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Endocrinology meets metabolomics: achievements, pitfalls, and challenges

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Dear Dr Shaw,

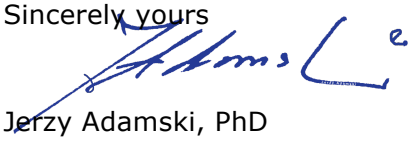
Please find attached an electronic submission of our revised manuscript "Endocrinology meets metabolomics: achievements, pitfalls, and challenges", by Janina Tokarz, Mark Haid, Alexander Cecil, Cornelia Prehn, Anna Artati, Gabriele Möller, and Jerzy Adamski, which you invited us to write for the Journal Trends in Endocrinology and Metabolism.

I attach a detailed rebuttal letter illustrating how we met the comments and requirements raised by the reviewers and the editorial comments.

All authors have agreed to the publication and the article has not appeared elsewhere nor is under consideration for a publication.

We look forward to a favorable response.

Sincerely yours



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Endocrinology meets metabolomics: achievements, pitfalls, and challenges

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Abstract

The metabolome, although very dynamic, is stable enough to provide specific quantitative traits related to health and disease. Metabolomics requires balanced use of state-of-the-art study design, chemical analytics, biostatistics, and bioinformatics to deliver meaningful answers for contemporary questions in human disease research. The technology is nowadays frequently employed for biomarker discovery and elucidation of mechanisms connected to endocrine-related diseases. Metabolomics has also enriched genome wide association studies (GWAS) in this area by functional data. The contribution of rare genetic variants to the metabolome variance and to the human phenotype has been underestimated until now.

Abbreviations

AAA: aromatic amino acids, AUC: area under the curve, BCAA: branched chain amino acids, BMI: body mass index, CE: capillary electrophoresis, CVD: cardiovascular disease, ER: estrogen receptor, FADS1: fatty acid desaturase 1, FIA: flow injection analysis, GC-MS: gas chromatography-mass spectrometry, GWAS: genome-wide association studies, HER2: human epidermal growth factor receptor 2, HPLC: high performance liquid chromatography, LC-FT-ICR-MS: liquid chromatography Fourier transform ion cyclotron resonance mass spectrometry, LC-MS: liquid chromatography mass spectrometry, LC-MS/MS: liquid chromatography tandem mass spectrometry , LD: linkage disequilibrium, LOD: limit of detection, LLOQ: lower limit of quantification, mGWAS: metabolomics combined with genome-wide association studies, NMR: nuclear magnetic resonance, PCA: principal component analysis, PLS-DA: partial least squares regression discriminant analysis, PR: progesterone receptor, ROC: receiver operating characteristics, SNP: single nucleotide polymorphism, TCA: tricarboxylic acid, T2D: type 2 diabetes, UHPLC: ultra-high performance liquid chromatography, ULOQ: upper limit of quantification

The scope of metabolomics

Metabolomics discovers patterns of metabolites in a biological system (cell, tissue, or organism) under a given set of conditions. The complete set of metabolites in this system is termed the metabolome. Metabolites are the final downstream products of biological processes and as such, metabolite pattern change upon the sum of gene effects, gene-gene interactions, and gene-environment interactions. Advanced analytical methods with a high level of sensitivity and reproducibility, wide metabolite coverage, and high sample throughput have led to a growing number of applications of metabolomics in human health and biomedical research, as well as applications in crop and food quality analyses.

Metabolomics methodology and application

Metabolomics (see Glossary) is based on a broad variety of instrumentations and requires extensive data analyses. Three analytical technologies are predominantly used in metabolomics: nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry coupled either to gas chromatography (GC−MS) or to liquid chromatography (LC−MS). Each technique provides a broad but specific coverage of metabolite classes including lipids, amino acids, sugars, biogenic amines, and organic acids, depending on the properties of the metabolites (Box 1 and 2). A flowchart of a typical metabolomics experiment is given in Figure 1. Metabolomics analyses require special sample preparation procedures. Pre-analytical aspects like the procedure of sample collection and storage strongly affect the preservation of the metabolome prior to measurement [1, 2]. Certainly, proper experimental designs are of high importance to ensure the reliability of the studies [3-5].

Basically, two approaches with different objectives are commonly used in metabolomics [6]. Targeted metabolomics aims to quantify a selected set of certain known metabolites (hypothesis-based approach). Non-targeted metabolomics aims to identify differences in the patterns of a broad spectrum of metabolites without their prior identification (hypothesis-free approach). The two approaches can be used complimentarily.

Targeted metabolomics has a high potential in clinical diagnostics, especially when patterns of metabolite concentrations are analyzed by multiplexed methods instead of quantifying single metabolite biomarkers. For example, some steroid related pediatric diseases (like congenital adrenal hyperplasia) remain undiagnosed if only a single marker among steroids is analyzed [7]. One of the most successfully used multiplexing metabolomics method is the Newborn Screen, where 43 metabolites are quantified simultaneously using

liquid chromatography tandem mass spectrometry (LC-MS/MS) [8]. In a single analytical run, multiple inborn errors of metabolism like phenylketonuria, maple syrup urine disease or medium-chain acyl-coenzyme A dehydrogenase deficiency and many others can be detected.

Considering all identified genetic effects known so far, studies investigating frequent human diseases with complex traits were able to explain less than half of the estimated trait heritability [9]. In case of type 2 diabetes (T2D) even rare variants derived from data for 111,548 patients do not explain predispositions to this disease [10]. For decades, the focus of studies on many chronic diseases was lying on genetics [11-13], but recently, both targeted and non-targeted metabolomics revealed the impact of the environment (including the effect of gut microbiota) on human health and disease [14-16]. Important disease genes or single nucleotide polymorphisms (SNPs) have been reported in some population studies using whole genome sequencing, transcriptomics, and SNP analyses. However, only few of the aforementioned genes and SNPs were convincingly replicated, e.g. the candidate genes or SNPs for atherosclerosis and coronary artery disease (CAD) [9, 16-19]. In contrast to the situation in genomics, biomarkers determined by metabolomics provide deep functional phenotyping and are able to predict for example the risk for cardiovascular diseases (CVD) with high confidence. Elevated plasma levels of trimethylamine N-oxide (TMAO) were shown to predict an increased risk for CVD by developing atherosclerotic plaques in a study cohort [20]. The frequency of variants in human exomes is very high (about 1 every 8 bases) and recent estimates name 3,230 genes with missense mutations from which above 70% do not have an established link to a human disease [21]. Such variants might be much faster analyzed for their physiological impact by metabolomics than by genetic approaches.

