Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry

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Metabolism has an essential role in biological systems. Identification and quantitation of the compounds in the metabolome is defined as metabolic profiling, and it is applied to define metabolic changes related to genetic differences, environmental influences and disease or drug perturbations. Chromatography–mass spectrometry (MS) platforms are frequently used to provide the sensitive and reproducible detection of hundreds to thousands of metabolites in a single biofluid or tissue sample. Here we describe the experimental workflow for long-term and large-scale metabolomic studies involving thousands of human samples with data acquired for multiple analytical batches over many months and years. Protocols for serum- and plasma-based metabolic profiling applying gas chromatography–MS (GC-MS) and ultraperformance liquid chromatography–MS (UPLC-MS) are described. These include biofluid collection, sample preparation, data acquisition, data pre-processing and quality assurance. Methods for quality control–based robust LOESS signal correction to provide signal correction and integration of data from multiple analytical batches are also described.

INTRODUCTION

Systems biology is focused on the study of biological components and, more importantly, their complex interactions to define the emergent properties of biological systems[1–3]. Metabolomics[2,4–6], and the associated field of metabonomics[7], are core areas of systems biology research that are focused on the holistic study of low-molecular-weight organic and inorganic (typically $<1{,}500$ Da) metabolites. Metabolites have an important role in biological systems. They are the building blocks for many other biological components (e.g., proteins, RNA, DNA and cell walls), they are central in intermediary metabolism, they provide many necessities for life (e.g., ATP for energy release) and they have an active role in regulation and signaling. Primary and rapid responses to environmental perturbations are generally, but not exclusively, metabolically focused and are followed by changes at the transcriptional and translational levels.

Metabolomics is applied to the study of microbes[8–10] and plants[11,12], and mammalian[13–16] and environmental systems[17]. In mammals these applications include the study of human diseases to define pathophysiological processes and discover biomarkers[16,18–21], the study of drug toxicity and efficacy[22–24], the study of the interaction of environment and genotype (e.g., nutrigenomics[25,26]) and the study of lipids (lipidomics)[27]. Typically, studies are inductive rather than deductive, and are designed for hypothesis generation or knowledge discovery. This approach starts from a position of limited biological knowledge with the objective to acquire and interrogate data related to a wide and diverse range of metabolites in the metabolome[28]. Holistic studies using MS or nuclear magnetic resonance (NMR) spectroscopy are typically defined as metabolic profiling. Metabolomics offers a number of benefits compared with other '-omic' strategies, with the most advantageous being its close biological proximity to the phenotype of the system and hence the rapid observation of system perturbations in the metabolome. Although analytical platforms are expensive (£100,000s), costs per sample are low (£10s) and, when combined with the high-throughput nature of metabolic profiling (analysis times are typically <30 min), these allow metabolic profiling to be applied to screen large sample sets in a high-throughput approach and at low total cost compared with other '-omic' platforms (e.g., transcriptomics or proteomics).

Metabolic profiling studies have been performed using a range of analytical platforms[29] including gas (GC) or liquid (LC) chromatography (and variants of LC such as UPLC) coupled to MS[30–35], capillary electrophoresis-MS[36,37], NMR spectroscopy[38–40], infrared and Raman spectroscopies[41], electrochemical detectors[42] and direct infusion (or direct injection) MS[9,43]. Of these, chromatography-MS and NMR spectroscopy are the most widely applied and offer different advantages and disadvantages in their application. Owing to the complexity and size of mammalian metabolomes and the diverse physical and chemical properties of metabolites, no single analytical platform can be applied to detect all metabolites in a biological sample[29]. The metabolomics community have realized that the application of multiple analytical platforms in metabolomics is an appropriate strategy to increase the coverage of detected metabolites. For example, the HUSERMET project is applying GC-MS, UPLC-MS and NMR spectroscopy to the epidemiological study of human serum followed by data integration (http://www.husermet.org/).

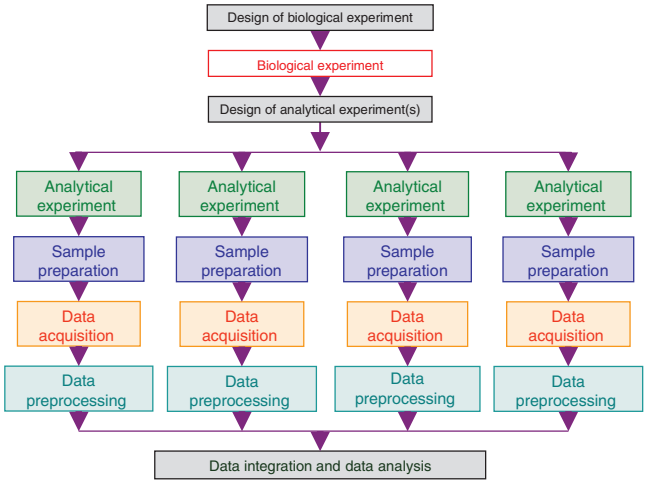

Figure 1 | The generalized workflow for the design of experiments, sample preparation, data acquisition, data preprocessing, integration of multiple analytical experiments and data integration/data analysis for the analysis of serum and plasma in a four-analytical-block biological experiment.

Even though the authors recommend the application of multiple analytical platforms in the study of mammalian systems, we are aware that not all laboratories have access to all of these analytical platforms. Therefore, the protocols have been constructed so that the user can apply the protocol for GC-MS only or the protocol for UPLC-MS only, without the necessity of analyzing samples on both analytical platforms. Data acquired in the HUSERMET project are being used as an example of the applicability of the methods described in this protocol. These data are being applied to define metabolic differences related to age, gender and ethnicity in the normal serum metabolome of 'healthy' subjects in the UK population and also to define biomarkers related to disease diagnosis or disease onset and progression, specifically for Alzheimer's disease and ovarian cancer. Further information can be found in the ANTICIPATED RESULTS section.

Serum/plasma analysis in large metabolomic studies

The study of mammals, particularly humans, can involve the collection, extraction and analysis of a diverse range of sample types. These include blood (serum, plasma)[33,44,45], urine[31,34,46], cerebrospinal fluid[21,47], lymph fluid[48], bile[49], feces[50], saliva[51], cells[52,53], tissues[54–56] and tissue or cell metabolic footprints[57]. Of these, blood and urine are the most frequently studied samples for a number of reasons. Sample collection is noninvasive (urine) or minimally invasive (blood) compared with the collection of cerebrospinal fluid and tissues. Blood and urine are integrative biofluids that incorporate the functions and phenotypes of many different parts of the body in a single sample, a 'metabolic footprint' of tissue metabolism[58]. However, this complexity can dilute small metabolic changes from a specific part of the body, and in these cases, tissues may be appropriate for knowledge discovery (e.g., kidney for renal diseases). These biofluids also contain many hundreds or thousands of metabolites (the human metabolome is estimated to contain 7,800 metabolites, not including many metabolites related to gut microflora, lipid and drug metabolism[59]), and hence provide an appropriate overview of many areas of metabolism in the body.

Humans are complex and diverse organisms[60]. The interaction of the genome and environment (diet, age, lifestyle, gender and many more factors) shape the phenotype as defined in the metabolome. The diversity of each of the environmental and genetic factors provides huge variety in the phenotype and consequently in the metabolome. To define the biological variation accurately and validly large sample sizes are required, to the extent of epidemiological investigations in which thousands of samples are studied. Until recently, NMR spectroscopy was the only analytical platform that had been routinely applied to these large-scale studies[61]. NMR spectroscopy is highly reproducible and, if the samples are analyzed in NMR tubes rather than via flow-through systems, the sample does not come into direct contact with the operational components of the platform. This minimizes contamination and maintenance issues, thus enabling the routine and high-throughput analysis of hundreds to thousands of samples. The coupling of chromatographic separations with MS platforms does not provide this low level of maintenance, as the sample comes in direct contact with many components of these platforms, contaminates surfaces and causes drift in the measured response and retention time over relatively short (tens of injections) analysis periods. Drift is also observed in the mass calibration due to changes in temperature and electrical circuitry, and this can have detrimental effects on instruments operating with high mass resolution and accuracy. However, recent studies have advanced the application of chromatography-MS platforms to large-scale studies of human biofluids[33,62]. These have assessed the appropriate length of analytical experiments before drift in response, mass accuracy and retention time become unacceptable. These authors have shown that the appropriate strategy is to perform small analytical experiments in which data acquired are robust and reproducible and then integrate data from multiple analytical experiments into a single data set related directly to a single biological experiment. This strategy is only possible by the use of standard quality control (QC) samples that are representative of the sample type under analysis and that are used over the whole time course of the study, which, in the case of an epidemiological investigation, may be several years[33,63–66]. This strategy has been applied for the GC-MS and UPLC-MS analysis of serum samples in the HUSERMET project. **Figure 1** shows how the separate procedures (collection, preparation, data acquisition and data pre-processing) in large-scale metabolomic studies are integrated into a single workflow.

Experimental design

Metabolomic studies of mammalian systems (e.g., *in vitro* tissue culture systems and animal models) and humans generally follow two distinct strategies. The first strategy operates in a well-controlled laboratory or experimental environment where the treatment or exposure is the only random variable and where the treatment or exposure is relatively extreme. The result is a large change in the metabolome that is easily measured; accordingly the sample size can be small and still provide statistical confidence to the results. Examples of controlled studies of mammalian systems include *in vitro* cell and tissue culture systems (e.g., see refs. 57,67) and animal models (e.g., see ref. 68) and examples in humans include well class-matched human case-control studies (e.g., see ref. 16).

In the study of the general population, a different strategy is required. To enable a greater understanding of the metabolic status of humans, medium-to-large-scale epidemiological studies are required in order to take into account the substantial diversity observed in physiology, metabolic status and lifestyle in the

general human population, as discussed by Broadhurst and Kell[69]. Metabolic changes are relatively subtle and therefore large-scale epidemiological studies are required to provide statistical confidence. These large-scale studies are required to boost the power of any subsequent statistical analysis, so that subtle differences within the subject cohort can be detected. For example, given an identical change in metabolite response, the statistical confidence interval (and therefore the *P* value) for a biomarker will become lower (further from 1.0) as the sample size increases; thus, providing the opportunity to reduce the number of false discoveries. Examples include the study of health and disease in large human populations to define pathophysiological changes related to a disease and to define biomarkers or risk indictors of diseases, drug efficacy and toxicity, and indicators of diet, lifestyle and age. Controlled experiments including case-control studies can be performed before large-scale studies of the general population are performed (see ref. 70 for further information).

Recent advances in analytical platforms and methodologies have enabled the expansion from small-scale controlled to large-scale studies, as described in this protocol. This provides the opportunity to scale up from biological experiments of <100 samples analyzed in a single day or week to the analysis of thousands of samples over periods of many months or years. This scaling-up requires that great care be taken in the selection of participants (study design), the collection of the biological samples and the design of the analytical experiment (design of experiment) in order to make subsequent data analysis unbiased and fit-for-purpose.

In large-scale epidemiological metabolomic studies, great care in the experimental design is necessary. Not all samples can be run in a single analytical batch because of issues ranging from instrument medium to long-tem reproducibility and necessary preventative maintenance. The issue of reproducibility is instrument dependent. In any chromatography-MS system, the sample unavoidably interacts directly with the instrument and this results in changes in measured metabolic feature response over time, both in terms of chromatography and MS. The degree, and timing, of signal attenuation is not consistent across all measured metabolic features and it is also dependent on the type of biofluid measured. For this reason, it is a necessary requirement that QC samples are periodically analyzed throughout an analytical run in order to provide robust quality assurance (QA) for each metabolic feature detected. A metabolic feature is defined as a detected chromatographic peak with associated retention index and electron-impact (EI) mass spectrum for GC-MS and with a retention time and unique accurate *m/z* for LC-MS. A single metabolite can be detected as multiple and different metabolic features. In GC-MS, two or more derivatization products (each with a different retention index and mass spectrum) can be formed and detected. In LC-MS, different metabolic features (each with the same retention time, but different accurate *m/z*) can be observed for a single metabolite as a result of isotopic peaks and different ionization products (e.g., different adducts). In data processing stages, data conditioning algorithms can use the QC responses as the basis to assess the quality of the data, remove peaks with poor repeatability, correct the signal attenuation and concatenate batch data together after data acquisition and before statistical analysis[33,64,65]. After signal correction and batch integration, each detected metabolic feature is required to pass strict QA criteria. This will be discussed later. Any peak that does not pass the QA criteria is removed from the data set and thus ignored in any subsequent data analysis.

It is important not to introduce any systematic bias (e.g., injection order matching sample preparation order) into a biological or analytical study, as signal correction and batch integration can never be perfect. To compensate for this, within-batch run order is assigned stochastically to each sample such that the sample order is random but stratified by exposure group. In addition, it is necessary that each batch is stratified comparably with the total experiment population. That is, each batch contains a representative cross section of the total study. Again, this will reduce bias in the data analysis.

Sample collection and storage. Probably the single most important part of any large-scale metabolomic study is the correct collection of the sample set. Thus, whereas a failed analytical run can be compensated for by reanalyzing the samples (or, if that is not possible, at least the data can be excluded), systematic failure to collect the samples correctly at the beginning of the investigation may invalidate the whole rationale for the study, with potential ethical implications. Serum is obtained by taking the blood sample and allowing it to clot naturally. The clot is then removed to leave the serum. Plasma is prepared by mixing blood with an anticoagulant followed by centrifugation at 4 °C to separate the plasma from the formed components of the blood (red and white blood cells and platelets). A number of anticoagulants are available, including potassium EDTA, citrate and lithium heparin. Both citrate and EDTA can interfere with subsequent metabolic profiling, either by introducing interfering peaks or, in the case of citrate, by obscuring the endogenous analyte. *Note:* For this reason, the preferred use of lithium heparin is recommended for preparing plasma samples for general analysis. EDTA is also commonly used as an anticoagulant, although we do not recommend it. Following the preparation of serum and plasma, aliquots (0.5 ml) should be rapidly frozen and stored at −80 °C until analyzed. No detailed studies have assessed the stability of the wide range of metabolites present in serum or plasma metabolomes during storage at −80 °C. *Note:* Stability can be expected to be dependent on each metabolite, and thus we recommend not applying multiple freeze/thaw processes to a single aliquot; rather, we suggest collecting multiple aliquots of serum or plasma for each subject and using each aliquot only once. In the HUSERMET study, samples have been analyzed within 2 years of sample collection. However, no detailed assessment of sample stability has been performed and therefore we cannot comment on suitable storage time periods for plasma and serum samples.

