD) or ulcerative colitis (UC). CD and UC have been previously shown to have different metabolic profiles

(33). At the time of sample collection, all patients had taken an experimental treatment targeting IBD, resulting in subpopulations of responders and non-responders. The dataset therefore consisted of four subgroups: CD responders ( $n = 21$ ), CD non-responders ( $n = 14$ ), UC responders ( $n = 18$ ), and UC non-responders ( $n = 16$ ). The nature of the treatment was not disclosed to biocrates.

## Aim

While the primary goal of the study was to investigate the effects of the treatment on small molecules and lipids in IBD patients, comparison to a control without IBD could shed light on the metabolic profile of each disease type, and on the potential beneficial

effects of the treatment. We therefore exported a control group from the QMDB matching the demographics of this dataset (shown in Figure 6) for use in group comparisons.



**Figure 6: Demographic breakdown of participants in the IBD study.** The IBD patients ( $n = 69$ ) were categorized by sex, age, BMI, diagnosis (CD: Crohn's disease; UC: ulcerative colitis), and treatment response.

## Methods

The IBD dataset was normalized to QC2 samples using target values for human plasma. This provides an optimal normalization to reduce variability between the IBD dataset and the QMDB control group.

The filter options in the QMDB were set to filter the database based on BMI and age range to identify the most relevant samples. Summary values (mean, median, standard deviation, first and third quartiles, interquartile range, and number of samples) were exported from the QMDB as natural values and as  $\log_2$ -transformed values.

The same summary values were calculated for each metabolite in the IBD dataset and pasted into the QMDB Excel template for visualizations and statistics provided to

QMDB users. To produce a more normal distribution, the concentration values were  $\log_2$ -transformed for all metabolites before use in group comparisons.

The dataset for analysis was consolidated by combining the IBD and QMDB summary data. The list of metabolites was limited to those that were present above the LOD in both datasets, resulting in a final set of 473 metabolites.

Univariate statistics were used to compare the levels of the 473 metabolites between the two groups (e.g., IBD vs. QMDB control) using the comparison of means in the QMDB Excel template to determine the  $p$  value. The false discovery rate (FDR) was calculated using the Benjamini and Hochberg method (29). Fold change (FC) was calculated from the natural values,

comparing the IBD group values to the QMDB control.

Unless otherwise stated, the lists of metabolites were filtered by three cut-off values to determine the significance of the results:  $p$  value < 0.001, FDR < 0.05, and  $\log_2$  FC  $\geq$  0.58 or  $\leq$  -0.58 (corresponding to a  $\pm$  1.5 FC in natural values). These comparatively strict thresholds were necessary, since the large number of samples in the QMDB would otherwise result in numerous statistically significant differences with a minimal concentration FC.

In addition to comparing the entire IBD dataset to the QMDB, we compared the metabolome of each IBD type separately to the QMDB control. Subgroup comparisons were performed using the same approach with smaller or different data subsets. In comparisons without QMDB involvement, the sample number was much lower, and the cut-off values could be set to the standard for metabolomics analysis ( $p$  < 0.05, FDR < 0.2).

## Results

### Differences in the plasma metabolome of IBD patients

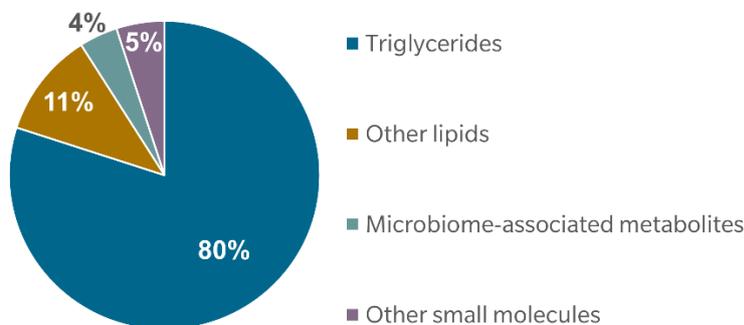
Looking at all 69 samples of the IBD dataset and 929 samples from the filtered QMDB control dataset, 97 metabolites were

significantly different between the IBD dataset and the QMDB control. These included 77 triglycerides (TGs), 11 other lipids, and 9 small molecules (Figure 7).

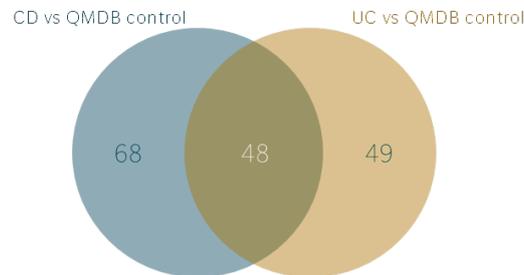
Interestingly, the TGs with higher levels in the IBD patients generally had saturated (SFA) or monounsaturated fatty acid (MUFA) side chains, whereas the TGs with lower levels in IBD mostly had polyunsaturated fatty acid (PUFA) side chains. The biggest differences were observed for the small molecule hypoxanthine, for which there was a 2.7 FC among the IBD patients. Of the nine small molecules with significantly different concentrations between the IBD and QMDB control datasets, five are known to be microbiome-associated: p-cresol sulfate, 3-indole propionic acid (3-IPA), proline betaine, 3-methylhistidine, and trimethylamine N-oxide (TMAO). They were all strongly and significantly decreased in the IBD dataset. These metabolites are related to microbiome metabolic activity and are likely to reflect the impact of IBD on the gut microbiome and its contribution to the host metabolome.