Statistics in Metabolomics

Metabolomics has a very high demand on data evaluation and biostatistics. In a typical metabolomics setting, a huge amount and a wide range of data are generated. To obtain reliable data that allow meaningful interpretation, rigorous data validation [22, 23] and several data processing steps [24] have to be conducted.

In targeted metabolomics, the first data validation step is to check whether the calculated metabolite concentrations lie within their linear range, i.e., between the upper and lower limit of quantification (ULOQ and LLOQ, respectively). The latter have to be determined during method development and validation. Furthermore, the coefficients of variance of the metabolites should be below 15%. The adherence to these stringent validation parameters are

typical for metabolomics in clinical settings, but entail the risk of discarding many metabolites that do not completely meet the specifications. During the discovery phase of new biomarkers, it might be prudent to use less stringent validation parameters to keep more metabolites in the data set. For instance, instead of using the LLOQ, the actual absolute limit of detection (LOD), below which the instrument cannot reliably detect and identify the molecule of interest, may be used as lower threshold for data validity. The above described data validation steps are not applicable to non-targeted metabolomics data, because validation parameters such as LOD, LLOQ, and ULOQ can usually not be determined in this measurement method.

In some cases, single data points might not be available [25, 26], not because of the absence of the metabolite but due to values being below the LOD. However, sample sets with some missing values should not be discarded immediately, because the data can probably be salvaged by imputation (refer to Box 3 for further details on imputation). In this review we will use data from analyses of metabolomics differences in gender dysphoric individuals under cross-sex hormone treatment to illustrate biostatistical processes [27].

Checking for outliers can be done descriptive statistics. One approach to detect these outliers is using boxplots. Most commonly, the whiskers of the plot extend to 1.5 times inter quartile range (1.5 x IQR) above and below the median of the data. All data points beyond these whiskers are considered to be outliers and are usually discarded. Box plots are very instrumental for example when analyzing phenotype-metabolite associations along a treatment even in complex phenotypes (Figure 2A).

After data processing, many different biostatistical methods [28-32] and tools [33-35] are available for data analysis. If only one variable is of interest, univariate tests should be used ("hypothesis driven approach"). If more than one variable shall be analyzed as for example in a discovery study for finding all differences between patients after cross-sex hormonal treatment, multivariate tests have to be performed ("non-hypothesis driven approach"). Depending on the data distribution, the uni- and multivariate tests, which can be applied, are different. On data sets with normally distributed values (normal distribution has to be confirmed by QQ-Plots [36] or bootstrapping [37]), univariate parametric tests like ANOVA or t-tests can be applied in order to evaluate group differences. For data with non-normal data distribution, the Kruskal-Wallis or Mann-Whitney-U tests have to be used.

Multivariate approaches like principal component analysis (PCA) or partial least squares regression discriminant analysis (PLS-DA) are also very popular. PCA allows for example a clear separation of values obtained from transgender individuals, which underwent

metabolomics analysis one year after cross-sex hormone treatment (Figure 2B) [27]. The results of the PCA analysis might be presented together with the variable vector map (Figure 2C). Vectors contributing to group separation of female-to-male and male-to-female transitions provided information on metabolites most responsible for the group differences.

A PCA is an unsupervised method, calculating group differences in the data without any prior grouping information. It utilizes the idea that observations with a large variance capture a large portion of information of the given data set, whereas observations with a small variance can be discarded, as they do not represent a lot of information [46]. These sets of observations with their possibly correlated variables are converted in a set of values of uncorrelated variables, the principal components (PC). These PCs may be visualized in score plots, or extracted as a text table. Usually, the first few principal components explain a sufficient amount of variance so that already with these first PCs, patterns in the data can be revealed. Samples with similar properties tend to form clusters in a score plot. A PCA is therefore suitable to identify groupings in the data as well as hidden properties [38].

PLS-DA is a supervised method in which prior knowledge of grouping in the data set is incorporated into the principal component calculations in order to maximize group separation. This approach always has the caveat of over-fitting the data, which will produce good looking plots with nonsensical data [39, 40] and PLS-DA data thus have to be thoroughly tested for over-fitting by cross-validation and permutation tests. For cross-validation, R2Y (the sum of squares) and Q2Y (the predictive performance) values can be extracted from PLS-DA score plots. PLS-DA data are generally reliable when R2Y values are close to, but not equal or above, 1 and the Q2Y value above 0.4. Also, the results for the permutation tests (pR2Y and pQ2Y) should be below 0.05. [41]

Receiver operating characteristic (ROC) curves (Figure 3) and the corresponding area under the curve (AUC) are also very valuable statistical analysis tools, especially in studies for biomarker detection or evaluation (see Box 4 for further explanations).

The dynamics of the human metabolome and its usefulness in endocrine related diseases

Confounder impact

The environment presents manifold challenges to the human body, which ultimately reflect on the human metabolome. As such, the human metabolome is highly dynamic. Among the obvious factors influencing the metabolome are age, gender, genome, body mass index (BMI), body fat distribution as well as lifestyle associated aspects like nutrition,

alcohol intake, smoking behavior, and physical activity. Less intuitive confounders include ethnicity (which reflects both the genetic background and lifestyle), circadian rhythms, hormonal status (e.g., stress, menstrual cycle and menopause in females), infections, diseases, medications and toxins, as well as the gut microbiota. Despite short-term flexibility, the human metabolome is relatively stable over months [42] and years [43] and thus, aberrations from conserved profiles are able to reflect a disease [2].

The impact of endocrine related diseases on the human metabolome is of particular interest, because many of them can be considered as being "metabolic diseases" like obesity and diabetes [44]. Since metabolic reprogramming is a major hallmark of cancer [45], endocrine related cancers like breast cancer can also be seen as a metabolic disease.

Breast cancer

Breast cancer is one of the most commonly diagnosed cancers worldwide [46] and is a highly heterogeneous disease with different subclasses based on the expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [47]. Despite these genetic classifications, breast cancer cells display widespread changes in many metabolic pathways to convert nutrients into biomass. Among these are enhanced aerobic glycolysis, increased flux through the pentose phosphate pathway and the tricarboxylic acid cycle (TCA), as well as elevated synthesis of glutamate, glutathione, and fatty acids [48-51]. Additionally, the levels of amino acids increase significantly in tumor tissues, but the magnitude of the change as well as the particular amino acid changed varies with tumor classification [52-54]. Thus, the metabolic changes in breast tumor tissue can be used for a metabolic discrimination of subclasses, which is demonstrated by the distinction of ER positive from ER negative tissues [52, 55], of triple-negative breast cancer from triple-positive breast cancer tissues [56], and of breast cancer tissues from other cancerous tissues [57]. Furthermore, it is possible to distinguish breast cancer subclasses (e.g., HER2 positive versus HER2 negative, ER positive versus ER negative) as well as breast cancer patients from healthy controls by metabolomics analyses in plasma samples [58, 59]. Metabolomics analyses in regard to breast cancer are considered as being almost ready for clinical use [60]. Once the analyses overcome the challenge of replication and once validation studies featuring large cohorts of patients were conducted [60, 61], metabolomics has the potential to detect malignancies before the disease is clinically detectable [62].