GC-MS in large-scale metabolomic studies. GC-MS, using capillary columns, provides appropriate chromatographic resolution, with peak widths of 2–5 s and reproducible retention times[29]. Its major limitation is that it is only capable of analyzing volatile compounds or those that can be made volatile by derivatization. Many of the metabolites found in serum and plasma need to be derivatized before analysis by GC-MS. A range of stationary phases can be applied to metabolome analysis in GC-MS, although methyl-phenyl columns are typically applied (e.g., 95:5 methyl/phenyl and 50:50 methyl/phenyl)[62,71]. GC provides separation of metabolites in a molecular weight range of 18 to ~350 Da (e.g., ammonium to cholesterol) and includes a range of relatively polar metabolite classes including amino acids, organic acids, amines and amides and sugars among others. The methods

described in this protocol enable the reliable detection of 100–200 metabolic features in a serum or plasma sample[62], although one metabolite can be detected as multiple metabolic features because of the formation of multiple derivatization products. Therefore, this number of peaks actually equates to a smaller number of metabolites.

Time-of-flight (TOF) and quadrupole mass analyzers are most frequently applied in metabolic profiling with GC-MS. *Note:* TOF mass analyzers are recommended, as they offer high sensitivity, high acquisition rates (which allow accurate determination of peak shape by the collection of many data points across the peak) and the option of collecting accurate mass data[62,71]. The mechanism of EI ionization has minimal instrument-to-instrument variability and provides highly reproducible and characteristic fragmentation patterns, enabling the resulting mass spectra to be used for determination of chemical structure. The coupling of highly reproducible GC retention times (or retention indices) with EI mass spectra allows the construction of mass spectral libraries that are transferable between instruments, regardless of manufacturer. These libraries can be applied for definitive metabolite identification.

Sample preparation involves a number of steps including deproteinization, freeze drying (lyophilization) and chemical derivatization (see protocol Steps 1–10 and 13–20).

The process of chemical derivatization is required to decrease the boiling point of many endogenous metabolites, thereby making them volatile enough to allow passage through GC columns at temperatures up to 350 °C. Chemical derivatization allows the GC/MS-based detection of many classes of metabolites in central metabolism, including mono- and disaccharides, organic acids, amino acids and amines. There are a multitude of different chemical derivatization reagents used, although a two-stage process of oximation followed by trimethylsilylation (TMS) is most frequently applied. This protocol provides chemical alteration of many different functional groups, including hydroxyls, ketones, carboxylic acids, thiols and amines, and thereby provides good coverage of a range of metabolites[11,31]. Oximation provides conversion of ketone groups to oximes, which are amenable to rapid derivatization with TMS reagents, while ketone bodies do not react rapidly with TMS reagents without previous oximation[72]. TMS replaces active hydrogens with a trimethylsilyl group through, for example, an esterification reaction for hydroxyl groups on carboxylic-acid-containing metabolites to form the TMS ester[73]. If not masked by derivatization, these active hydrogens allow intra- and inter-molecular hydrogen bonding, which increases the boiling points of these metabolites and makes then unsuitable for analysis by GC-MS. A range of artifacts can also be present[74]. Other methods are also applied, e.g., chloroformate derivatization reactions[75].

A range of temperatures and derivatization reaction times are reported in the literature[31,62,71]. The authors apply a rapid derivatization time of 15 min and a temperature of 80 °C. Automated derivatization systems are becoming available (e.g., Anatune) and are independent of the derivatization method used. In this protocol, a single analytical batch of 30 subject samples, 15 QC samples and one saline blank sample are derivatized together and analyzed on a single day.

To compensate for variation in retention time over the long lifetimes of GC columns (1–6 months), retention indices rather than retention times are used. During instrument maintenance, a small section of column is removed from the inlet end. This has an effect of reducing the retention time as the column is shorter and metabolites, therefore, elute from the column more quickly.

To formulate a retention index, a homologous series of chemicals (e.g., *n*-alkanes) are spiked into each sample to compensate for retention time drift over time. Each compound has a defined point on the retention index scale. For example, the retention index for *n*-alkanes is calculated by multiplication of the carbon number by 100 (e.g., C_{10} has a retention index of 1,000). The retention index of a metabolite bracketed by two retention index standards will remain consistent even if the retention times of all three analytes change. For application in multiple laboratories, the accuracy of the retention time requires that the oven temperature or temperature gradient be constant in the elution range of the retention indices. Although the retention indices may co-elute with metabolic features of interest, the mass spectra of the retention indices are significantly different to those of metabolic features. Distinction of co-eluting retention indices from metabolic features, on the basis of their mass spectra, is achievable with data preprocessing software, as applied in this protocol.

UPLC-MS in large-scale metabolomic studies. The introduction of UPLC substantially increased the available chromatographic resolution and number of metabolites detected when compared with traditional LC[76]. The coupling of UPLC to mass spectrometers of high mass resolution (typically > 5,000 full width at half maximum (FWHM)) and high mass accuracy (typically < 5 p.p.m.) provides high chromatographic resolution coupled with high mass accuracy as a tool for putative metabolite identification. However, typical chromatographic peak widths of 1–7 s require fast acquisition rates or scan times, and TOF or Fourier transform/Orbitrap mass spectrometers are the most commonly applied[33,63,77,78]. The linear dynamic range of these instruments operates over three to five orders of magnitude. Metabolites at a concentration of <0.01% of the metabolite of highest intensity can be detected. Similar specifications are observed for GC-MS. The application of hybrid mass spectrometers (e.g., quadrupole-TOF, ion trap-TOF and linear trap quadrupole (LTQ)-Orbitrap) provides access to multiple functionalities, including accurate mass measurements and collision-induced dissociation (CID) mass spectra, both being used for metabolite identification.

However, unlike GC-MS, retention time and CID mass spectra are not reproducible between different systems because of differences in LC column chemistries and mass spectrometer designs; hence, transferable mass spectral libraries are not currently available. The quality of CID and EI mass spectra are dependent on the intensity of the precursor or molecular ion. Low-intensity ions result in poor quality mass spectra from which identification is difficult.

Most applications use reversed-phase column chemistries[33,34,79], although other chemistries (e.g., hydrophilic interaction chromatography) are also used[80,81]. Reversed-phase chemistries start with a high aqueous content mobile phase (with modifiers, e.g., formic acid) and the organic phase fraction is increased, typically operating from 100% aqueous to 100% organic. The organic solvent is typically either methanol or acetonitrile. Acetonitrile is a more non-polar (or lipophilic and hence 'stronger') organic solvent and operates at lower back-pressures, although in our experience it can present problems with elevated background and reduced sensitivity at high acetonitrile concentrations. For this reason, methanol was

chosen in the instrument optimization procedure, and it provided appropriate chromatographic separation[33]. Shortages for methanol have not been observed as for acetonitrile. The gradient elution methods that we apply for electrospray positive (ES+) and electrospray negative (ES−) ion modes have been derived from a closed-loop, multi-objective optimization process[33]. The optimization process defined two separate optimal sets of conditions for ES+ and ES− ion modes because of the differences in the metabolites detected and metabolic profiles. The application of the same gradient elution program in both ES+ and ES− offers advantages in metabolite identification, as a single metabolite will be recorded with the same retention time in both ion modes, and, when feasible, source polarity switching can be carried out. However, a simple post-data acquisition method has been developed to match the same metabolites in both ion modes by calibration of the retention times observed for a group of metabolites detected in both ion modes.

The use of reversed-phase chemistries provides efficient retention and separation of relatively nonpolar metabolites across a large molecular weight range (50 to >1,500) and includes high-molecular-weight lipid species (e.g., phospholipids and triglycerides) and nonpolar amino acids (e.g., tryptophan) as examples. Polar metabolites elute in the column void volume or early in the chromatographic run wherein efficient retention and separation is not achieved.

GC-MS and reversed-phase UPLC-MS are complementary, as each provides detection of diverse sets of metabolites with only a limited number detected by both platforms. GC-MS methods provide the detection of low-molecular-weight metabolites with a boiling point (either before or after chemical derivatization) low enough to allow elution through a GC column. The boiling points of these metabolites are typically <300 °C. Metabolites detected include amino and organic acids, fatty acids, carbohydrates, phosphorylated metabolites (e.g., glucose-6-phosphate) and cholesterol. UPLC-MS reversed-phase methods provide the complementary detection of higher-molecular-weight compounds of medium-to-high lipophilicity, including many classes of lipids (glycerolipids, phospholipids, fatty acids, bile acids and sterols). Polar metabolites are not well retained on reversed-phase columns. Some metabolites detected in GC-MS studies are also detected in UPLC-MS studies (e.g., tryptophan and phenylalanine); however, many metabolites are only detected on a single platform. Some metabolites require specific sample preparation and analytical methods and will not be detected when applying the methods described in this protocol, for example, adenosine phosphates. This is the reason why both methods should be applied in metabolic profiling studies, combined with NMR spectroscopy. Samples are typically analyzed in positive ion mode (ES+) and negative ion mode (ES−) in metabolic profiling studies as they provide complementary data. A range of metabolites are only detected in a single-ion mode, dependent on their acid/base properties. In targeted analysis, a choice of ion mode is made that allows detection of all targeted metabolites.

Sample preparation for UPLC-MS is simpler compared with GC-MS, as, generally, no derivatization is required. It involves deproteinization, lyophilization and reconstitution in a suitable aqueous/organic solvent mixture (see protocol Steps 1–12 and 21–24). Reconstituted samples contain high concentrations of detectable lipids including phospholipids. These can interfere with retention time, and response precision and methods have been described to remove these high-concentration lipids[82,83]. This is the researcher's choice, as lipids are observed to be biologically important in a range of processes involving health, disease and drug metabolism.

In a typical application, as defined in this protocol, 2,000–7,000 metabolic features are detected in a serum or plasma sample, with a greater number detected in positive rather than negative ion mode. A metabolic feature is defined as a detected chromatographic peak with associated retention time and unique accurate *m/z* for LC-MS. A single metabolite can be detected as different ion types, including protonated and deprotonated ions, adduct ions, isotopomers (predominantly ^{13}C and ^{34}S), fragment ions, dimers, trimers and instrument-specific ions[84]. Therefore, this number of features relate to a fewer number of actual metabolites.

Choice of serum or plasma samples. Serum and plasma are similar, but not equivalent. Serum is what remains after the normal clotting process has occurred, and results in the removal of the formed components of the blood (red and white blood cells and platelets), together with those proteins involved in the formation of the clot. Blood plasma is produced by the removal of cells by centrifugation after treatment of the blood sample with an anticoagulant to prevent clot formation. These two methods of removing cells from the blood will lead to slightly different compositions of the resulting fluid. Serum is relatively easy to prepare and does not require particularly sophisticated equipment at the site of collection, making it ideal for multicentre collection facilities (e.g., general practitioner's surgeries or in the subject's home). Preparation of plasma on the other hand does require access to refrigerated centrifuges, which may be readily available in hospitals, but not in the wider medical community. Clearly, therefore, the choice of plasma versus serum has to be based on practical considerations. The choice of serum or plasma is often a pragmatic one, and will usually be based on a combination of the facilities available to the investigator and personal preferences. However, given the likely differences in sample composition between plasma and serum, care should be taken to ensure that only one sample type is used in any particular study. Clearly, care should also be taken in extrapolating the results obtained, for example, plasma to serum and vice versa, without first performing a 'bridging' study.

Serum/plasma sample preparation

A serum, or plasma, sample is composed of a complex matrix of low-molecular-weight organic and inorganic chemicals (metabolites) combined with other higher-molecular-weight species, including proteins and RNA. The matrix is more complex than urine, in which these higher molecular weight species are present at much lower concentrations.

The preparation of serum and plasma samples for LC or GC analysis includes a step to remove the high-molecular-weight species, which involves the addition of an organic solvent/solvent mixture to precipitate these species followed by a centrifugation step to separate the precipitate and the metabolite-containing supernatant. This is described as the deproteinization step, and a range of methods have been described in metabolomic applications involving different solvents/solvent mixtures and temperatures[33,85–87]. Methanol, in a ratio of 3:1 (vol/vol) to the sample, has been shown to be highly efficient in protein removal at room

temperature (15–20 °C) and is simple to use, and was, therefore, chosen by us after development and validation steps. Typically, 30 subject samples are prepared every day to produce 120 extracts to lyophilize per day. This allows 120 subject samples to be prepared in a single week. These solutions are then lyophilized and chemically derivatized (for GC-MS applications), reconstituted in a high aqueous solvent (for reversed-phase UPLC-MS applications) or used directly without lyophilization in hydrophilic interaction chromatography applications (in which a high organic solvent is used). In highly aqueous solutions, incomplete solubilization of lipids may be observed. To solve the solubilization problem, methanol can be added to the reconstitution solution at a concentration that provides full dissolution.

Unlike urine, serum and plasma contain high levels of enzymes, and therefore the temperature at which sample preparation is performed is important. The optimal temperature for activity of most enzymes is 37 °C (body temperature). Reduced temperatures will decrease enzyme activity, although the activity is not completely inhibited until temperatures below −56 °C are reached. Therefore, sample preparation to the step involving addition of solvent for protein precipitation should be performed at temperatures just above the freezing point of water, and thawing/preparation on ice is recommended.

In the protocols described here, 120 samples are prepared in a single week for an analytical block. For each serum or plasma sample (400 µl) a single deproteinization sample is prepared and four separate aliquots (of 370 µl volume) are lyophilized for GC-MS, UPLC-MS (positive ion mode) and UPLC-MS (negative ion mode) analyses. A reserve sample remains, which can be used in the case that re-analysis is required (e.g., if there is a major failure of an analytical platform after sample reconstitution). The preparation of all four samples for analysis at the same time removes the variables that might be introduced from preparing samples separately, and thus reduces potential errors that can be observed in replicate sample preparation procedures. After preparation, all lyophilized samples are stored for a maximum of 3 months at 4 °C.

Sample preparation order is randomized from sample picking and re-randomized from sample analysis order to ensure no systematic biases are present (e.g., to ensure that analysis order does not correlate with sample preparation order). Each subject sample is labeled with a unique identifier with the general construction of 'DDMMYYnnn' where DDMMYY refers to block preparation start date and nnn refers to the sample run order (001–120). In this example, sample XXXXXX41 may be the first and sample XXXXXX87 the second sample prepared, as consecutive numbers relate to sample run and not sample preparation order.