### Distinct plasma metabolomes in IBD subtypes

Separating the CD and UC patients provided a more detailed picture of how each disease differs from healthy controls. The overlapping and unique differences are shown in Figure 8.



**Figure 7: Breakdown of significantly different plasma metabolite concentrations in the IBD dataset vs. the QMDB control.** In total, the mean concentrations of 97 metabolites, including triglycerides (blue), other lipids (yellow), microbiome-associated metabolites (green), and other small molecules (purple), were significantly different between the EDTA plasma samples from the IBD dataset ( $n = 69$ ) and the QMDB control ( $n = 929$ ).



**Figure 8: Overlap of significantly different plasma metabolite concentrations in the CD and UC subgroups compared to the QMDB control.** Compared to the QMDB control ( $n = 660$  compared to CD,  $n = 866$  compared to UC), significantly different mean concentrations were detected in 117 and 98 metabolites in the EDTA plasma samples from CD patients ( $n = 35$ ; blue) and UC patients ( $n = 34$ ; yellow) respectively, with an overlap of 48 metabolites.

For the CD patients, there were 116 metabolites with concentrations that were significantly different from the QMDB control: 90 TGs, 19 other lipids including several PUFAs, and 7 small molecules. The pattern of TG saturation observed in the CD subgroup was identical to that observed in the IBD vs. QMDB comparison: higher levels of TGs with SFA and MUFA chains, and lower levels of TGs with PUFA side chains compared to the QMDB control.

Interestingly, in the CD patients, the levels of the PUFA eicosapentaenoic acid (EPA) were even lower than in the total IBD group. The omega-3 PUFAs arachidonic acid (AA) and docosahexaenoic acid (DHA) were also reduced based on the  $p$  value but with an FC of slightly below 1.5.

As in the previous comparison, hypoxanthine concentrations were significantly elevated, while p-cresol sulfate, 3-IPA, and 3-methylhistidine were significantly lower compared to the control.

For the UC dataset, there were significant differences from the QMDB control in the concentration of 97 metabolites. These consisted of 72 TGs, 15 other lipids including cortisol, and 10 small molecules. The same TG saturation pattern was also present in this comparison. However, in the UC patients, the higher levels of SFA- and MUFA-TGs compared to the control were often significant, while the reduction in PUFA-TGs was only rarely significant. A significant increase in cortisol levels was

observed in the UC patients, but not in the CD patients compared to the control.

### Plasma metabolomes in response to IBD treatment

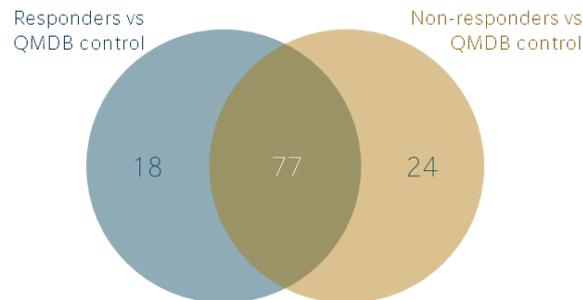
Participants in both disease subgroups received an experimental treatment that showed efficacy in a subset of each group. In this subsection we focus on the comparison of the profiles of these treatment response subgroups.

Of the 35 CD patients, 21 were responders and 14 non-responders to the treatment. No significant differences were identified from a direct comparison of these subgroups, even with more permissive significance thresholds ( $p < 0.05$  and FDR  $< 0.2$ ). There was a non-significant trend toward higher levels of the two conjugated primary bile acids glyco- and taurochenodeoxycholic acid (GCDCA and TCDCA) in responders, as well as lower levels of sarcosine and cortisol ( $p < 0.07$ ). A follow-up study with higher sample numbers might yield more conclusive and robust results.

Comparing the subgroup of responders (CD and UC combined) to the QMDB control group found 95 significant differences, while there were 101 differences between non-responders and the QMDB control (Figure 9). Of these changes, 77 were common to both subgroups, and all were changed in the same direction, including a majority of TGs. The PUFA EPA was lower in both subgroups than in the control, while

DHA was lower only among the responders. Cortisol and aspartic acid were lower only among the responders, and proline betaine

and citrulline were lower only among the non-responders.



**Figure 9: Overlap of significant differences in plasma metabolite concentrations in the IBD treatment response subgroups compared to the QMDB control.** Compared to the QMDB control ( $n = 929$ ), significantly different mean concentrations were detected in 95 and 101 metabolites in the EDTA plasma samples from responders ( $n = 39$ ; blue) and non-responders ( $n = 30$ ; yellow) respectively, with an overlap of 77 metabolites.