Obesity

Obesity results of the interaction of predisposing genetic factors and environmental factors like excessive calorie intake and sedentary lifestyle [63]. Adult obese subjects display a

specific metabolic pattern in blood with increased levels of branched-chain amino acids (BCAA), aromatic amino acids (AAA), carnitine esters from BCAA catabolism, and an altered fatty acid profile with higher levels of saturated fatty acids [64-69]. Metabolite profiles from obese children and adolescents have been described to be similar to adults [70, 71], but other studies demonstrated deviating profiles [72, 73], which might be explained by a higher metabolic flexibility in young obese subjects, which is gradually lost with persisting obesity [72]. Despite the described metabolite profile in obese subjects, the metabolome allows for stratification of subgroups. Discrimination of metabolic healthy obesity from metabolic abnormal obesity [74, 75], of insulin sensitive obesity from insulin resistant obesity [76], of obesity from obesity associated with metabolic syndrome [63], and of obesity from obesity associated with type 2 diabetes [66, 77] can be achieved. Furthermore, the most effective therapy for obesity, bariatric surgery [78], has been shown to modify the obesity-specific metabolic profile into a healthier direction [77, 78]. Metabolomics studies in the context of obesity have so far been almost exclusively case-control or cross-sectional studies with no causal explanatory power [69].

Type 2 diabetes

Type 2 diabetes (T2D) is a progressive disease characterized by increasing insulin resistance accompanied by increasing blood glucose levels, which typically precede the onset of overt diabetes for several years [66, 79]. Patients with manifest T2D show a metabolic profile in blood with increased levels of BCAA, AAA, α-hydroxybutyrate, several carbohydrates, and some ketone bodies. On the other hand glycine, lysophosphatidylcholine C18:2, and linoleoyl-glycerophosphocholine show decreased levels [66, 79-84]. A similar panel of metabolites, namely BCAAs, AAAs, glycine, lysophosphatidylcholine C18:2, linoleoyl-glycerophosphocholine, acetylcarnitine, α-hydroxybutyrate, and 2-aminoadipic acid, can distinguish patients with pre-diabetes from healthy controls [79, 83-86]. Many of the aforementioned metabolites were found to be powerful predictors for the progression of T2D up to 14 years before the onset of overt diabetes [79, 83, 85, 87-90], which enables the identification of at-risk individuals and allows for timely intervention [91]. Prediction power, determined by the area under the curve (AUC) of receiver operating characteristic (ROC) curves (see Figure 3 and Box 4 for definitions), was significantly improved upon addition of metabolite profiles to established clinical risk factors [79, 80, 83, 89]. However, since obesity, insulin resistance, and T2D are diseases all associated with elevated blood concentrations of BCAAs [69, 76], their use as biomarker for a single condition should be

handled with care. Although BCAA levels gradually increase in obesity and exacerbate further in T2D [77], additional studies are needed for a stringent stratification of disease subtypes and validation in larger cohorts [92]. Similarly, correlation analyses of certain metabolic markers to specific diabetic complications and the prediction of the progression of T2D complications [93, 94] need further investigation.

Endometriosis

Another endocrine related disease that urgently require a sensitive and robust non-invasive diagnostics is endometriosis, which effects around 10% of women in their reproductive years [95]. Endometriosis is known to be estrogen dependent [96] and characterized by the appearance of endometrium like tissue in places outside the uterine, in most cases in the pelvic cavity but also in other places [97]. The resulting symptoms are heterogeneous. Some patients show no symptoms at all, while others suffer from e.g., pelvic pain or infertility [97]. Usually, it takes several years between the time point the patients complain about symptoms and the time of diagnosis [98]. Up to now, reliable non-invasive or minimal invasive diagnosis methods are not available and diagnosis usually takes place upon an invasive surgery, the laparoscopy.

Many attempts were already made to find endometriosis indicators in easily accessible body fluids or tissue samples using different analytical technologies [99, 100]. Only recently, metabolomics has been used to identify biomarkers for endometriosis and to learn about the disease mechanism. Several studies were performed using the different sample materials serum [101-108], plasma [109-111], urine [112], peritoneal fluid [106, 113], endometrial curretage [106], or follicular fluid [114].

Most of the studies found lipids and lipid associated components to be changed upon endometriosis [101, 103-109, 111, 113, 114]; however, the classes of the regulated lipids were different between the studies and included phospholipid components [101, 104, 114], eicosanoids [103, 107], fatty acids [104, 105, 108], medium- to very-long-chain glucosylceramides [106], sphingolipids [106, 108, 111, 114], phospholipids [108, 111, 113], triglycerides [108], carnitine [109], acylcarnitines [109], steroidal hormones [102], cholesterol [102], bile acid [102] alone or in combination with other lipids or with metabolites of other classes. Beside the lipids, other metabolite classes were found to be regulated: lactate [101], glycolysis and TCA metabolites [101, 104], amino acids [101, 104], sugars [101, 104, 110], 2-hydroxybutyrate [101, 104], creatine [104] and creatinine trimethylamine-N-oxide [109], adipic acid [104], and choline containing metabolites [110]. The metabolites found to be significantly regulated are often described to be comparably

regulated in other diseases. For example higher amounts of lactate and lower levels of glucose and arginine as seen by Dutta *et al.* can be found as well in cancer [101]. When ROC curves were calculated, sensitivity and specificity values ranged from 81.8% to 92.83% and 81.5% to 100%, respectively [101, 104, 111, 113, 114]. However, Nisenblat *et al*. evaluated beside other studies the metabolomics studies published until July 2015 [101, 104-106, 111] and concluded that the meta-analyses were not unequivocal because different matrices were analyzed or insufficient descriptions on quality assurance were provided [99]. Thus, a biomarker that could reliably substitute diagnostic surgery could not be identified [99]. To our opinion, the outcomes of the successive metabolomics studies are also far from presenting a reliable biomarker. In future, more standardized study designs are required, especially with respect to patient information, sample collection, sample storage, and statistical evaluation [99, 115-117].

Genetic impact on metabolomics phenotypes

Genome wide association studies (*GWAS)*

With the advent of cost efficient genotyping and high-throughput sequencing technologies, the number of genome wide association studies (GWAS) and reported biomarkers exploded in the past ten years [118]. GWAS make use of SNPs that are in linkage disequilibrium (LD) to a gene to identify genetic markers that are associated with a certain phenotypic trait or a disease. By evolutionary selection, gene variants with large effect sizes have minor allele frequencies below 0.5% (rare variants) and are typical for Mendelian diseases (Figure 4). On the other hand, common diseases like type 2 diabetes (T2D) are determined by a larger number of common variants with small effect sizes [10].