The application of internal standards is recommended for GC-MS platforms in which a greater number of processing steps (e.g., chemical derivatization) and associated errors of low sample volume injections (typically 1 µl) are present compared with UPLC-MS. The data acquired in the HUSERMET project have shown that the technical variability is greater for GC-MS compared with UPLC-MS[33,62]. An internal standard (or standards) is spiked into all samples so as to be present at the same concentration and is applied to compensate for variability in sample processing and analytical platform operation. For example, for two adjacent injections of 1.00 and 1.10 µl, the internal standard peak area for the second injection will be 10% greater than that of the first. As the peak area (or height) ratios of metabolite to internal standard peaks are applied, variation throughout the process from the point of internal standard addition will be compensated for. Ideally, the internal standard would be chemically similar to the analyte of interest; for example, $^{13}C_6$ glucose would be applied as an internal standard for glucose. However, for cases in which hundreds to thousands of metabolites are detected, this is not achievable and a mix of internal standards should be applied, wherein each internal standard acts to compensate for multiple metabolites, a metabolite class or a subset of metabolites (e.g., $^{13}C_6$ glucose for all monosaccharides).

Sample analysis. As described above, large-scale biological experiments are broken down into smaller analytical experiments in which reproducible and robust analytical data can be acquired[33,62]. The multiple analytical blocks of data are processed through a QA procedure and integrated into a single data set. Following optimization and validation of the GC-MS and UPLC-MS methods, an appropriate sample size for a single analytical block was deemed to be 120 samples[33,62]. Each UPLC-MS sample requires 22 (ES+) or 24 (ES−) min for analysis, and each GC-MS sample requires 25 min. Thus, when combined with QC samples, this occupied 4.5 d of GC-MS and UPLC-MS instrument time and allowed all samples to be deproteinized and lyophilized in a single week. Each GC-MS analytical block is composed of four analytical batches with 30 samples per batch, 2 blank samples and 15 QC samples. This is to ensure that all samples are analyzed before a reduction in stability of TMS derivatives is observed[62]. Each UPLC-MS analytical block is composed of two analytical batches with 60 subject samples per batch, 30 QC samples, 2 column mix chromatography test samples and 4 blank samples[33]. The typical block and batch run files for GC-MS and UPLC-MS are shown in **Supplementary Methods 1–3**.

Instrument maintenance is performed at the end of each analytical batch for UPLC-MS and involves mass spectrometer ion source and LC column cleaning. Instrument maintenance is performed at the start of each analytical block for GC-MS and involves replacement of injector liner, septum and gold seal and cutting a 5 cm length from the top of the GC column. Tuning and mass calibration for GC-MS and UPLC-MS is performed at the start of each analytical block (rather than each analytical batch) to ensure no large step changes in the data from the two or four analytical batches, which can be detrimental in data pre-processing steps.

In large-scale studies, an appropriate file naming system is essential to provide unique and descriptive file names. In the HUSERMET project, the authors used a specific naming system as follows:

(i) UPLC-MS—DDMMYYpos_XX and DDMMYYneg_XX, where DDMMYY relates to the day, month and year of sample preparation commencement and XX relates to the sample type and number (i.e., SigmaQC, sample 1 to sample 120, test solution and blank).
(ii) GC-MS—DDMMYY_1_DDMMYY_2_XX, where first DDMMYY_1 relates to the derivatization date and DDMMYY_2 relates to the sample preparation date and XX relates to the sample type and number (i.e., SigmaQC, sample 1 to sample 120 and blank).

QC samples. QC samples are theoretically identical biological samples, with a metabolic and sample matrix composition similar

to those of the biological samples under study. Two types of QC sample are available: pooled QC in which small aliquots of each biological sample to be studied are pooled and thoroughly mixed, and commercially available biofluids composed of multiple biological samples not present in the study. Pooled QC samples offer a number of advantages, including being closest to the composition of the biological samples, and are well suited to small, focused, studies in which all of the samples are available before analysis (e.g., small clinical trials or animal studies). However, pooled QC samples are not always applicable in large-scale studies in which there are many thousands of samples to analyze and in which sample collection is not completed before sample preparation and analysis begin.

In the HUSERMET project, sample preparation and data acquisition commenced before the completion of sample collection. Therefore, a surrogate QC sample was required. It was decided to apply a commercially available serum sample, purchased from Sigma-Aldrich. During the project, it has been observed that the metabolites present and their relative concentrations are different between the serum sample purchased from Sigma-Aldrich and the distribution of serum collected from the subject population. Some metabolites are only detected in the subject population and some are only detected in the commercial serum sample. This was observed for all analytical platforms. During data pre-processing, these metabolites (all associated metabolic features) are removed from the data set, as the methods applied require detection of all metabolites in QC and subject population samples. This provides loss of metabolic information in this data set. This loss related to ~20% of metabolic features detected on all the analytical platforms. *Note:* We recommend using a pooled QC sample deriving from all or a subset of the subject population, to ensure that no or minimal metabolic information is lost. For example, aliquots from the first 1,000 subject samples of a 5,000-subject study could be applied to prepare a pooled QC sample as a compromise to applying samples from all 5,000 subjects.

QC samples provide a measure of the repeatability within an analytical batch and allow metabolic features with excessive drift in signal, retention time or accurate mass to be removed before data analysis in a QA process, as described below in this section. These measures are applied to each individual detected metabolic feature and also to signal correction for the same 'metabolic feature' within and between analytical blocks.

The QC sample is applied for three reasons. The first is to 'condition' or equilibrate the analytical platform before important samples are analyzed to ensure that reproducible data are acquired. Data reported for GC-MS and UPLC-MS have shown that data acquired for serum and plasma in the first four and eight injections for GC-MS and UPLC-MS, respectively, are not reproducible[33,62,83]. This is an effect after preventative maintenance in which active sites are not equilibrated (or 'blocked'), with the sample matrix and multiple injections providing this equilibration.

The second reason is to provide data to calculate technical precision within each analytical block, i.e., a QA procedure. The US Food and Drug Administration (FDA) suggests a range of criteria that should be applied. In the guidance for bioanalytical method validation for drugs in industry, the FDA recommends that for single analyte tests, the tolerance limits are set such that the measured response detected in two-thirds of QC samples is within 15% of the QC mean, except for compounds with concentrations at or near the limit of quantification, wherein a tolerance of 20% is acceptable[88]. For biomarkers, the FDA guidance allows up to 30% coefficient of variation, but clearly, the objective of any analytical methodology should be to aim for the best achievable repeatability. In our case, the methods are not specific for one metabolic feature of interest, but instead, we aim to detect thousands of metabolic features (or metabolites); therefore, an acceptance tolerance of 20% would seem to be appropriate for UPLC-MS. For GC-MS, we apply an acceptance tolerance of 30%, because variation associated with chemical derivatization and injection is higher than for UPLC-MS.

The third reason for using QCs is to provide data to use for signal correction within and between analytical blocks. We apply a univariate approach termed quality control–based robust LOESS (locally estimated scatterplot smoothing) signal correction (QC-RLSC). Here, drift in the signal for each metabolic feature in each subject sample is corrected for by observing the change in signal for the same metabolic feature in 'bracketing' QC samples and correcting in the subject samples for the temporal shift in signal related to that observed for QC samples. This is essential in metabolic profiling experiments. In methods providing absolute quantification using calibration curves, changes in response are 'normalized' in the calibration process. This is not available in metabolic profiling and, therefore, a separate method for signal correction of data to remove biases in response related to analysis order is required. The method provides data for each block that can be easily integrated. We apply a univariate approach[16], although multivariate approaches have also been reported[66].

The frequency of QC sample injections has been assessed in relation to the accuracy and robustness of the signal correction process applying the QC-RLSC algorithm. It has been observed for the Waters LCT system and ThermoFisher Scientific LTQ-Orbitrap system that for stable data with the absence of large fluctuations in response (as is observed in our data), three to five samples bracketed by two QC samples can be analyzed to produce robust signal correction. The analysis orders described in **Supplementary Methods 1–3** apply three and four samples bracketed by QC samples, although this can be altered as appropriate.

Data preprocessing. Raw data acquired using GC-MS and UPLC-MS are typically pre-processed to provide structured data in an appropriate format for data analysis; that is, the chemometric analyses are generally performed on metabolic features with a (relative) concentration rather than directly on the chromatograms. Chromatography-MS data for a single sample are a matrix of *m/z* versus retention time (or index) versus ion current or intensity. These data are processed with a range of software to construct a 3D matrix of chromatographic peak (with related retention time or index, EI fragmentation mass spectrum and/or accurate mass) versus response versus sample ID. This process provides alignment of drift (retention time and accurate mass) in data related to run order and ensures that a chromatographic peak (i.e., metabolic feature) is identified with the same parameters in each sample (i.e., chromatographic peak for methionine in sample 1 is accurately identified as methionine in sample 120 or 5,000).

A range of software is available from instrument companies (e.g., Waters MarkerLynx, ThermoFisher SIEVE, Agilent MassHunter, Applied Biosystems MarkerView, Shimadzu Profiler AM+ and LECO ChromaTOF) or as open-source and freely available software (e.g., XCMS[89], MZmine[90], Metalign[91] and MathDAMP[92]).

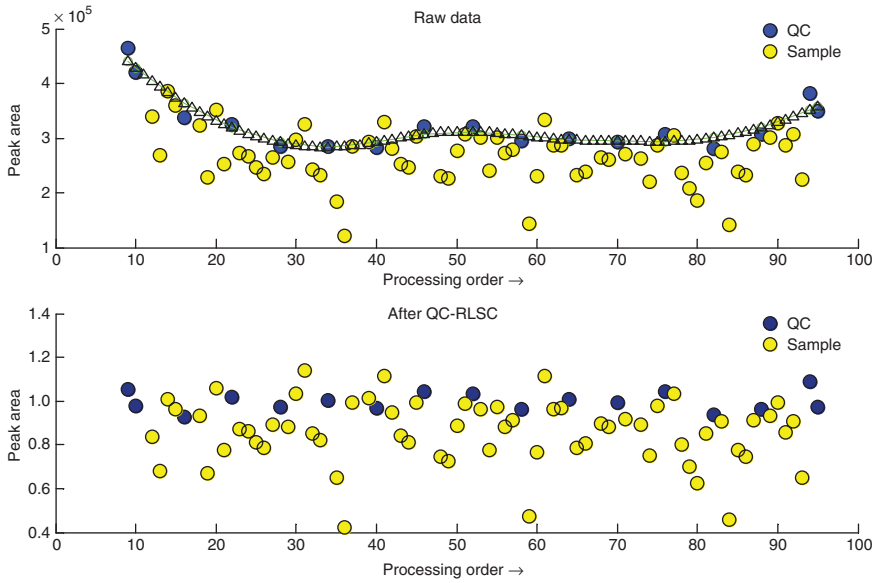

Figure 2 | The QC-RLSC protocol for a metabolic feature detected in UPLC-MS (ES+) with signal attenuation across a given analytical batch. A cross-validated LOESS curve (upper plot) is fitted to the QC samples, the correction curve interpolated (triangles), to which the total data set for that peak is corrected (lower plot).

We use the LECO ChromaTOF software for GC-MS data[62] and the XCMS software for UPLC-MS data[33,77]. Each has been optimized for the data sets acquired. We currently apply the original XCMS option ('matchFilter') for UPLC-MS data, as this was the only XCMS algorithm available during the optimization process. However, for new users of XCMS, we recommend the new option called 'centwave'[33,77].

Quality control–based robust LOESS signal correction

As discussed earlier, the issue of signal intensity drift over time is a major confounding factor in long-term metabolomic studies, particularly when using GC-MS and UPLC-MS platforms. To this end, we include the periodic analysis of a standard biological QC sample together with the subject samples. At the end of the experimental run, and after chromatographic deconvolution, each detected metabolic feature was normalized to the QC sample using QC-RLSC. Here, a low-order nonlinear locally estimated smoothing function (LOESS)[93] is fitted to the QC data with respect to the order of injection. A correction curve for the whole analytical run is then interpolated, to which the total data set for that feature is normalized. Using this procedure, any attenuation of peak response over an analytical run (i.e., any confounding factor due to injection order) is minimized[33,65].

LOESS curve fitting. LOESS curve fitting combines much of the simplicity of classical linear least squares regression with the flexibility of nonlinear regression. It does this by fitting simple models to localized subsets of the data to build up a function that describes the deterministic part of the variation in the data, point by point. In this way, the data analyst is not required to specify a global function of any form to fit a model to the data, but only to fit segments of the data. In this implementation, the local polynomials that are fit to each subset of the data are constrained to be either first or second degree (i.e., either locally linear or locally quadratic). The polynomial is fitted using weighted least squares[94]. In this implementation, the standard tri-cubic weight function was used[93]. To calculate the regression curves to the data with high accuracy, another parameter needs to be optimized. This parameter, known as the 'span' or 'smoothing parameter', determines how much of the data is used to fit each local polynomial. The smoothing parameter, α, is a number between $(\lambda + 1) / n$ and 1, with λ denoting the degree of the local polynomial and n denoting the total number of QC samples in the whole analytical run. The value of α is the proportion of data used in each fit; such that, the subset of data used in each weighted least squares fit comprises the $n\alpha$ (rounded to the next largest integer). Using too small a value for the smoothing parameter is not desirable, as the regression function will eventually start to capture the random error in the data. To stop this over fitting, leave-one-out cross validation was used over the integer range of $n\alpha$ for each degree of polynomial ($\lambda = [1,2]$). Once the LOESS curve is fitted to the QC data, a correction curve for the whole analytical run is constructed using

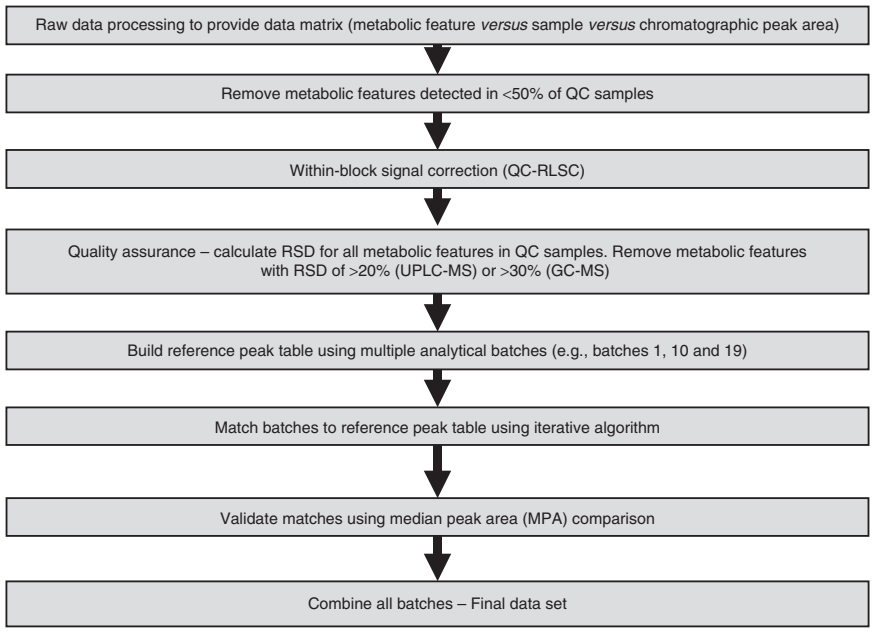

Figure 3 | The data preprocessing workflow for UPLC-MS data. The workflow incorporates QC samples for quality assurance, QC-RLSC and block integration.

cubic-spline interpolation[95], to which the total data set for that peak is normalized. **Figure 2** illustrates the QC-RLSC procedure in practice for a feature in which drift in the signal across a given analytical batch was observed.