## Discussion

### Hypoxanthine

The metabolomics results clearly show that hypoxanthine was by far the most different metabolite between the IBD (both CD and UC) and QMDB datasets, reaching a remarkably high statistical significance even after correction for multiple testing ( $FDR < 2 \times 10^{-87}$ ). The increased levels in treated IBD patients strongly suggest that the treatment affects purine metabolism. It seems likely that the patients received a thiopurine drug, which is widely used in the treatment of IBD. Thiopurines affect the activity of hypoxanthine-guanine phosphoribosyltransferase (HPRT), a purine salvage enzyme that catalyzes the conversion of hypoxanthine and guanine to their respective mononucleotides (34). The IBD treatment is thus the probable cause of the most prominent metabolic difference observed.

### Polyunsaturated fatty acids

In a cohort of 99 patients with IBD (96% CD, 4% UC) compared to 51 controls, Guan et al. identified decreased levels of PUFAs (AA, DHA, and EPA) (35). This is consistent with our study, where EPA was significantly lower in the IBD group, and DHA and AA were

lower based on two out of three criteria. When stratifying the IBD group per disease type, there was a trend toward lower levels for all three PUFAs compared to the controls. In the CD patients, DHA and EPA were significantly lower than the control values, while only EPA was significantly lower among the UC patients. In line with this, supplementation with DHA has been suggested as a way to decrease the risk of developing CD in a prospective study with over 200,000 participants from Europe (36).

However, blood levels of PUFA have been shown to correlate with disease activity and inflammatory mediators in CD (37), which may be a source of variability in groups with a broad range of disease activity levels.

### Triglycerides

Different studies have reported total circulating TG levels as being the same, (38), or lower (39), or higher in IBD (40) than in the respective control group. External factors such as lipid-lowering drugs are a likely reason for these discrepancies.

In a study of nearly 10,000,000 Korean subjects, CD incidence was associated with low serum low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels

(lipoprotein particles poor in TGs compared to chylomicrons and very-low-density lipoprotein), but not with serum TG levels, while UC incidence was associated with low serum TG levels, but not with lipoprotein particle levels (39). In another study, serum TG levels in 701 IBD subjects (54% CD, 46% UC) were especially high in CD patients compared to those with UC (40).

In this case study, all IBD group and subgroup comparisons to the QMDB control showed a pattern of increased concentrations of TGs with SFA and MUFA side chains, and decreased TG concentrations with PUFA side chains. This can be explained at least in part by the overall decrease in the PUFAs that contribute to TG synthesis.

#### Microbiome-derived metabolites

Several microbiome-associated metabolites were lower in the IBD group than in the QMDB control. These include 3-IPA, an intermediary of tryptophan metabolism by the gut microbiome; and TMAO, indoxyl sulfate, and p-cresol sulfate, which are derivatives of the microbial metabolites TMA, indole, and p-cresol, respectively.

IBD is a group of inflammatory intestinal diseases that have a major impact on the balance of the gut microbiome and therefore the concentrations of metabolites synthesized exclusively by microbial populations. IBD also affects gut transit time. Together, this may have a strong influence on metabolite levels and contribute to the observed decrease in gut microbiome-related metabolites.

## 5 Summary

The QMDB provides quantitative EDTA plasma concentration ranges for human adults derived from analysis with the biocrates standardized MxP® Quant 500 kit. In this application note, we explain how the QMDB was developed and the samples included in the dataset. We also describe

#### Metabolome differences between responders and non-responders

One of the goals of the analysis was to determine whether the metabolome of the responders was more similar to the normal healthy metabolome than the metabolome of the non-responders. The fact that there were more marked and significant differences between the non-responders and the QMDB than when comparing the responders to the QMDB suggests that this may indeed be the case. However, the number of significantly and markedly different metabolite concentrations differed only by 6%, preventing us from reaching a definitive conclusion.

#### Conclusions

By comparing the metabolic profile of IBD patients to a representative subpopulation of the QMDB, we were able to identify some of the hallmarks of IBD. Differences in the concentrations of PUFAs, TGs, and microbiome-associated metabolites were consistent with the differences between IBD patients and healthy controls previously described in the literature. The treatment-specific increase in hypoxanthine also confirmed a metabolic response to the treatment in line with expectations.

Overall, this example shows that the QMDB is able to provide a healthy control group for statistical analysis in studies with EDTA plasma samples from human adults with disease.

the demographics of the QMDB and how the data was validated. The validation shows that the QMDB metabolite concentration ranges are comparable to those of other healthy control datasets obtained with biocrates technology, demonstrating that the QMDB can be

combined with independent datasets measured at different sites. Finally, we list use cases for the QMDB and report in detail a case study in which the QMDB was successfully used as an external control in a study without a healthy control group. The statistical analysis confirmed the suitability of the QMDB as a source of healthy external control datasets.

The evidence compiled in this application note thus suggests that the QMDB is suitable for use as a quantitative metabolomic reference database providing representative metabolite concentration ranges for EDTA plasma samples from the healthy adult population and customized subpopulations. The added value provided by this resource will be extremely beneficial to biocrates clients when analyzing and interpreting their data.

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