Several issues can arise that can hinder the identification of a causal relationship between a SNP and a trait or disease. First, unless a SNP is not located within the coding region of a gene, the assignment of a SNP to a nearby gene might be incorrect [119, 120]. Second, even in case of a statistical significant association between an exonic SNP and a trait or disease, it remains a statistical association that may have been created by a confounder and does not imply a causal nexus.

GWAS in combination with metabolomics (mGWAS)

The use of metabolites or ratios of metabolites as intermediate phenotypes in combination with GWAS (mGWAS) can augment the explanatory power of a causal variant significantly [121, 122]. In mGWAS, the impact of the discovered genetic variant is in many cases linked

to known enzymatic conversions or to the transport of the associated metabolites and thus strengthening a causal relationship [123].

The genotype-dependent differences in metabolite concentrations are termed "genetically influenced metabotype" (GIM) [122, 124]. A classic example is the association of the gene coding for fatty acid desaturase 1 (FADS1, SNP rs174547), which introduces a double-bond at position 5 in fatty acids, with metabolite ratios of biochemically linked glycerophospholipids that differ by one double-bond [122]. Depending on the FADS1 genotype (homozygote with major allele, heterozygote, homozygote with minor allele), people display different concentration levels of the enzyme products. Interestingly, the FADS1 locus showed significant positive association with low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels and a negative correlation with triglyceride levels, establishing a connection with clinical endpoints such as CVD [123]. This FADS1 gene polymorphism based risk for coronary artery disease has been confirmed in a Chinese population by Li *et al*. [125].

mGWAS in pathway annotation

The in mGWAS often observed functional connection between a genetic variant and the associated protein can be exploited [122, 124] to infer the biological function of poorly characterized proteins [126]. For instance, Draisma *et al.* discovered significant associations of SNPs in LD to the MFSD2A gene with plasma concentration levels of lysophosphatidylcholines [123]. Indeed, Ngyuen *et al.* later confirmed MFSD2a to act as a transporter for lysophosphatidylcholines in the brain [127].

The known function of a gene can also be used to elucidate the identity of unknown metabolites in GWAS combined with non-targeted metabolomics [124]. In a publication by Long *et al*., significant associations were found between NAT8 (*N*-acetyltransferase 8) and a hitherto unknown metabolite that was later confirmed to be the *N*-acetylated product of 2-aminooctanoic acid (*N*-acetyl-2-aminooctanoic acid) [128].

Heritability of phenotypes

An often-occurring phenomenon in GWAS is the large proportion of missing heritability, i.e., the large difference between the explained variance from the sum of significantly associated SNPs and the estimated heritability derived from twin studies [129]. This is particularly true for complex traits or diseases like T2D, where many gene variants with small effect sizes aggregate to explain only a small proportion of the heritability [10, 129]. To date many reasons for this missing heritability are discussed [130-132]. However, recent studies reveal that a considerable amount of missing heritability in complex traits can be

explained by a huge number of variants each exhibiting only tiny effect sizes that do not reach genome-wide significance [133, 134]. Based on these (and other) findings, Boyle, Li and Pritchard proposed the so called "omnigenic model" [135]. The model states that a certain amount of heritability in complex traits is based on a few ”core” genes that are directly involved in the trait relevant pathways. These core genes are part of a broad cellular network of ”peripheral” genes each contributing with a tiny effect size to the heritability. In this network, the ”peripheral” genes are linked either by near or long range connection to a ”core“ gene and as such may contribute only indirectly to the observed phenotype.

Using metabolite concentrations as intermediate phenotypes in combination with GWAS yields much higher explained variance of the genetic heritability than GWAS alone [136, 137], i.e., mGWAS have a greater capability to identify significant associations in studies with a lower cohort size. By combining metabolomics with whole genome sequencing, Long *et al*. were able to detect even rare genetic variants associated with blood metabolite levels of common diseases [128]. In future, the amalgamation of metabolomics and GWAS information may assist to reveal the structure of the regulatory networks implicated in the “omnigenic model”.

Genetic impact on the human metabolome

The low penetrance of genetic variants (i.e., the proportion of individuals with the variant who exhibit clinical symptoms) shows that the presence of a certain allele variant does not necessarily translate into a diseased phenotype. Beside the genetic predisposition, environmental effects (e.g., nutrition) may also decide whether a disease will occur phenotypically. Thus, complementary methods are necessary to gain new insights into disease etiology or for detection of biomarkers. Because metabolite levels integrate information from both endogenous (genetic) and exogenous (nutrition, medication, environment) origins, metabolomics is in particular suited to assess the biochemical status of a person [138].

Whole exome and whole genome sequencing data have been used to estimate the impact of the genome on the human plasma metabolome [139]. Using discovery (Framingham Heart Study) and replication (Atherosclerosis Risk in Communities Study) cohorts, Rhee *et al*. demonstrated that beside common variants also rare variants associate with specific metabolites (Figure 5). For example, the heritability of allantoin (purine and uric acid metabolite) or glutamate levels are explained by rare variants, while the heritability of anthranillic acid (tryptophan metabolite) or arginine levels are explained by both rare and common variants in equal proportions. Furthermore, novel metabolite changes caused by

genetic variants were lately discovered displaying that variants in GMPS (guanine monophosphate synthase), HAL (histidine ammonia-lyase), PAH (phenylalanine hydroxylase) and UPB1 (beta-ureidopropionase 1) cause changes in xanthosine, histidine, phenylalanine and ureidopropionate levels, respectively [139].

Conclusions

Over the years, metabolomics has been proven to be a powerful tool in biomarker discovery and in the study of mechanisms of health and diseases but many questions remain unanswered (see Outstanding Questions Box). The strength of metabolomics is its ability to provide a comprehensive snapshot of the biochemical state in a given biological system. Combined with genomics, transcriptomics, and proteomics studies, metabolomics provides comprehensive insights into biological processes. Both genetic and environmental impact is visible in the metabolome. On the overall, the contribution of rare variants to the metabolome variance has been underestimated so far. It is to be expected that the molecular basis of syndromes comprising rare disease phenotypes will be explained by metabolomics. In the near future, we may expect the discovery of metabolic biomarkers allowing early disease diagnosis, dependable risk stratification, and provision for clinical tools for the precision therapies of endocrine-related diseases.

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Figure Legends

Figure 1. Flowchart of a metabolomics experiment.