After QC-RLSC, each metabolic feature was required to pass strict QA criteria. Any feature that did not pass the QA criteria was removed from the data set and, thus, ignored in any subsequent data analysis (see protocol Steps 33A and 33B).

The workflow for data pre-processing using QC samples for QA, signal correction and block integration is shown in **Figure 3**. Block integration is discussed in more detail in METHODS.

Metabolite identification. Metabolite identification is a complex process in metabolomics and does not provide 100% coverage. Currently, there are chromatographic peaks that are not identified in metabolomic data sets for chromatography-MS (and NMR spectroscopy) acquired data. This is because the human metabolome has not been completely and experimentally characterized yet and the libraries and databases of experimental data applied for identification are not yet completed to reflect all known metabolites. The human metabolome is composed of endogenous metabolites, exogenous metabolites (e.g., drugs, food components, phytochemicals), metabolic products of endo- and exogenous metabolites and metabolites from gut microflora. Not all of those metabolites known to be present can be purchased to construct mass spectral libraries to aid identification processes[85]. Therefore, different levels of identification are available and the reporting procedures for metabolite identification in metabolomics has been described by the Metabolomics Standards Initiative (MSI)[96]. These are classified below.

(1) Putative identification (defined as 'putatively annotated compounds' and 'putatively characterized compound class' in the MSI reporting standards) matches a single measured parameter (e.g., accurate mass or fragmentation mass spectrum) to a metabolite present in a database or library. The confidence of a correct identification is generally lower when compared with definitive identification.

(2) Definitive identification (defined as 'Identification' in the MSI reporting standards) uses two or more measured orthogonal parameters of a metabolite present in a sample that are matched to those of an authentic chemical standard analyzed under identical analytical conditions. However, the possibility of false-positive identification can still be high in metabolic profiling without appropriate method development. For example, two metabolites with similar structure and chemical properties (e.g., structural or stereoisomers) will have similar retention times and fragmentation mass spectra and so can be identified as both. Chromatographic separation of these isomers is required, but this is not readily achievable for all structural or stereoisomers detected in metabolic profiling studies.

(3) Identification of metabolites detected by GC-MS using mass spectral libraries, which includes those commercially available (e.g., NIST/EPA/NIH), those freely available (e.g., Golm Metabolite Database[97]) or laboratory-specific libraries (e.g., Manchester Metabolomics Database (MMD) and associated libraries[84]). The fragmentation mass spectrum of the sample-derived metabolite is matched to fragmentation mass spectra in libraries and scored with a match probability. This provides putative metabolite identification as a single measured parameter. When the mass spectrum is combined with the retention time or retention index measured from an authentic chemical standard, the identification becomes definitive.

(4) Identification of metabolites by UPLC-MS uses, as a preliminary step, the accurate mass only. We recommend the matching of the experimentally determined accurate mass within a specific mass error for a single metabolic feature to the theoretical accurate mass related to a single or multiple molecular formulae. The molecular formulae can then be searched for in databases (e.g., HMDB (http://www.hmdb.ca/), KEGG (http://www.genome.jp/kegg/), ChemSpider (http://www.chemspider.com/) METLIN[98] and MMD[84]) to define putative metabolite identifications. However, if no metabolite matches are found, the molecular formula provides a starting point for further experimental metabolite identification. However, the possibility of false positives and negatives is relatively high, as the type of ion detected is also required (i.e., $[M+H]^+$, $[M+Na]^+$, etc.). This is not always achievable, as a single metabolite can be detected as multiple types of ions

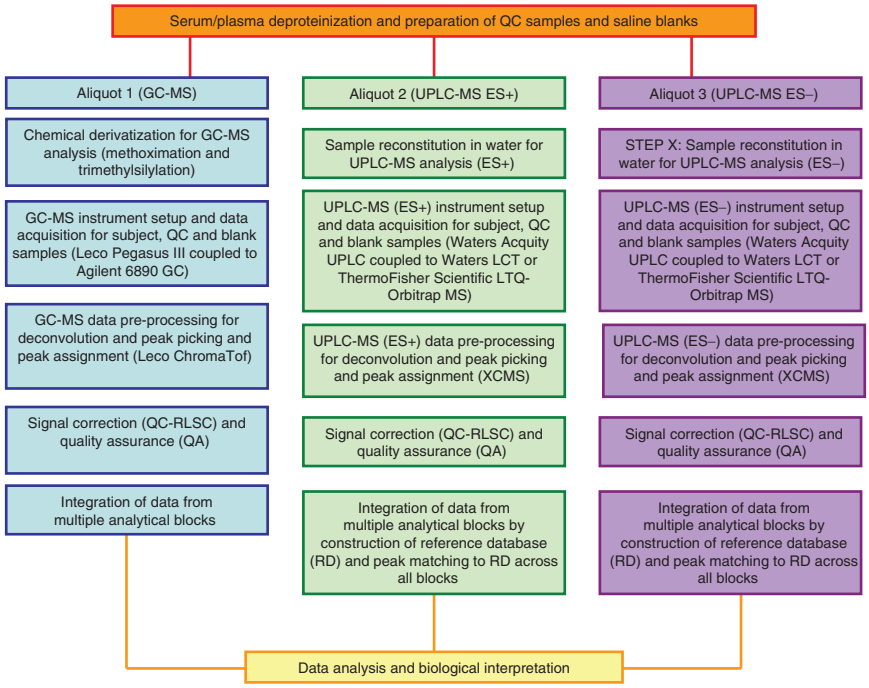

Figure 4 | The experimental workflow followed in the HUSERMET project. Four sample aliquots are prepared from a single serum or plasma sample, with three aliquots processed forward for GC-MS, UPLC-MS (ES+) and UPLC-MS (ES–) analysis. Separate workflows for sample preparation, data acquisition, data preprocessing, signal correction and quality assurance are available for data analysis.

(or metabolic features)[84,99]. Recent advances have provided the capability to perform automated and high-throughput putative identification using the strategy described above[100]. Previously, this was a semi-automated process. Fewer transferable mass spectral libraries are available for UPLC-MS data. This is due to retention time differences observed when different analytical methods or columns are applied and differences in fragmentation mass spectra acquired on different instrument types (e.g., ion trap versus tandem) or instruments of the same mass analyzer type, but of differing instrument design or manufacturer.

Therefore, definitive identification of all detected metabolites is not currently achievable and is a significant limitation in metabolic profiling. For definitive identification, the retention time and fragmentation mass spectra are acquired for sample and authentic chemical standard, but on a one-by-one basis, as libraries are not available as seen for GC-MS. Software for automated annotation of detected chromatographic peaks is more readily available for GC-MS compared with UPLC-MS.

This manuscript will describe integrated standard operating protocols for the sample collection, sample preparation and GC-MS and UPLC-MS analysis of serum and plasma samples. The manuscript will include procedures for QA and data integration for large-scale metabolic profiling studies, as has been developed during the HUSERMET project. The workflow for samples preparation, data acquisition, data pre-processing, signal correction and QA is shown in **Figure 4**.

MATERIALS

REAGENTS

- Human blood sample for serum or plasma or plasma **! CAUTION** Adhere to all relevant ethical regulations and guidelines for the collection and use of human blood. **! CAUTION** To avoid potential contact with bloodborne pathogens, perform all work with appropriate personal protection equipment including gloves and glasses.
- HPLC-grade methanol (CHROMASOLV, Sigma-Aldrich, cat. no. 34860) **! CAUTION** Methanol is toxic and highly flammable and should be handled in a fume hood.
- HPLC-grade water (CHROMASOLV, Sigma-Aldrich, cat. no. 34877)
- HPLC-grade acetonitrile (CHROMASOLV, Sigma-Aldrich, cat. no. 34851) **! CAUTION** Acetonitrile is harmful and highly flammable and should be handled in a fume hood.
- Hexane ('Extra Dry'—water < 20 p.p.m.; Acros Organics, cat. no. 326920010) **! CAUTION** Hexane is harmful and highly flammable and should be handled in a fume hood.
- Pyridine (Acros Organics, cat. no. 131980010) **! CAUTION** Pyridine is harmful and highly flammable and should be handled in a fume hood.
- Formic acid (VWR, cat. no. 450122M) **! CAUTION** Formic acid is corrosive and volatile, and should be handled in a fume hood.
- *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA; Acros Organics, cat. no. 221580250) **! CAUTION** MSTFA is an irritant and flammable and should be handled in a fume hood.
- *O*-methoxylamine hydrochloride (Acros Organics, cat. no. 21049025C) **! CAUTION** *O*-methoxylamine hydrochloride is corrosive and harmful.
- Helium (99.9999%, BOC Specialty Gases, cat. no. 285365) **! CAUTION** Helium is an asphyxiant.
- Nitrogen (99% +, supplied by a nitrogen generator) **! CAUTION** Nitrogen is an asphyxiant.
- Leucine-enkephalin (LE; Sigma-Aldrich, cat. no. L9133)
- Malonic acid d_2 (Sigma-Aldrich, cat. no.175854)
- Succinic acid d_4 (Sigma-Aldrich, cat. no. 293075)
- Glycine d_5 (Sigma-Aldrich, cat. no. 175838)
- Citric acid d_4 (Cambridge Isotope Laboratories, cat. no. DLM-3487-0)
- D-fructose $^{13}C_6$ (Cambridge Isotope Laboratories, cat. no. CLM-1553-0)
- L-tryptophan d_5 (Cambridge Isotope Laboratories, cat. no. DLM-1092-0)
- L-lysine d_4 (Cambridge Isotope Laboratories, cat. no. DLM-2640-0)
- L-alanine d_7 (Cambridge Isotope Laboratories, cat. no. DLM-251-0)
- Stearic acid d_{35} (Cambridge Isotope Laboratories, cat. no. DLM-379-0)
- Benzoic acid d_5 (Cambridge Isotope Laboratories, cat. no. DLM-122-0)
- Octanoic acid d_{15} (Cambridge Isotope Laboratories, cat. no. DLM-619-0)
- Sodium chloride (VWR, cat. no. 452142B)
- Docosane (Sigma-Aldrich, cat. no. D4509) **! CAUTION** Docosane is an irritant and should be handled in a fume cupboard.
- Nonadecane (Sigma-Aldrich, cat. no. N28906) **! CAUTION** Nonadecane is an irritant and should be handled in a fume cupboard.
- Decane (Sigma-Aldrich, cat. no. D90-1) **! CAUTION** Decane is an irritant and should be handled in a fume cupboard.
- Dodecane (Sigma-Aldrich, cat. no. D22110-4) **! CAUTION** Dodecane is an irritant and should be handled in a fume cupboard.
- Pentadecane (Sigma-Aldrich, cat. no. P-7385) **! CAUTION** Pentadecane is an irritant and should be handled in a fume cupboard.
- Dodecanamide (Sigma-Aldrich, cat. no. S408344)
- Benzoyl leucine (Sigma-Aldrich, cat. no. 75813)
- 11-Deoxycorticosterone (Sigma-Aldrich, cat. no. D6875)
- Cortisone (Sigma-Aldrich, cat. no. C2755)
- Thyroxine (Sigma-Aldrich, cat. no. T2376)
- Epitestosterone (Sigma-Aldrich, cat. no. E5878) **! CAUTION** Epitestosterone is harmful.
- *N*-benzoyl-D-phenylalanine (Sigma-Aldrich, cat. no. S782971)

EQUIPMENT

- GC-TOF-MS—autosampler (Gerstel MPS-2L, Gerstel) and gas chromatograph (Agilent 6890 GC with split/splitless injector, Agilent) coupled to a TOF mass spectrometer (LECO Pegasus III, LECO). All instruments are controlled through a single software package (LECO ChromaTOF software, v2.x or greater)
- GC column (VF-17MS column, 0.25 mm ID × 30 m × 0.25 µm film thickness or similar, Varian, cat. no. CP8982)
- Low pressure drop liner with wool GC liner (Thames Restek, cat. no. RE20994)
- GC vials and inserts (2 ml vials with screw caps; Thames Restek, cat. nos. RE21142 and RE21723) and 200 µl vial inserts (Fisher Scientific, cat. no. VGA-100-504D)
- UPLC-TOF-MS—Autosampler and Ultra-Performance Liquid Chromatograph (Waters Acquity system, Waters) coupled to a TOF mass spectrometer (Waters LCT mass spectrometer range, Waters). All instruments are controlled through a single software package (Waters MassLynx v3.x or greater).
- UPLC-LTQ-Orbitrap-MS—Autosampler and Ultra-Performance Liquid Chromatograph (Waters Acquity system, Waters) coupled to a hybrid linear ion-trap-Orbitrap XL mass spectrometer (ThermoFisher Scientific LTQ-Orbitrap range, ThermoFisher Scientific). All instruments are controlled through a single software package (ThermoFisher Scientific XCalibur, v2.× or greater).
- UPLC column—ACQUITY UPLC BEH C_{18} 2.1 mm × 100 mm, 1.7 µm (Waters, cat. no. 176000864) with precolumn in-line filter (Waters, cat. no. 700002775)
- UPLC vials—total recovery screw cap glass vials (Waters, cat. no. 186000385C)
- LECO ChromaTOF software (v2.x or greater for instrument control, data acquisition and GC-MS data processing; LECO)
- Waters MassLynx (v3.x or greater for instrument control and data acquisition; Waters)
- ThermoFisherScientific XCalibur (v2.x or greater for instrument control and data acquisition; ThermoFisher Scientific)
- XCMS software (v1.10 or greater) for UPLC-MS data processing