A flowchart of a typical metabolomics experiment is depicted in Figure 1. The study design has a huge impact on the clarity of data interpretation. The most robust designs are based on longitudinal collections of samples where a phenotype development or disease progression is followed in the same individual. Case-control or cross sectional studies require high numbers of samples to meet power requirements. Pre-analytical aspects like the procedure of sample collection and storage strongly affect the preservation of the metabolome prior to measurement. During metabolite measurements, different analytical steps are performed and monitored (Figure 1 shows the example of mass spectrometry) and if required, they might be repeated to meet quality requirements. After metabolite annotation (verification of analyte identity), the data are processed using biostatistical tools. This step is quite challenging, especially in the case of huge data sets (e.g., hundreds of samples each revealing changes in hundreds of metabolite levels). Only when significant discrimination between the phenotypes of interest can be achieved or biostatistical models explaining the observed changes in metabolite concentrations are provided, further data interpretation can be approached.

Figure 2. Biostatistical analyses of a metabolomics data set.

A. Boxplots depicting the value distribution of one metabolite in different categories. The concentrations of the metabolites are plotted against clinical diagnoses at different time points. Numbers outside the boxes correspond to individuals considered as outliers. Whiskers represent 1.5 times inter quartile range (1.5 x IQR) above and below the median of the data within the box, the box itself contains 75% of the data, black bars in the boxes represent the median. Modified after [27] with permission of the publisher.

B. PCA analysis of all metabolites in different categories. Metabolomic data of the same group of patients as in A underwent principal component analysis. Red or green triangles correspond to transgender individuals of female-to-male (FtM) or to male-to-female (MtF) change, respectively. Red and green ellipses circumventing FtM or MtF groups are well separated. Numbers represent individual patients. Modified after [27] with permission of the publisher.

C. PCA analysis with variable vector map. Vectors contributing to FtM and MtF group separation are given in blue. These vectors include BMI, age, total fat mass, and distinct metabolites. M0 - baseline, M12 – value after 12 months of cross-sex hormone treatment. Modified after [27] with permission of the publisher.

Fig 3. Biomarker performance analysis using ROC curves.

Different models based on successive addition of separate variables are used to analyze the quality of biomarkers in a diagnostic procedure. Metabolomics-derived biomarkers for the endometriosis diagnosis were evaluated. Different models are coded by lines with distinct colors. The dotted line represents the random model. A model combining age, BMI, sphingomyelin (SM), and phosphatidylcholine (PC) metabolites differentiates endometriosis patients and healthy controls with a sensitivity of 90.0% and a specificity of 84.3%. Model 1 = age, Model 2 = age + BMI, Model 3 = age + BMI + SMOHC16:1, Model 4 = age + BMI + SMOHC16:1 + PCaaC36:2/PCaeC34:2. Modified after [111] with permission of the publisher.

Figure 4. Explanation of metabolite variability by genome variability of different frequency.

The observed heritability is given only for metabolites with h^2q values between 4.9E-06 (choline) and 0.60 (dimethylglycine). Minor allele frequency (MAF) is color coded. Modified after [139] under Creative Commons license.

Figure 5. Relationship between effect size and minor allele frequency.

The black curve describes the effect size as a function of the minor allele frequency (MAF) with $y = x^{-0.39}$. While common variants with MAF > 5% (blue) display only small effect sizes, rare variants with MAF < 0.5% (orange) usually have much larger effect sizes. Low-frequency variants with 0.5% < MAF < 5% (gray) take a medial position between the two extremes. The selection against variants with large effect sizes is a consequence of evolutionary processes. Modified after [128] under Creative Commons license.

Outstanding Questions

- Which confounders impact the metabolome? Although major confounders like sex, age, nutrition, and genetics were already identified, further confounders are still anticipated.
- Which new elements of study design shall be developed for metabolomics? Although a huge impact from genomics and epidemiology already facilitate the improvement of metabolomics-oriented studies, there is further need to address e.g., dynamic changes and longitudinal variability of the metabolome.
- How to increase the coverage of the metabolome by analytical techniques? In contrast to genome analyses only a part of all metabolites present in the living system is accessible by current techniques yet.
- How to standardize metabolite identification and quantification among different laboratories? Data of samples analyzed by different procedures in distinct laboratories are usually difficult to compare.
- How to increase sample throughput in metabolomics? At present, large human cohorts studies e.g., required for analyses of impact of rare genetic variants on metabolism, are hampered by a low processing speed of individual samples.
- How to better visualize interactions between affected metabolic pathways? Present approaches are hampered by the lack of specific identifiers for several metabolites and the still limited knowledge on how the pathways are interacting with each other.
- How to facilitate *omics* data integration? Genome and transcriptome data are more and more publicly available while the accessibility to metabolome resources does not reach this level yet. This makes comprehensive integrative comparisons of metabolomics data with other *omics* data still challenging.

Rebuttal Letter for TEM-D-17-00048

We would like to thank the reviewers and editor for their valuable comments and requests. We provide point-to-point answers for the changes made. We further provide a copy with changes traced and highlighted in color to facilitate monitoring of our corrections.

EDITORIAL COMMENTS:

1. At 4,590 words and >140 references, your manuscript as at the upper bound for word limit and reference number. Please consider replacing some of the text with boxes and summarizing certain key points as a figure.
REPLY: We have shortened the text, which has now 4,230 words. Some parts of the text were moved to text boxes. Reference number is now 148 instead of 151.

2. Figures 2-5 would be better suited for a primary research paper. Please consider revising these figures to summarize key concepts rather than providing details into the data and methodology.
REPLY: Figures 2-5 originate from research papers published already. We have changed corresponding chapters in the text and legends to illustrate what specific requirements for the metabolomic data presentation in individuals after a hormonal therapy (Figure 2), metabolic biomarker evaluation in an endocrine-related disease (figure3) and impact of genetic variants on metabolites (Figures 4 and 5) have to be met. Please note that figures 2-5 have been modified to facilitate the messages from this review.

REVIEWER

Reviewer #1: The manuscript entitled "Endocrinology and metabolomics: genetic impact and functional phenotyping" by Tokarz J, Haid M, Cecil A, Prhn C, Artati A, Möller G, Adamski J. is a very comprehensive and well-written review on the topic, citing up-to-date references, and will be of great interest to the readers of Trends in Endocrinology and Metabolism.

Minor comments:
1. The title as well as the abstract point towards the use of metabolomics, in conjunction with genetics, to overcome the shortcomings of GWAS studies. However, only pages 12 to 14 are specifically about this aspect. Under this perspective, the overview of statistic methods that could be used in metabolomics appears out of place. If this is a general review of metabolomics, the title and abstract should be modified, otherwise, the article should be shortened and the last section should be expanded.
REPLY:

- We have changed the title to “Endocrinology meets metabolomics: achievements, pitfalls, and challenges” which reflects more closely the content of the review
- The abstract has been rewritten to facilitate understanding of main messages from this manuscript and reads now: “The metabolome,

although very dynamic, is stable enough to provide specific quantitative traits related to health and disease. Metabolomics requires balanced use of state-of-the-art study design, chemical analytics, biostatistics, and bioinformatics to deliver meaningful answers for contemporary questions in human disease research. The technology is nowadays frequently employed for biomarker discovery and elucidation of mechanisms connected to endocrine-related diseases. Metabolomics has also enriched genome wide association studies (GWAS) in this area by functional data. The contribution of rare genetic variants to the metabolome variance and to the human phenotype has been underestimated until now."

- The biostatistics part has been shortened and rewritten with referencing to hormone-dependent effects depicted by metabolomics. The changes are too numerous to cite them here. Please refer to the manuscript version with the changes made visible.

2. In pages 8 to 11, it is strange to end the presentation of each "discovery" by showing that nothing has been discovered or confirmed yet.
REPLY: This aspect has been amended and the messages from this chapter are more positive and clear.

3. p. 6 "The PCA method is based on the idea that variables capturing a large amount of variance ... ": this sentence should read "principal components" rather than "variables". The variables are often scaled to unit variance anyway.
REPLY: We understand the comment of the reviewer. However, we suggest another solution. We changed the sentence to illustrate more specifically what we would like to say. The sentence reads now." A PCA is an unsupervised method, calculating group differences in the data without any prior grouping information. It utilizes the idea that observations with a large variance capture a large portion of information of the given data set, whereas observations with a small variance can be discarded, as they do not represent a lot of information [46]."

4. Figure 2C is very difficult to read, with a lot of the text overlapping. The clarity of this figure should be improved.
REPLY: Done; metabolite assignment has been revised and is shown with different spacing avoiding overlapping.

5. The section entitled "The Dynamics of human metabolome and its usefulness in endocrine related disease" would benefit from having sub-titles.
REPLY: Done; subtitles were introduced, however, not only in the respective but also in the other chapters of the manuscript.

Miscellaneous:
1. We completely rephrased the earlier submitted Glossary and included Abbreviations in the body text.

Trends Box

- Metabolomics provides versatile functional phenotyping
- The dynamics of the metabolome is highly reflecting gene-gene as well as gene-environment interactions
- Distinct environmental challenges like nutrition, medication or age reveal specific metabolomics signatures
- Multiple endocrine disorders reveal unique metabolite signatures
- Metabolomics derived biomarkers have high potential for the use in precision medicine

Manuscript with changes visible

Text Boxes

Text Box 1. Common approaches in metabolomics

- Targeted metabolomics. This approach is used to quantify the absolute concentrations of a predefined set of metabolites. A standard calibration curve of a concentration range of each metabolite of interest should be generated for accurate quantification. Internal standards should be added in constant amounts to samples, blank, and calibration standards to enable compensation for any interference due to sample preparation or matrix effects. Especially for flow injection analysis mass spectrometry (FIA-MS), the usage of stable isotope labeled internal standards is of highest importance. Targeted metabolomics has several advantages. First, the analysis provides results in absolute concentrations of metabolites in the sample. Second, all measurements can be validated for analytical performance. Third, due to the usage of internal standards and specific metabolite extraction protocols, matrix effects can be minimized. Therefore, targeted measurements are used to validate possible biomarkers. A drawback of targeted analysis is the limited set of simultaneously quantified metabolites, giving the possibility of missing certain biological processes.
- Non-targeted metabolomics. This approach aims to simultaneously detect as many metabolites as possible. Although depending on the analytical platform, the solvent, the column chemistry, and the ionization technique used, non-targeted metabolomics can provide a detailed assessment of the metabolites in a sample by revealing a wide range of metabolites of different chemical classes. Thus, the result of this approach can generate new hypotheses and drive the next step of research. Another advantage is that it offers an unbiased means to examine the relationship between interconnected metabolites from multiple pathways. However, it is not yet possible to obtain all metabolite classes simultaneously, since many factors affect metabolite detection and recovery depending on functional groups of the analytes. Additionally, non-targeted metabolomics does not allow for absolute but at the best for semiquantitative quantification. The detection of a high number of unknown metabolites not yet annotated in metabolite databases is a common phenomenon in non-targeted metabolomics analysis.

Text Box 2. Essential technological aspects in metabolomics

- Matrix:
  - Matrix in metabolomics analyses is the sample material, in which metabolites appear. Metabolomics methods have already been validated and applied to a wide range of biological matrices including body fluids (plasma, serum, urine, cerebrospinal fluid, saliva) as well as tissues (and biopsies), stool, lung lavage, and exhaled air from humans and other species.
- Critical steps in the metabolomics workflow:
  - Proper experimental design (e.g., number of replicates, group size).
  - Proper sample collection: Metabolites in the sample must remain exactly the same as at the time of sampling. This is usually achieved by a "collect and snap freeze" procedure.
  - Suitable sample extraction: The metabolite extraction (e.g., liquid-liquid or solid phase extraction) will greatly influence the variety of metabolites detected by the analytical methods. The use of aqueous buffer will result in an extraction of hydrophilic metabolites like amino acids, whereas the use of organic solvent will foster the extraction of more hydrophobic compounds like lipids.
- Analytical platforms available:
  - Targeted metabolomics is mostly based on LC-MS, FIA-MS (Flow Injection Analysis Mass Spectrometry), UHPLC-MS (Ultra-High Performance Liquid Chromatography Mass Spectrometry), or GC-MS. Separate analytical methods are developed for groups of chemically different substances, i.e., different methods would be needed for steroids and amino acids.
  - Non-targeted or profiling metabolomics is mostly performed by NMR, CE-MS (Capillary Electrophoresis Mass Spectrometry), GC-MS, LC-FT-ICR-MS (Liquid Chromatography-Fourier Transform-Ion Cyclotron Resonance-Mass Spectrometry), or UHPLC-MS. Implementation of high-resolution mass spectrometry is very effective to facilitate structural characterization of the metabolites. Non-targeted metabolomics often requires a high degree of parallel analyses (i.e., standardized simultaneous analyses on LC- and GC-MS) to cover as many metabolites as possible and to avoid a bias on specific chemical classes.