- Heater block (Techne Dri-Block heaters, Thermo Fisher, cat. no. BLD-715-010G) and aluminum alloy blocks (Thermo Fisher, cat. no. BLD-715-010G)
- Vortex mixer (Thermo Fisher, cat. no. MPR-558-010F)
- Centrifuge (Thermo Fisher, cat. no. CFA-114-010G)
- Refrigerator (4 °C)
- Freezer (−80 °C)
- Centrifugal vacuum evaporator (Fisher Scientific, cat. no. DTF-600-010L)
- Centrifuge tubes (15 ml, Fisher Scientific, cat. no. FB55951)
- Centrifuge tubes (2 ml, Fisher Scientific, cat. no. TUL-150-370W)
- Serum collection tubes (Greiner Vacuettes, cat. no. 455092)
- Lithium heparin plasma collection tubes (Greiner, cat. no. 455084)
- Cryovials (Greiner, cat. no. 122261/122263)
- Microcentrifuge tubes (Eppendorf or equivalent)
- R statistical scripting language (version 2.6.0)

REAGENT SETUP

Serum collection

- Acquire appropriate ethical approval from the local research ethics committee (or appropriate group) before collection of any blood sample. **! CAUTION** Apply all relevant ethical guidelines during collection of all samples. Samples should be collected by a clinician or study nurse.
- The devices used for obtaining the samples together with the containers used for sample collection and storage can be a rich source of unwanted contaminants, particularly of polymers such as polyethylene glycol and impurities including phthalates; these can interfere in the subsequent analysis. For this reason, it is good practice to screen these containers before the study starts using the available analytical platforms. Once you have identified suitable contaminant-free consumables, if at all possible, purchase sufficient quantities of these important consumables to last for the duration of the study. If this is not possible, then any new batches of these consumables should be screened before being deployed, as manufacturing processes can change over time.
- Blood (typically 10 ml) is drawn from a suitable vein into suitable serum collection tubes and allowed to clot for a minimum of 1 h at 4 °C on ice. The clotting time should be recorded. The serum fraction is prepared by centrifugation of the blood collection tube at 2,500*g* for 15 min at 4 °C. Samples are then immediately divided into aliquots (0.5 ml) in cryovials and frozen at −80 °C until sample preparation procedures are carried out. Collect enough blood (typically >4 ml) so that there will be enough serum for at least four aliquots. **! CAUTION** Serum provides a potential infection risk; perform all work with appropriate personal protection equipment including gloves and glasses.
- Samples were stored for a maximum of 2 years before analysis in the HUSERMET project. However, no detailed data are available to define a maximum storage time for serum at −80 °C.

Plasma collection Acquire appropriate ethical approval from the local research ethics committee (or appropriate group) before collection of any blood sample. **! CAUTION** Apply all relevant during the collection of all samples. Samples should be collected by a clinician or study nurse.

The devices used for obtaining the samples together with the containers used for sample collection and storage can be a rich source of unwanted contaminants, particularly polymers such as polyethylene glycol and impurities including phthalates that can interfere in the subsequent analysis. For this reason it is good practice to screen these containers before the study starts using the available analytical platforms. Having identified suitable contaminant-free consumables, if at all possible, purchase sufficient quantities of these important consumables to last for the duration of the study. If this is not possible, then any new batches of these consumables should be screened before being deployed, as manufacturing processes can change over time.

Plasma samples are obtained from blood freshly drawn from a suitable vein and placed in lithium heparin plasma collection tubes in which it is mixed with lithium heparin as the anticoagulant. The plasma fraction is prepared immediately by centrifugation at 3,000*g* for 20 min at 4 °C. Samples are then immediately divided into aliquots (0.5 ml) in cryovials and frozen at −80 °C until sample preparation procedures are carried out. Collect enough blood (typically >4 ml) so that there will be enough plasma for at least four aliquots. **! CAUTION** Plasma provides a potential infection risk; perform all work using appropriate personal protection equipment including gloves and glasses.

Samples were stored for a maximum of 2 years before analysis in the HUSERMET project. However, no detailed data are available to define a maximum storage time for plasma at −80 °C.

***O*-methoxylamine in pyridine** Prepare a working solution (20 mg ml^{-1}) every day by dissolving 30 mg (±3 mg) of *O*-methoxylamine hydrochloride in 1.5 ml dry pyridine.

Internal standard (IS) solutions **▲ CRITICAL** All solutions are stored at 4 °C and must be prepared fresh every week. **! CAUTION** Chemicals are irritants, wear appropriate personal protective equipment and perform the experiments in a fume hood.

MSG IS1: Accurately weigh and record 10.0 ± 0.5 mg quantities of malonic acid d_2, succinic acid d_4 and glycine d_5 into a single 15 ml centrifuge tube, add 10 ml of water and vortex mix for 1 min to provide full dissolution. Label as MSG IS1.

CFT IS1: Accurately weigh and record 10.0 ± 0.5 mg quantities of citric acid d_4, D-fructose $^{13}C_6$ and L-tryptophan d_5 into a single 15 ml centrifuge tube, add 10 ml of water and vortex mix for 1 min to provide full dissolution. Label as CFT IS1.

LA IS1: Accurately weigh and record 10.0 ± 0.5 mg quantities of L-lysine d_4 and L-alanine d_7 into a single 15 ml centrifuge tube, add 10 ml of water and vortex mix for 1 min to provide full dissolution. Label as LA IS1.

SBO IS1: Accurately weigh and record 10.0 ± 0.5 mg quantities of stearic acid d_{35}, benzoic acid d_5 and octanoic acid d_{15} into a single 15 ml centrifuge tube, add 10 ml of methanol and vortex mix for 1 min to provide full dissolution. Label as SBO IS1.

IS2 Solution: A working internal standard solution 'IS2' is prepared fresh each day by combining 2 ml aliquots of each of the four IS1 stock solutions (MSG IS1, CFT IS1, LA IS1 and SBO IS1) and adding 4.0 ml of water to produce a final volume of 12.0 ml. The nominal concentration of each component is 0.167 mg ml^{-1}.

RI solution **▲ CRITICAL** All solutions are stored at 4 °C and must be prepared fresh every month. **! CAUTION** Chemicals are irritants, wear appropriate personal protective equipment and perform the experiments in a fume hood.

RI1 solution: Accurately weigh 30 mg (± 3 mg) each of docosane and nonadecane into a 15 ml centrifuge tube. Add 40 µl each of decane, dodecane and pentadecane. The tube must be weighed after addition of all five alkanes and the weight of each component recorded, this should be 30 mg (± 5 mg) each. Add 10 ml hexane to form the retention index marker solution 1 (RI1).

RI2 solution (working solution): The working retention index solution 2 (RI2) is prepared by adding 2.0 ml RI1 to 8.0 ml pyridine. RI2 may be stored in a sealed container at 4 °C for up to 4 weeks.

Mobile phase solutions **▲ CRITICAL** All solutions should be prepared fresh, although they can be stored at room temperature for up to 48 h.

Mobile phase A is prepared by addition of 1.0 ml of formic acid to 1,000 ml of HPLC-grade water and mixing thoroughly.

Mobile phase B is prepared by addition of 1.0 ml of formic acid to 1,000 ml of HPLC-grade methanol and mixing thoroughly. **! CAUTION** Methanol is toxic and highly flammable and should be handled in a fume hood.

Needle wash solutions **▲ CRITICAL** All solutions should be prepared fresh, although they can be stored at room temperature for up to 120 h.

! CAUTION Methanol is toxic and highly flammable and should be handled in a fume hood.

Weak needle wash solution: A weak needle wash solution is prepared by adding 50 ml of HPLC-grade methanol to 950 ml of HPLC-grade water and mixing thoroughly.

Strong needle wash solution: A strong needle wash solution is prepared by adding 800 ml of HPLC-grade methanol to 200 ml of HPLC-grade water and mixing thoroughly.

Leucine-enkephalin solution **! CAUTION** Methanol is toxic and highly flammable and should be handled in a fume hood.

LE1 solution: Accurately weigh 25 mg (± 3 mg) of leucine-enkephalin and dissolve in 100.0 ml of 50:50 methanol/water containing 0.1% (vol/vol) formic acid. This is defined as LE1.

LE2 (working solution): To prepare a working solution of concentration 2.5 ng $µl^{-1}$, dissolve 1.0 ml of LE1 solution in 99.0 ml of 50:50 methanol/water containing 0.1% (vol/vol) formic acid. This is defined as LE2 and is infused into the TOF mass spectrometer for online mass correction.

EQUIPMENT SETUP

▲ CRITICAL This section contains information regarding the maintenance, setup and mass calibration of the analytical equipment.

GC-TOF-MS During long-term studies in which a single block of 120 samples is analyzed every week, a representative indication of the quality of data acquired from an instrument can be observed from the data acquired in the previous set of sample injections. Load data for five QC samples from the start, middle and end of the last day of the previous week and check that peak widths, heights, retention times and chromatographic resolution do not vary significantly. Check the instrument's log file and confirm that no malfunctions (indicated by red bullet points) have been recorded. Also check the instrument log book for any observed changes or errors in performance or operation. If any are found, either rectify or defer analysis until the instrument is serviced.

If the data are reproducible, replace the septum, inlet liner and gold seal. Remove 2–5 cm from the front of the GC column and re-install. These components collect the contaminating components of the sample upon injection and therefore require regular replacement. Check the autosampler syringe and needle for damage or jamming, and clean, replace or repair if necessary. With the 'clean syringe' command on the Gerstel MPS-2L control panel, check that the syringe draws solvent correctly without entraining air bubbles.

Confirm that the vacuum is at 3×10^{-7} Torr or lower; if not, find and rectify leaks in the mass spectrometer housing and column connections. ▲ **CRITICAL** Using the full diagnostics procedure, you should obtain a combined air leak and calibration gas mass spectrum, and then confirm that the nitrogen peak height at *m/z* 28 is <15% of the *m/z* 69 peak height, and oxygen < 5% of the *m/z* 69 peak height. ▲ **CRITICAL** On the first day of a block analysis, the instrument should be tuned. For the LECO Pegasus III mass spectrometer described in this protocol, perform an 'Acquisition System Adjust', 'Filament Focus', 'Ion Optics Focus' and 'Mass Calibration' set of tuning and mass calibration operations. Set the detector voltage in the mass spectrometer method to a value 50 V greater than the tune file detector voltage. Replace the wash solvents (pyridine) and dispose of waste solvent.

During sample analysis, chromatographic separations are performed on a Varian VF-17MS column. Gas saver flow (25 ml min^{-1}) is switched on 15 s after sample injection. The temperature program begins at 70 °C with a hold time of 4 min, followed by a linear temperature ramp of 20 °C per min up to 300 °C, followed by a hold time of 4 min. The oven temperature is then allowed to cool to 70 °C before the next injection. The transfer line temperature is held at 240 °C. The mass spectrometer source is operated at a temperature of 250 °C in EI mode, with an electron energy of 70 eV. Data are acquired over the range of *m/z* of 45–600, at an acquisition rate of 20 Hz. The detector is operated in the range 1,400–1,800 V, typically 50 V greater than the voltage determined during the LECO-defined tuning checks.

UPLC At the start of an experimental block, prepare a fresh set of UPLC mobile phase and needle wash solutions, as described in the reagents section.

Prime the system, wash the autosampler needle and sample syringe.

Install a fresh, unused UPLC column and pre-line filter, and then condition the column by operating with 100% mobile phase B and starting the flow at 0.05 ml min^{-1}; next, leave for 3 min and then increase to 0.1 ml min^{-1}; next, leave for 3 min and then increase to 0.20 ml min^{-1}; next, leave for 3 min and increase to the operating flow rate (0.36 or 0.40 ml min^{-1}). Set the column temperature to 50 °C. Leave for 10 min.

Change the mobile phase composition to 50% mobile phase A/50% mobile phase B and monitor the operating pressure. This should be <12,000 p.s.i. ▲ **CRITICAL** If this is >12,000 p.s.i., check the column, pre-line filter and post-UPLC tubing for blockages. If <12,000 p.s.i., change the mobile phase composition to 100% mobile phase A and monitor the system pressure. This should be <6,000 p.s.i. when stabilized. Allow the column to equilibrate for a minimum of 30 min.

Following each of the analytical batches using the same UPLC column, pump 100% acetonitrile at a flow rate of 0.40 ml min^{-1} through the system for a minimum of 30 min to clean the column of highly nonpolar metabolites.

Change the mobile phase composition to 50% mobile phase A/50% mobile phase B and monitor the operating pressure. ▲ **CRITICAL** This should be <12,000 p.s.i. Following the cleaning procedure, re-equilibrate the system with 100% mobile phase A for a minimum of 30 min at a flow rate of 0.40 ml min^{-1}.

Chromatographic separations are performed on an ACQUITY UPLC BEH column (C_{18}, 2.1 × 100 mm, 1.7 µm) operating at 50 °C and applying a binary mobile phase system. Mobile phase A is water containing 0.1% (vol/vol) formic acid and mobile phase B is methanol containing 0.1% (vol/vol) formic acid. Different gradient elutions are performed for positive and negative ion mode detection, as described in **Table 1**, with flow rates of 0.36 and 0.40 ml min^{-1} for positive and negative ion mode detection, respectively.

TOF-MS During long-term studies in which a single block of 120 samples is analyzed every week, a representative indication of the quality of data acquired from an instrument can be observed from the data acquired in the previous set of sample injections. Load data for five QC samples from the start, middle and end of the last day of the previous week and check that peak widths, heights, retention times and chromatographic resolution do not vary by >20%. If the observed shift in retention time from the data acquired at the beginning and the end of the block vary significantly (by >0.2 min), check the UPLC system for leaks.

Check whether the vacuum pressures are as typically observed and check that all voltages are as set in the Tune page of MassLynx. The instrument logbook should be checked for any observed changes or errors in performance or operation. If any are found, either rectify immediately or defer analysis until the instrument is serviced.