Text Box 3. Data imputation

Data imputation is mandatory for non-targeted metabolomics, as these results usually contain a high amount of missing values. Imputation may be done in several ways [140-142]:

- Minimum value imputation:
  One way to obtain imputed values is to use a value below the minimum (min) value which was measured for the given metabolite for all samples and replacing missing data points (mis) as follows: mis = min/X, where X is most commonly "2" to represent that mis≠min) [143].
- LOD imputation:
  Another feasible, and very similar route, is to use the metabolites' specific LOD value, and replace the missing values in a similar fashion: mis=LOD/X (X=2 or X=√2) [144, 145].
- Other methods:
  These include, and are not limited to, k nearest neighbours (KNN) imputation, zero value imputation, mean value imputation, median value imputation, and random forest imputation [141].

Important note on data imputation: The imputation is not reasonable, if the measurements for a metabolite show too many missing values, and thus, certain cut-off levels are suggested. The cut-off level can vary depending on the tool used for data evaluation. For example, a metabolite with more than a pre-set 50% missing values will be discarded from the data set, when the web-server MetaboAnalyst is used [146], whereas the package "statTarget" for R sets this threshold at 20% by default [147].

Text Box 4. ROC and AUC

A ROC curve is a graphical representation of the performance of a binary classifier system. The latter separates a given data set into two groups (e.g., patients and control probands) on the basis of a classification rule (e.g. body temperatures > x °C characterize patients, body temperatures < x °C characterize controls). After classifying the patients into their respective groups - based on the chosen criterion or biomarker - the true positive (TPR) and true negative (TNR) rates for group affiliation are calculated as follows:

TPR (also called Sensitivity):

$$\frac{True\ positive}{True\ Positive + False\ negative}$$

TNR (also called Specificity):

$$\frac{False\ positive}{False\ Positive + True\ negative}$$

In order to obtain a ROC curve, the TPR is plotted against "1- Specificity" (also called false positive rate FPR) for pre-defined thresholds T in dependence of criterion x (temperature in our example).

$$TPR(T) = \int_{T}^{Inf} f_1(x)\,dx \ versus \ FPR(T) = \int_{T}^{Inf} f_0(x)\,dx$$

Each point on the ROC curve therefore represents a sensitivity/specificity pair corresponding to a given criterion. The AUC is the calculated area under the ROC curve and can be used to compare different ROC evaluations for different thresholds.

In the field of biomarker discovery, the use of ROC curves is superior to the comparisons of fold changes or odds ratios, because a ROC curve provides unequivocal information on both the sensitivity and selectivity of the biomarker [148].

Glossary

Figure 1

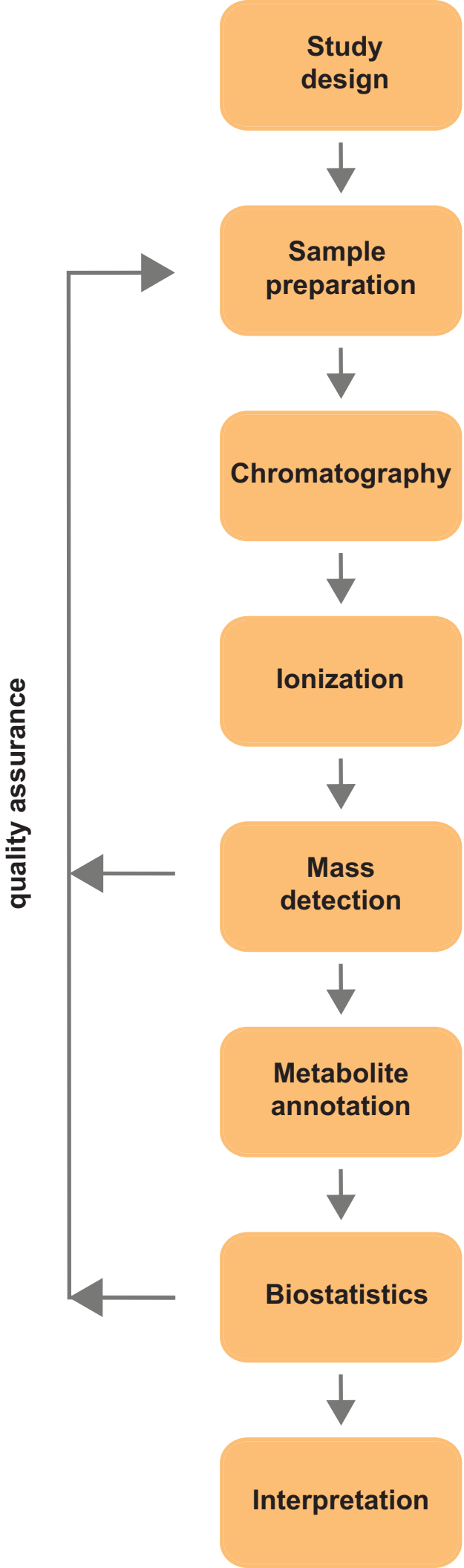
Study design
Sample preparation
Chromatography
Ionization
Mass detection
Metabolite annotation
Biostatistics
Interpretation
quality assurance