At the end of each analytical batch, the source is cleaned to remove residues that can reduce the instrument sensitivity. Sonicate the sample cone in a 50:50 (vol/vol) methanol/water solution containing 1% (vol/vol) formic acid for 15 min and dry with nitrogen before replacing.

TABLE 1 | Waters Acquity UPLC gradient elution program applied for UPLC-MS analysis for ES+ and ES– modes.

Time (min)	Flow rate (ml min^{-1})	Mobile phase A (%)	Mobile phase B (%)	Curve
Positive ion mode (ES+)				
Initial	0.4	100	0	5
1.0	0.4	100	0	5
16.0	0.4	0	100	5
20.0	0.4	0	100	5
22.0	0.4	100	0	1
Negative ion mode (ES–)				
Initial	0.36	100	0	4
2.0	0.36	100	0	4
17.0	0.36	0	100	4
22.0	0.36	0	100	4
24.0	0.36	100	0	1

Infuse leucine-enkephalin (LE2) solution into the mass spectrometer through the reference probe and check the MS response. ▲ **CRITICAL** The response should be <300 counts in continuum mode for the ^{12}C isotope peak in attenuated mode and for the ^{13}C isotope peak in normal mode. Tune the instrument manually (using ion source parameters first and detector voltages second) until the required ion count is observed.

The MS system should be calibrated according to the manufacturer's instructions with a sodium formate solution. The intensities of all calibrant peaks should be around 300 counts per scan in continuum mode (if the intensity of any of the calibrant peaks is substantially higher (by >50%), this peak should be manually rejected from the calibration before calibration acceptance). The residual error of an acceptable calibration must be within 10 p.p.m.

Switch the photo diode array lamp on for at least 1 h before data acquisition.

Load the appropriate MS tune method, setting the appropriate values for the parameters on the ES− or ES+ page and wait for the read-backs to reach the set values. The source and desolvation temperatures should be set to 100 and 200 °C, respectively, for nominal mass correction and calibration. Set these temperatures to 140 and 480 °C, respectively, for sample analysis.

LTQ-Orbitrap-MS During long-term studies in which a single block of 120 samples is analyzed every week, a representative indication of the quality of data acquired from an instrument can be observed from the data acquired in the previous set of sample injections. Load data for five QC samples from the start, middle and end of the last day of the previous week and check that peak widths, heights, retention times and chromatographic resolution do not vary significantly (by >20%). If the observed shift in retention time from the data acquired at the beginning and the end of the block vary significantly (by >0.2 min), check the UPLC system for leaks.

Check whether the vacuum pressures are as typically observed and check that all voltages are as set in the Tune page of LTQTune and green ticks are in position against each parameter. If any errors are found, either rectify immediately or defer analysis until the instrument is serviced.

At the end of each analytical batch, the source is cleaned to remove residues that can reduce the instrument sensitivity. This involves sonication of the sample cone and transfer lens in a 50:50 (vol/vol) methanol/water solution containing 1% (vol/vol) formic acid for 15 min.

Tune the mass spectrometer according to the manufacturer's specifications, applying the peaks at *m/z* 514.2 (taurocholate, negative ion) and 524.2 (MRFA peptide, positive ion).

Switch the photo diode array lamp on for at least 1 h before data acquisition.

The MS system should be calibrated according to the manufacturer's instructions, with a calibration solution containing SDS, sodium taurocholate, MRFA peptide, Ultramark 1621 and caffeine. ▲ **CRITICAL** If the instrument is being switched into operate mode from standby, wait a minimum of 2 h before performing a mass calibration. ▲ **CRITICAL** The intensities of all calibrant peaks should be between 10^4 and 10^7. Set the scan time to 100 ms and average three microscans. The error for all calibrant peaks should be within 5 p.p.m. If the mass error is greater, perform a second mass calibration. If the mass calibration error is still >5 p.p.m., either rectify immediately or defer analysis until the instrument is serviced.

Load the appropriate MS tuning method, setting the appropriate values for the parameters, and then wait for green ticks to be in position against each parameter. The parameters are instrument-specific and should be defined by the instrument operator. For the described instrument, these parameters have been described previously[33].

PROCEDURE

Prepare serum and plasma samples ● TIMING 2–3 h

1| Allow plasma/serum samples to thaw on ice at 4 °C for 30–60 min.

2| Aliquot 400 µl of plasma/serum into a labeled 2.0-ml microcentrifuge tube and add 200 µl of internal standard solution (IS2) and then 1,200 µl of methanol.

3| Thoroughly mix on a vortex mixer for 15 s and pellet the protein precipitate in a centrifuge operating at room temperature and at 15,800*g* for 15 min.

4| Transfer 370-µl aliquots into four separate labeled 2.0-ml microcentrifuge tubes and dry down (lyophilize) each sample in a centrifugal vacuum evaporator for 18 h. Apply no heating during the drying process.

■ **PAUSE POINT** Store the samples at 4 °C for up to 12 weeks.

Prepare QC samples ● TIMING 2–3 h

5| Allow plasma/serum samples to thaw on ice at 4 °C for 30–60 min.

6| Aliquot 400 µl of plasma/serum into a labeled 2.0-ml microcentrifuge tube and add 200 µl of internal standard solution (IS2) followed by 1,200 µl methanol.

7| Thoroughly mix on a vortex mixer for 15 s and pellet the protein precipitate in a centrifuge operating at room temperature and at 15,800*g* for 15 min.

8| Transfer 370-µl aliquots into four separately labeled 2.0-ml microcentrifuge tubes and dry down (lyophilize) each sample in a centrifugal vacuum evaporator for ~18 h. Apply no heating during the drying process.

■ **PAUSE POINT** Store the samples at 4 °C for up to 12 weeks.

Prepare saline blank samples ● TIMING 15 min

9| Aliquot 100 µl of 0.7% (wt/vol) sodium chloride into a 2.0-ml microcentrifuge tube and add 50 µl of internal standard solution followed by 300 µl methanol.

10| Thoroughly mix on a vortex mixer for 15 s and dry down (lyophilize) each sample in a centrifugal vacuum evaporator for ~18 h. Apply no heating during the drying process.
■ **PAUSE POINT** Store the samples at 4 °C for up to 12 weeks.

Prepare chromatography check solution ● TIMING 15 min
11| Accurately weigh 10 mg of each chemical (± 3 mg) of dodecanamide, benzoyl leucine, 11-deoxycorticosterone, cortisone, thyroxine, epitestosterone and *N*-benzoyl-D-phenylalanine into a 15-ml centrifuge tube. Dissolve in 10.0 ml of solvent (50:50 (vol/vol), methanol/water) to prepare a 1.0 mg ml^{-1} solution and label the tube as CCS1.

12| Aliquot 2.0 ml of CCS1 solution into a 15-ml centrifuge tube, add 8.0 ml of solvent (50:50 (vol/vol) methanol/water) and thoroughly mix to prepare a solution of 200 µg ml^{-1}. Label this as CCS2.
■ **PAUSE POINT** Store the samples at 4 °C for up to 2 weeks.

Chemical derivatization for GC-MS analysis ● TIMING 45–60 min
13| Lyophilize dried samples for 1 h; switch on the Dri-Block heater and allow it to reach a set-point temperature of 80 °C.

14| Add 50 µl of a 20 mg ml^{-1} *O*-methoxylamine in pyridine solution to the dried extract, thoroughly mix for 15 s on a vortex mixer, and then heat in the Dri-Block heater at 80 °C for 15 min.

15| Remove samples from block heater, add 50 µl of MSTFA to each solution, vortex for 15 s and heat in a block heater at 80 °C for 15 min.

16| Remove samples from the block heater and allow them to cool for 5 min. To each sample, add 20 µl of working retention index solution (RI2) and vortex for 15 s.

17| Centrifuge each sample at 15,800*g* for 15 min and transfer 100 µl of the supernatant to a 200-µl vial insert placed in a 2-ml vial; seal with a screw cap.

GC-TOF-MS analysis ● TIMING 30 min per sample
18| Analyze samples applying the following instrument parameters. A volume of 1 µl of derivatized sample solution is injected through a split/splitless injector operating at a temperature of 280 °C, at a split ratio of 4:1 and with a helium carrier gas flow rate of 1 ml min^{-1} in constant flow mode. Chromatographic separations are done as described in the EQUIPMENT SETUP section.
? TROUBLESHOOTING

19| Analyze the samples in a predetermined order. The typical analysis order is composed of QC samples, subject samples and saline blank samples. Five QC samples are injected at the start of each analytical batch and then one QC sample is analyzed at every fourth injection. The sixth injection is the saline blank and subject samples are randomly analyzed between each QC sample in sets of three injections. Typically, 47 injections are performed in a 24-h period; these 47 injections comprise 15 QC samples, 2 saline blank samples and 30 subject samples. This allows the analysis of 120 subject samples in a working week of 5 d (first sample injected on Monday morning and last sample injected on Friday morning). A typical analysis order is shown in **Supplementary Method 1**.
? TROUBLESHOOTING

20| At the end of each analytical batch, assess six metabolites (lactic acid, alanine, glutamine, fructose, tryptophan and octadecanoic acid). Ensure that the peak shapes, peak heights and retention times are reproducible with no systematic drift.
■ **PAUSE POINT** Archive raw analytical data for future use.

Sample reconstitution for UPLC-MS analysis ● TIMING 30–45 min
21| Add 100 µl (TOF-ES− and Orbitrap ES+ and ES−) or 200 µl (TOF-ES+) of water to dried samples, vortex for 15 s and centrifuge at 15,800*g* for 15 min.

22| Transfer 90 µl (TOF-ES− and Orbitrap ES+ and ES−) or 180 µl (TOF-ES+) of supernatant to low-recovery-volume 2-ml vials and seal with screw caps.

23| Tap the bottom of each vial to release air bubbles present at the bottom.

24| Place in UPLC autosampler/sample manager operating at 4 °C.

BOX 1 | TYPICAL ANALYSIS ORDER FOR UPLC-MS DATA ACQUISITION

1. For UPLC-MS analysis on a Waters TOF instrument, 96 injections or less are recommended; more injections may cause signal stability to fall below acceptable levels. QC samples, saline samples, chromatography check solution and subject samples are analyzed in a predetermined order. Typically (but not exclusively), 96 injections are carried out for each analytical batch in a 35–40-h period. Ten QC samples are injected at the start of each analytical batch and then one QC sample is injected at every fourth injection. Injections 11 and 96 are chromatography check solutions and injections 94 and 95 are saline blank samples. The remaining injections are composed of a randomized order of 60 subject samples. A typical analysis order is shown in **Supplementary Method 2**. Two analytical batches are completed in a working week of 5 d, equating to 120 subject samples. Multiple injections of a single QC sample in a single vial can be carried out, and we use three or four multiple and consecutive injections.
2. For UPLC-MS analysis on a ThermoFisher Scientific LTQ-Orbitrap instrument with robust QC-RLSC, 120 injections or less are recommended before signal stability falls below acceptable levels. QC, saline and subject sample and chromatography check solution are analyzed in a pre-determined order. Typically (but not exclusively), 120 injections are carried out for each analytical batch in a 44–49-h period. Ten QC samples are injected at the start of each analytical batch and then one QC sample at every fifth injection. Injections 11 and 120 are chromatography check solutions and injections 97 and 98 are saline blank samples. The remaining injections are composed of a randomized order of 84 subject samples. A typical analysis order is shown in **Supplementary Method 3**. Two analytical batches are completed in a working week of 5 d, equating to 168 subject samples. Multiple injections of a single QC sample in a single vial can be carried out and we use three or four multiple and consecutive injections.

UPLC analysis ● TIMING 22 or 24 min per sample

25| Inject 10 µl of reconstituted sample on to the UPLC column from sample vials stored at 4 °C. Chromatographic separations are performed as described in the EQUIPMENT SETUP section.

? TROUBLESHOOTING

TOF-MS analysis ● TIMING 22 or 24 min per sample

26| If the UPLC system is coupled to a Waters LCT TOF-MS instrument, perform the following procedures. If a different manufacturer's TOF-MS system or a different Waters TOF-MS model is used, please develop and validate the methods described, taking into account the manufacturer's instructions.

? TROUBLESHOOTING

27| Acquire accurate mass data in 'V mode' as centroid data in the *m/z* range of 50–1,000 and with dynamic range enhancement activated. A scan time of 0.4s (0.35 s and 0.05 s dwell time) is applied. Half (50%) of the UPLC eluent is directed to the mass spectrometer and the other 50% is diverted to waste. Instrument-specific parameters have been recorded and described previously[33].

? TROUBLESHOOTING

28| At the end of each analytical batch, assess seven metabolites (dodecanamide, benzoyl leucine, 11-deoxycorticosterone, cortisone, thyroxine, epitestosterone and *N*-benzoyl-D-phenylalanine). Ensure that the peak shapes, peak heights and retention times are reproducible with no systematic drift. The typical analysis order is composed of QC samples, subject samples, chromatography check solution and saline blank samples, analyzed in a predetermined order as described in **Box 1**.

■ **PAUSE POINT** Archive raw analytical data for future use.

LTQ-Orbitrap-MS analysis ● TIMING 22 or 24 min per sample

29| If the UPLC system is coupled to a ThermoFisher LTQ-Orbitrap XL instrument, perform the following procedures. If a different model of the Orbitrap system (ThermoFisher Scientific) is applied, please develop and validate the methods described, taking into account the manufacturer's instructions.

? TROUBLESHOOTING

30| Acquire accurate mass data in the Orbitrap mass analyzer in the *m/z* range of 50–1,000 (in centroid mode), with a mass resolution of 30,000 at mass 400 (FWHM), and with a scan time of 0.4 s. The UPLC eluent is split, with 50% directed to the mass spectrometer and 50% diverted to waste.

? TROUBLESHOOTING

31| At the end of each analytical batch, assess seven metabolites (dodecanamide, benzoyl leucine, 11-deoxycorticosterone, cortisone, thyroxine, epitestosterone and *N*-benzoyl-D-phenylalanine). Ensure that the peak shapes, peak heights and retention times are reproducible with no systematic drift. The typical analysis order is composed of QC samples, subject samples, chromatography check solution and saline blank samples, analyzed in a pre-determined order, as described in **Box 1**.

■ **PAUSE POINT** Archive raw analytical data for future use.

Data preprocessing

32| Preprocess the data by following the steps in option A for GC-TOF-MS data and those in option B for UPLC-MS data.

(A) GC-TOF-MS data analysis ● TIMING 4–6 h for target list generation, 2–3 h for raw data processing of data acquired over 5 d

(i) Using LECO's terminology, perform a 'peak find' data processing method with a single QC sample injected in the middle of the block experiment. The data processing method should have 'Baseline', 'Peak Find', 'Calculate Area/Height' and 'Retention Index' functions activated. Key parameters in this method are the baseline offset, data points to be averaged for smoothing, expected chromatographic peak width, maximum number of unknown peaks to find and the minimum signal-to-noise ratio for the (automatically selected) quantitation mass. All parameters are sensitive to the chromatographic performance obtained and must be selected to reflect this. From representative chromatograms acquired in the HUSERMET project, in which we analyzed thousands of human serum samples with GC-MS, baseline offset was set at 0.5, data points to be averaged for smoothing was set at automatic, peak width was set at 1.8 s and the maximum number of unknown peaks to find was set to 400. A signal/noise (S/N) threshold of 100:1 was used; this was an informed compromise between comprehensive reporting and the collation of spectra of sufficient quality to be reliably found subsequently. A retention index method is prepared in the software by compiling a method table containing the retention indices (1,000, 1,200, 1,500, 1,900 and 2,200), the observed retention time and the quantitation ions used to confirm the detection of each retention index compound.

(ii) Step 32A(i) produces a table of potential candidates for inclusion in a reference table and annotated with a retention index, mass spectrum and single quantitation ion. From this table, delete candidates whose mass spectrum does not contain fragment ions expected for TMS derivatives at *m/z* 73 and 147, and whose quantitation ion chromatogram indicates that a single mass spectral feature has been reported as multiple features ('peak splitting'). In these cases, delete the features with lowest S/N while retaining the feature with the highest S/N. Manually edit the mass spectrum for the isotopically labeled internal standards to remove ions present in the unlabeled endogeneous metabolite. Assess the automatically chosen quantitation masses for accuracy, a high S/N ratio and no interference to peak shape from co-eluting derivatized metabolite peaks. Amend the quantitation mass if necessary. The metabolite peaks are then exported to a reference file created before Step 32A(i). Parameters in the reference table are set at 100,000,000 for tolerance (to ensure all peaks are matched and reported independent of peak area), 20 for RI deviation, 700 for match threshold, 2,500 for minimum area and 5.0 for S/N threshold.

(iii) A separate study sample can then be processed through the deconvolution software, as described in 32A(i), with the 'Compare' function also enabled. To do this, set the mass threshold setting at 50. Derivatized metabolic features uniquely detected in this sample are marked, the mass spectrum and quantitation masses are assessed as described above in Step 32A(ii) and then exported to the reference file. This process is performed for a range of samples from the study.

▲ **CRITICAL STEP** In large-scale studies, we recommend performing Step 32A(iii) on samples from different experimental blocks to ensure that all derivatized metabolite peaks are present in the reference file.

(iv) Each peak in the reference file is named with a unique label (e.g., internal standard succinic d_4 acid, sample peak X). At this stage, definitive identification of each peak can be performed. To do this, compare the retention index and mass spectrum of each metabolite with those recorded for authentic chemical standards and present in in-house libraries (e.g., Golm metabolome database or MMD in-house library) or in commercially available mass spectral libraries (e.g., NIST, EPA or NIST05 libraries) (see Experimental design). If a match to a retention time/index (± 10) and mass spectrum (match >70%) is observed, the identification can be described as definitive and the peak can be labeled metabolite name_definitive. If a match to only a mass spectrum is observed, the identification can be described as putative and the peak can be labeled 'metabolite name_putative'.

(v) The final stage is used to define the most appropriate internal standard for each peak. This can be performed by analyzing 60 QC injections in a single block. Calculate the peak area ratio (peak area metabolite/peak area internal standard) for each metabolite peak associated with each internal standard and calculate the relative standard deviation (RSD) for each of these peaks for injections 6–60. The internal standard providing the lowest RSD is chosen as the internal standard for that metabolite.

(vi) Perform raw data processing using the reference table described above (Step 32A(i–v)) for all samples to reliably find and report the selected metabolic features in all samples. Process all the blocks using the appropriate set of parameters and internal standard selections. As noted, automatic feature detection and measurement achieves a high success rate (estimated to be in excess of 98%), which was further improved by manually inspecting the peak area measurements for each internal standard in each sample, and manually correcting where required. Further outlier rejection tests can be performed on a block basis before accepting data. This has led to the rejection of <1% of the injections performed.

■ **PAUSE POINT** Archive processed data for future use.

(B) UPLC-MS data analysis ● TIMING 6–8 h for processing of data acquired over a 5-d period

(i) Perform conversion of instrument-specific data format to NetCDF format. Data preprocessing is performed using the open-source XCMS software that requires data in a specific format. The NetCDF format is appropriate as many (but not all) mass spectrometer manufacturing companies provide software with instruments to convert to NetCDF format. For the Waters TOF instrument the software is called DataBridge, which operates in the MassLynx software, and for the ThermoFisher Scientific LTQ-Orbitrap instrument the software is called FileConverter, which operates in the Xcalibur software. All data can then be easily transferred as NetCDF files to the XCMS processing PC for data processing.

(ii) Perform XCMS data processing. In UPLC-MS data, semiautomated reference spectra selection and target list generation are not required. The deconvolution software performs this action in an automated manner. Data are deconvolved into a usable data matrix using XCMS (an open-source deconvolution program available for LC-MS data[89]). XCMS is run using the R statistical scripting language (version 2.6.0) and produces a matrix of features with associated retention time and accurate mass and chromatographic peak area calculated with a single accurate mass. The XCMS settings we applied for UPLC-LCT MS data are 'step' (0.10), 'S/N threshold' (3), 'bw' (10) and 'time limit' (15 s). All other parameters are set to 'default' settings. The XCMS settings we applied for UPLC-LTQ Orbitrap data are 'step' (0.02), 'S/N threshold' (3), 'mass limit' (0.05 amu), 'bw' (10) and 'mzwid' (0.05). All other parameters are set to 'default' settings. Preprocessed data are then exported as a .csv file for further data processing, univariate and multivariate data analysis procedures.

■ **PAUSE POINT** Archive processed data for future use.

Data processing, signal correction and QA procedures for multiple analytical blocks

33| Perform data alignment and normalization for the complete data set, composed of multiple analytical blocks, as described below for GC-MS (option A) and UPLC-MS (option B).

(A) Data processing, signal correction and QA procedures for GC-MS data ● TIMING 6–8 h for processing of data acquired over a 5-d period

(i) Remove data related to the first three QC sample injections in each analytical batch. Perform signal correction for each data acquired in each analytical block using the QC-RLSC method to fit a LOESS polynomial curve to the QC data for each metabolic feature. In this implementation, the local polynomials that are fitted to the data are constrained to be either first or second degree (i.e., either locally linear or locally quadratic). The polynomial is fitted using weighted least squares[94] with a standard tri-cubic weight function[93]. To stop overfitting, use leave-one-out cross-validation over the integer range of $n\alpha$ for each degree of polynomial ($\lambda = [1,2]$), where α is the smoothing parameter. Once the LOESS curve is fitted to the QC data, construct a correction curve for the whole analytical run using cubic-spline interpolation[95], to which the total data set for that metabolic feature is normalized. **Figure 2** illustrates the QC-RLSC procedure in practice for a metabolic feature in which signal drift across a given analytical batch was observed.

(ii) Perform a QA procedure to remove metabolic features with poor repeatability. Data for all detected metabolic features for all QC sample injections from injection four to the last injection of the QC sample are applied. Remove all metabolic features that are detected in <50% of QC samples and all metabolic features with a RSD, as calculated for the QC samples, of >30%. The higher RSD value for GC-MS compared with UPLC-MS (see Step 33B) reflects the increased variability that can be introduced in the chemical derivatization procedure as well as the low injection volume applied (1 μl) compared with UPLC-MS sample preparation.

(iii) Combine data from the separate analytical batches for all blocks into a single data set. Include relevant information on blocks, subjects, sample types and injection order.

(B) Data processing, signal correction and QA procedures for UPLC-MS data ● TIMING 6–8 h for processing of data acquired over a 5-d period

(i) Remove data related to the first eight QC sample injections in each analytical batch. Perform signal correction for each analytical block using the QC-RLSC method, as described in Step 33A(i).

(ii) Perform a QA procedure to remove metabolic features with poor repeatability. Data for all detected metabolic features for all QC sample injections from injection nine to the last injection of the QC sample are applied. Remove all metabolic features that are detected in <50% of QC samples and all metabolic features with a RSD, as calculated for the QC samples, of >20%.

(iii) An important consequence of separate analytical block experiments for UPLC-MS data is that metabolic features are not aligned across blocks; matching the same metabolic features across multiple blocks is required. Construct a reference database, composed of unique metabolic features—as defined by accurate mass and retention time—in a chosen subset of the analytical blocks. For example, for the HUSERMET data, three of ten analytical blocks were chosen. The reference database will contain all unique features detected in the biological experiment. The data describe the metabolite identifier, *m/z* and retention time, and the estimated median peak area (MPA) for the QC samples for that instrument. Subsequently, and for each block separately, match the features in each block to the

reference peak database. Only match a metabolic feature if the error in *m/z* is less than a specific range (e.g., ± 5 p.p.m.) and the error in retention time is less than a specific range (e.g., ± 10 s). For matched metabolic features, include an identifier related to the reference peak database. For example, if a metabolic feature in block 1 matched to metabolic feature 76 in the reference database, then label this metabolic feature as 76. Following the matching of metabolic features, combine data from all blocks into a single data set.

(iv) As a secondary check, or validation, of the matching process, compare each of the peaks in the new 'matched' data set with the reference table with respect to the expected QC MPA (before QC-RLSC). Using the MPA tolerance formula: $\zeta_i = |(MPA_{b,i} - MPA_{ref,i}) / MPA_{ref,i}|$ where *b* is batch number and *i* is matched peak number; if a value of $\zeta < 4$ is found then the candidate peak is assumed to be within an acceptable peak area tolerance to be correctly matched. *Note*: Once the data are normalized using QC-RLSC, small differences in between-batch MPA will be corrected for. Any peak failing the final QA process is removed from the data set.

■ **PAUSE POINT** Archive processed data for future use.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Instrument	Step	Problem	Potential reason	Solution
GC-MS	18,19	Absence or low number of peaks	Failed injection	Reinject sample
			Blockage in syringe	Replace syringe
		Poor chromatographic peak shape	GC column degradation or contamination	Replace the GC column
			Sample injection volume is too high	Optimize injection volume and concentration of components in sample
		Carryover	The sample is too concentrated	Optimize injection volume and concentration of components in sample
			Too high injection volume	Optimize injection volume and concentration of components in sample
		Unstable GC gas flow or pressure	Leak in GC system	Work through connections on GC to check for gas-tight seals
		Unsteady baseline	Detector or electronics fault	Arrange engineer visit
		Drop in response	Detector or electronics fault	Arrange engineer visit
		High mass spectrometer pressure	Gas infusion from atmosphere	Work through connections on MS for gas-tight seals; call the engineer
UPLC-MS	25–27,29,30	High back pressure	Blockage in capillary or tubing of MS source	Replace MS source tubing or capillary
			Blockage in capillary tubing of UPLC	Replace UPLC tubing
			Blockage in UPLC injection system	Replace UPLC injector tubing
			Build-up of material in UPLC column	Replace UPLC column
		Low number or absence of peaks	Failed injection	Reinject sample
			Blockage in UPLC injection system	Replace UPLC injector tubing
		Poor chromatographic peak shape	Column contamination or degradation	Replace UPLC column

(continued)

TABLE 2 | Troubleshooting table (continued).

Instrument	Step	Problem	Potential reason	Solution
			Sample injection volume too high	Optimize injection volume and concentration of components in sample
		Carryover	Inappropriate wash solutions	Choose appropriate wash solutions
			Too concentrated a sample	Optimize injection volume and concentration of components in sample
			Too high injection volume	Optimize injection volume and concentration of components in sample
			Chromatography not optimized	Optimize gradient elution program
		Periodic sensitivity loss	Matrix suppression	Optimize sample preparation with salt-removal step (e.g., SPE)
			Poor recovery	Optimize sample preparation with salt-removal step (e.g., SPE)
		Sudden sensitivity loss	Component failure of MS system	Arrange engineer visit
		Gradual sensitivity loss	MS source contamination	Clean MS source
		Drops in baseline	Ion suppression, high salt levels	Optimize sample preparation with salt-removal step (e.g., SPE)
		Unsteady ion response	Ion source parameters (voltages and gas flow rates) not optimized	Optimize ion source parameters; consult instrument manual
			Capillary needle protruding too far from probe	Retract capillary needle in to probe
			Probe too close to MS entrance	Retract probe away from MS entrance
			Unstable liquid flow	Check UPLC and connecting systems for leak
			Solvents have not been degassed	Degas solvents
		High noise levels (chemical/electronic)	Detector damaged and producing discharges	Arrange engineer visit
			Inappropriately set threshold levels	Consult manual and apply appropriate thresholds
		High mass spectrometer pressure	Gas infusion from atmosphere	Work through connections on MS for gas-tight seals; call the engineer

Other problems or an inability to solve the problem should lead to a discussion with a qualified instrument engineer.

● TIMING

Steps 1–4, Prepare serum and plasma samples: 2–3 h
Steps 5–8, Prepare QC samples: 2–3 h
Steps 9 and 10, Prepare saline blank samples: 15 min
Steps 11 and 12, Prepare chromatography check solution: 15 min
Steps 13–17, Chemical derivatization for GC-MS analysis: 45–60 min
Steps 18–20, GC-TOF-MS analysis: 30 min per sample
Steps 21–24, Sample reconstitution for UPLC-MS analysis: 30–45 min
Step 25, UPLC analysis: 22 or 24 min per sample
Steps 26–28, TOF-MS analysis: 22 or 24 min per sample
Steps 29–31, LTQ-Orbitrap-MS analysis: 22 or 24 min per sample
Step 32, Data preprocessing: MS 6–9 h; UPLC-MS 6–8 h
Step 33, Data processing, signal correction and QA procedures for multiple analytical blocks: 6–8 h for processing data acquired over a 5-d period for each analytical platform

ANTICIPATED RESULTS

The serum and plasma extraction protocol uses polar solvents (i.e., methanol and water) and is therefore expected to be biased to relatively polar metabolites. However, the data has shown that a wide range of nonpolar metabolites are also detected (phospholipids, glycerides, fatty acids), even without the application of nonpolar solvents such as chloroform. Therefore this method is appropriate for ease of operation and metabolite coverage in serum and plasma samples. The analysis of samples on different analytical platforms (GC-MS and UPLC-MS, as described above, and NMR spectroscopy, as performed in the HUSERMET project) also provides complementary data, with many metabolites being detected on only one or two of the platforms.

The role of QC samples has been shown, for the first time, to be essential in long-term metabolomic studies using MS as the analytical platform. Without the use of QC samples, the reproducibility of data is substantially lower and does not allow biological comparisons to be made. The importance of QC samples is reflected by the length of instrument time—approximately 30% of total instrument time—dedicated to QC samples.

The protocols have been applied to two large-scale (n > 3,000 subject samples) studies. The HUSERMET project for GC-MS[62] and UPLC-MS (Waters UPLC coupled to a Waters TOF instrument operating in positive and negative ion modes)[33] and in a biomarker validation study for pre-eclampsia which is validating discovery data acquired in a smaller study (Waters UPLC coupled to a ThermoFisher LTQ-Orbitrap mass spectrometer, positive ion mode only)[16]. The results for the HUSERMET project when the first ten blocks (n = 1,200 samples) were analyzed are shown below. Typical chromatograms for GC-TOF-MS and UPLC-MS (positive ion mode) are shown in **Figure 5**. A total of 259, 7,813 and 7,914 metabolic features were present in the raw data for GC-MS, UPLC-MS+ and UPLC-MS−; there were 157, 2,181 and 2,283 metabolic features in the data after QA, signal correction and block integration. Each of these features was present in a minimum of 80% of the samples analyzed. **Table 3** shows the

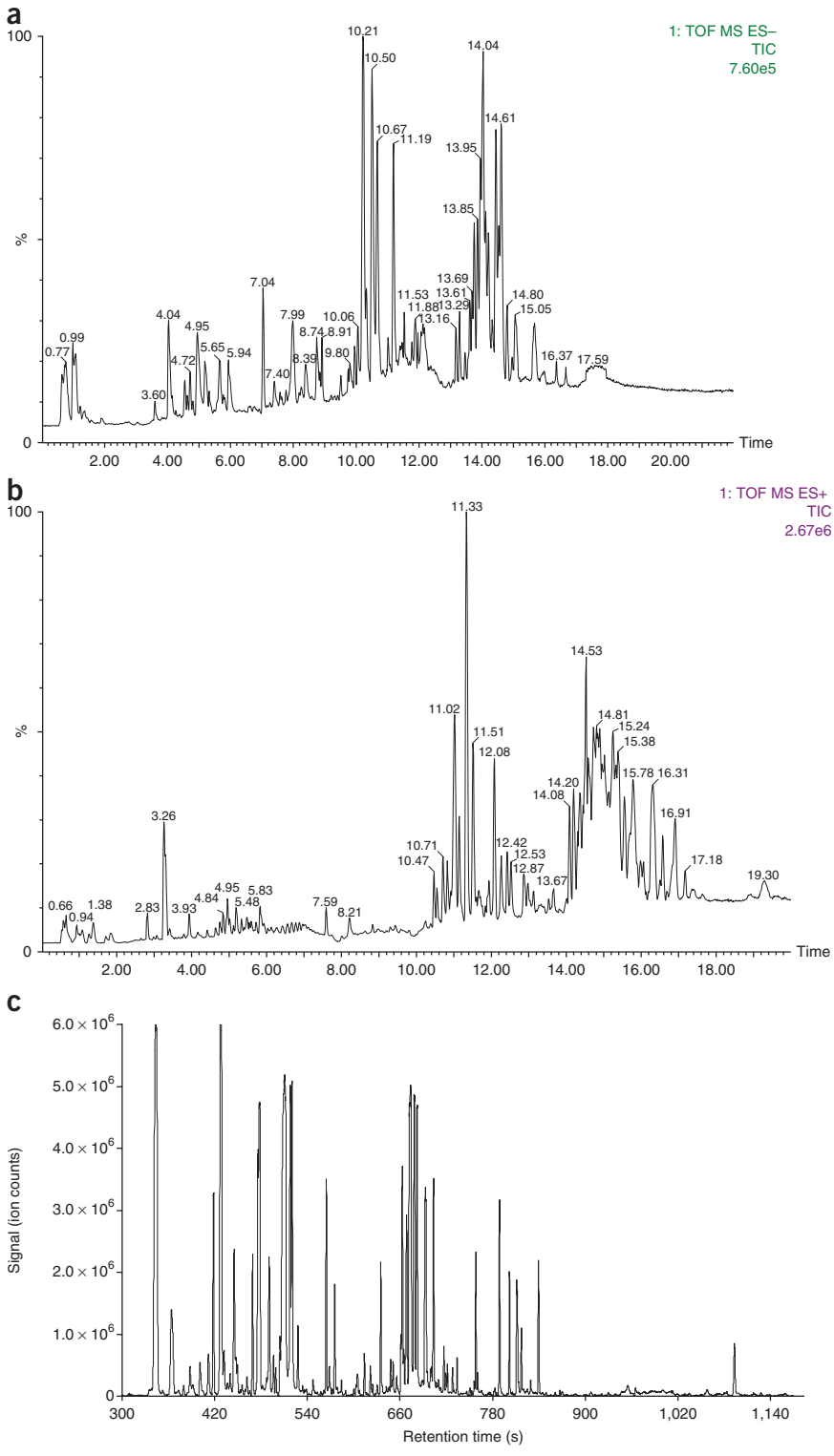

Figure 5 | Typical chromatograms observed for serum. (**a**–**c**) Shown are chromatograms for UPLC-TOF (ES−) MS (**a**), UPLC-TOF (ES+) MS (**b**) and GC-TOF-MS (**c**). The base peak intensity chromatogram is depicted for UPLC-TOF-MS data for both ES+ and ES−. The single-ion chromatogram for m/z 73 is depicted for GC-TOF–MS data. m/z 73 is a fragment ion characteristic of the trimethylsilyl-derivatized products of metabolites.

results of the metabolic feature matching process for ten analytical blocks (20 analytical batches) acquired on the UPLC-TOF-MS system. The performance of each batch is quite similar. Approximately 5,600 metabolic features are consistently detected. Approximately 84% of peaks pass the QA process. Approximately 84% of the quality-assured peaks match to the reference table. Within the matching process, 99.5% of the matches are unique (the 0.5% that are not uniquely matched are easily removed). Of the matched peaks, 98% pass the MPA test. The peak area for QC and subject samples before and after signal correction and block integration for a single metabolic feature is shown in **Figure 6** for UPLC-MS+. Before signal correction, changes in peak area can be observed between blocks. However, after signal correction and block integration, the peak area distribution for each block is similar to the distribution for ten blocks combined. This indicates the robustness of the QC-RLSC method.

In the HUSERMET project, 118 metabolite peaks (related to 86 unique metabolites) were identified in the 159 reproducibly detected peaks reported for GC-MS. Of 2,283 and 2,181 reproducibly detected features applying UPLC-MS− and UPLC-MS+, respectively, 1,451 and 1,519 were putatively identified based on accurate mass, with a mass error <10 p.p.m. These data

TABLE 3 | Table showing the results of the feature-matching process for ten analytical blocks acquired on the UPLC-ToF-MS.

Idx	Block	Number of peaks >0% QCs	Number of peaks passing QA	Peaks passing QA (%)	Matched to REF	Unique matches	Unique matches (%)	Pass MPA test	Pass MPA test (%)	Quality assured peaks (%)
1	4A	5,703	4,829	85	4,817	4,804	99.7	4,731	98.2	84.7
2	4B	5,766	4,795	83	4,225	4,207	99.6	4,110	97.3	83.2
3	5A	5,853	4,423	76	3,555	3,531	99.3	3,417	96.1	75.6
4	5B	5,520	4,173	76	3,398	3,378	99.4	3,245	95.5	75.6
5	6A	5,825	4,861	83	3,779	3,751	99.3	3,667	97.0	83.5
6	6B	5,762	4,979	86	3,816	3,794	99.4	3,684	96.5	86.4
7	8A	5,734	4,825	84	3,900	3,877	99.40	3,833	98.3	84.1
8	8B	5,608	4,763	85	3,883	3,860	99.4	3,813	98.2	84.9
9	13A	5,719	4,862	85	4,410	4,395	99.7	4,371	99.1	85.0
10	13B	5,778	4,901	85	4,907	4,898	99.8	4,869	99.2	84.8
11	14A	5,815	4,894	84	4,444	4,430	99.7	4,415	99.3	84.2
12	14B	5,671	4,942	87	4,457	4,440	99.6	4,426	99.3	87.1
13	15A	5,658	4,854	86	4,114	4,193	99.5	4,091	99.4	85.8
14	15B	5,595	4,828	86	4,163	4,144	99.5	4,142	99.5	86.3
15	16A	5,533	4,733	86	4,128	4,101	99.3	4,106	99.5	85.5
16	16B	5,436	4,699	86	4,185	4,167	99.6	4,155	99.3	86.4
17	17A	5,769	4,709	82	3,767	3,733	99.1	3,710	98.5	81.6
18	17B	5,754	4,811	84	3,793	3,760	99.1	3,755	99.0	83.6
19	18A	5,440	4,655	86	4,661	4,655	99.9	4,661	100.0	85.6
20	18B	5,471	4,622	84	4,297	4,284	99.7	4,286	99.7	84.5
	Mean	5,671	4,758	84	4,135	4,115	99.5	4,074	98	83.9
	s.d.	131.88	186.52	3.13	405.87	411.50	0.21	434.83	1.30	3.1

The table shows the combined results for positive and negative ion mode. A and B (in Block column) relate to the analytical batch in which the data were acquired, with data for two analytical batches collected for each analytical block. The number of features reproducibly detected in >50% of all QCs is relatively consistent for each batch and block. Of the detected features, 84% pass the quality assurance process after QC-RLSC. Approximately 84% of the quality assured features match to the reference table with a mass window of ± 5 p.p.m. and a retention time window of ± 25 s. Of the matched peaks, 98% pass the MPA test. More than 4,000 features detected in >80% of all subject samples were passed forward for data analysis.

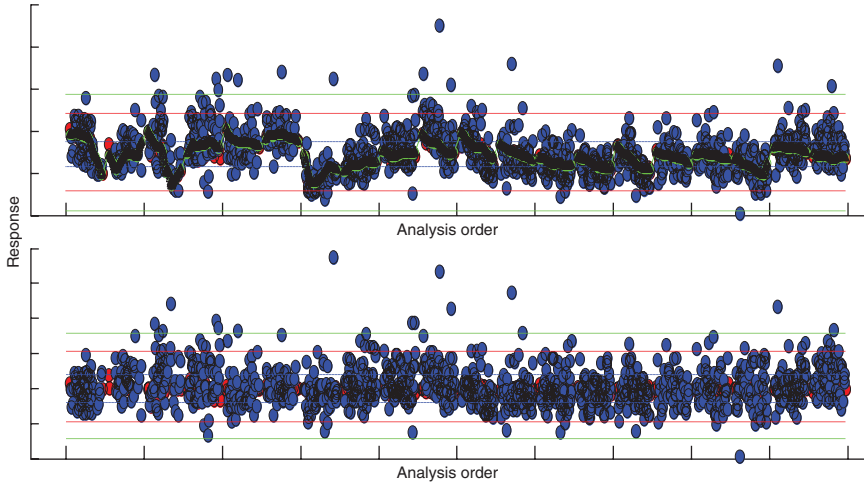

Figure 6 | Peak area data for 1-methylnicotinamide before and after QC-RLSC. The data represents ten experimental blocks and a total of 1,200-subject sample and 600 QC sample injections. Blue circles represent the subject samples and red circles represent the QC samples.

show that similar numbers of features were detected in positive and negative ion mode for the LCT platform.

The data are being applied to define the normal serum metabolome of 'healthy' subjects in the UK population and to define metabolic differences related to age, gender, ethnicity and disease (specifically Alzheimer's disease and ovarian cancer). An example of the data generated is shown in **Figure 7,** which described the gender-specific creatinine distribution as determined using clinical biochemistry methods and GC-TOF-MS for 1,183 subject samples. The distributions are very similar and the correlation coefficient is 0.661. As would be expected because of increased muscle mass, the distribution for males is shifted to a higher creatinine concentration compared with females. The same biological conclusion can be inferred from both the clinical biochemistry and metabolic profiling data.

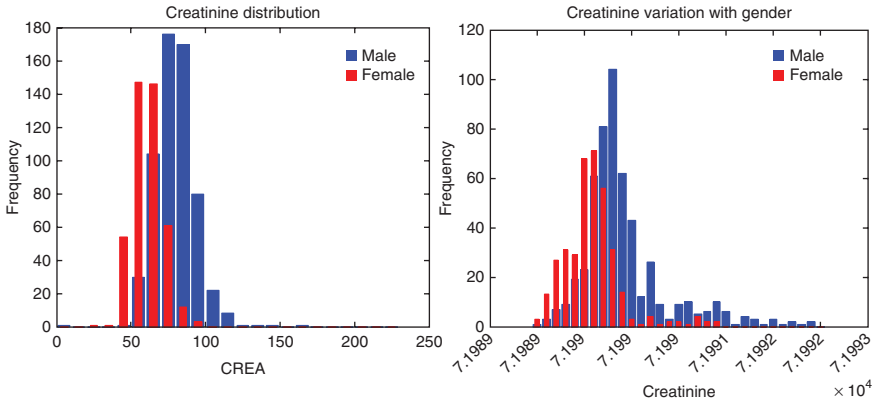

Figure 7 | Gender-specific creatinine distribution. Determined by applying a biochemical quantification assay (left) and GC-TOF-MS in metabolic profiling mode (right). CREA, creatinine.

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AUTHOR CONTRIBUTIONS W.B.D. developed the experimental design strategy, the quality control strategy, the analytical methods and co-wrote the paper. D.B. developed the experimental design, sample scheduling and quality control strategies, and developed the QC-RSLC algorithm, performed data analysis and co-wrote the paper. P.B. and E.Z. developed the experimental design strategy and methods and acquired data. S.F.-M. and N.A. acquired data. M.B. developed the XCMS deconvolution strategy and performed data analysis. J.D.K. developed the sample stratification algorithm. A.H., J.N.H. and A.W.N. developed the experimental design strategy. I.D.W., D.B.K. and R.G. developed the experimental design strategy and co-wrote the paper.

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