Figure 2

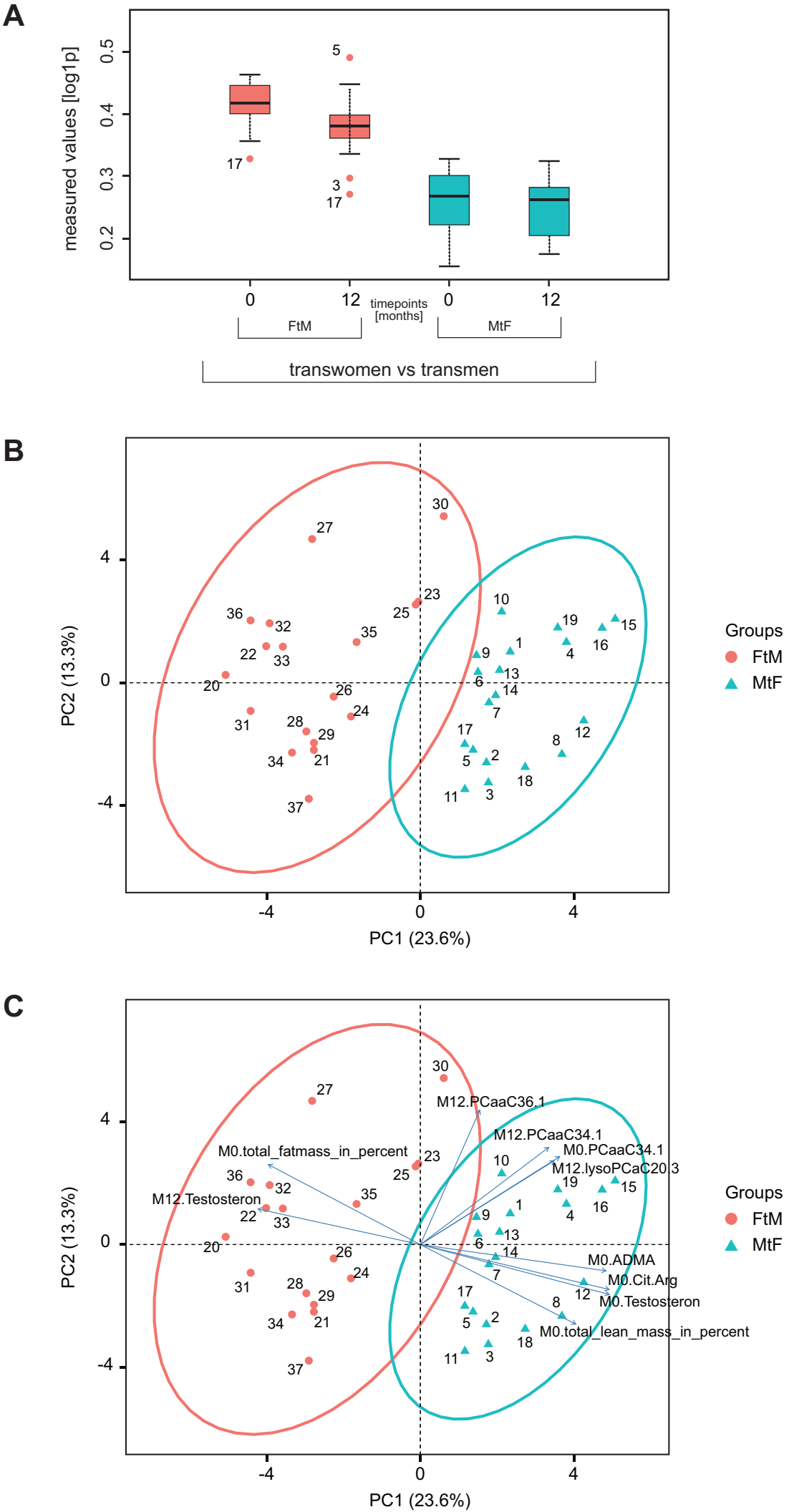
A
measured values [log1p]
0.2
0.3
0.4
0.5
0
12
timepoints [months]
0
12
FtM
MtF
transwomen vs transmen
B
PC2 (13.3%)
PC1 (23.6%)
Groups
FtM
MtF
C
PC2 (13.3%)
PC1 (23.6%)
Groups
FtM
MtF
M0.total_fatmass_in_percent
M12.Testosteron
M12.PCaaC36.1
M12.PCaaC34.1
M0.PCaaC34.1
M12.lysoPCaC20.3
M0.ADMA
M0.Cit.Arg
M0.Testosteron
M0.total_lean_mass_in_percent

Figure 3

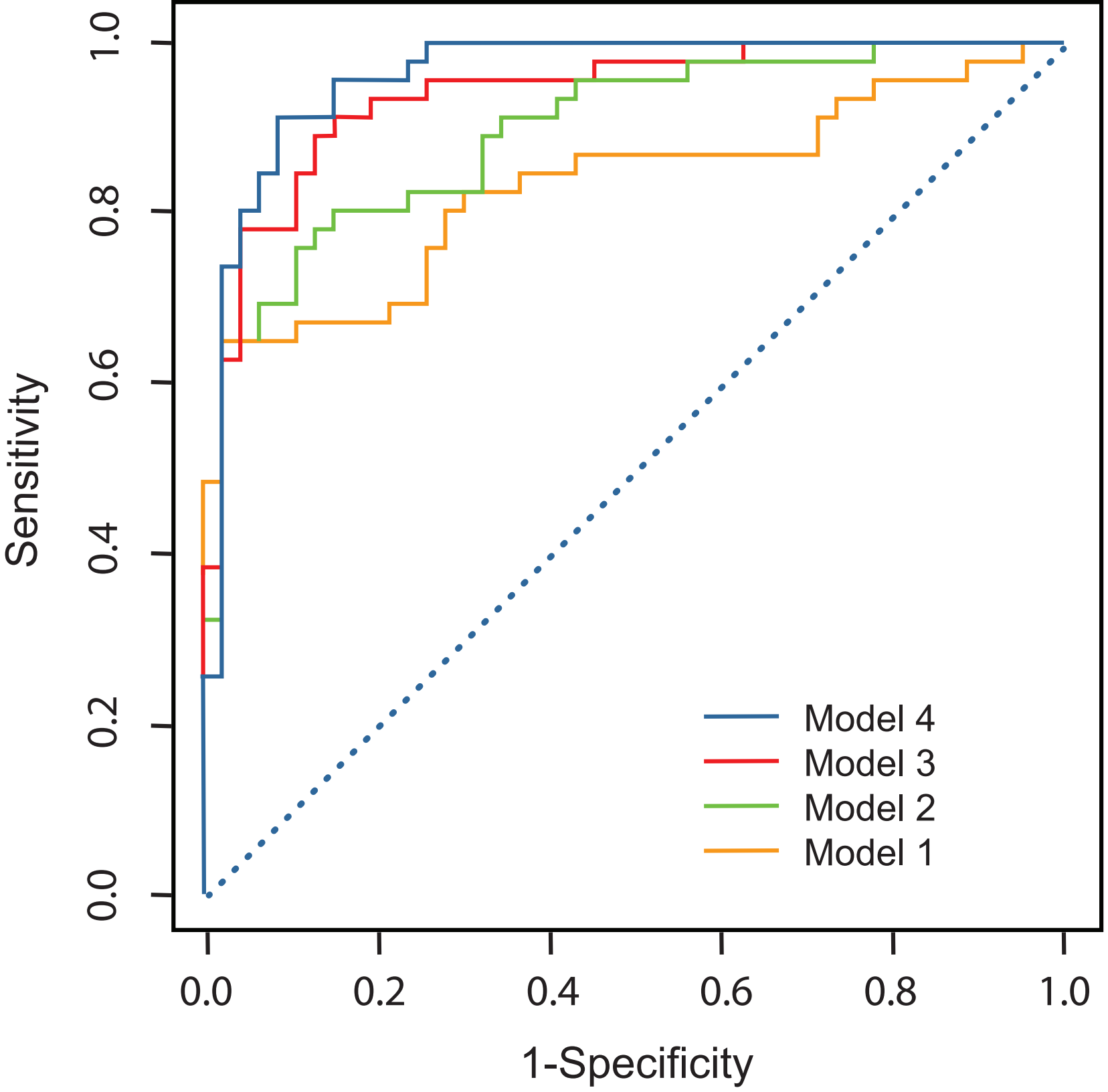
Sensitivity
1-Specificity
0.0
0.2
0.4
0.6
0.8
1.0
Model 4
Model 3
Model 2
Model 1

Figure 4

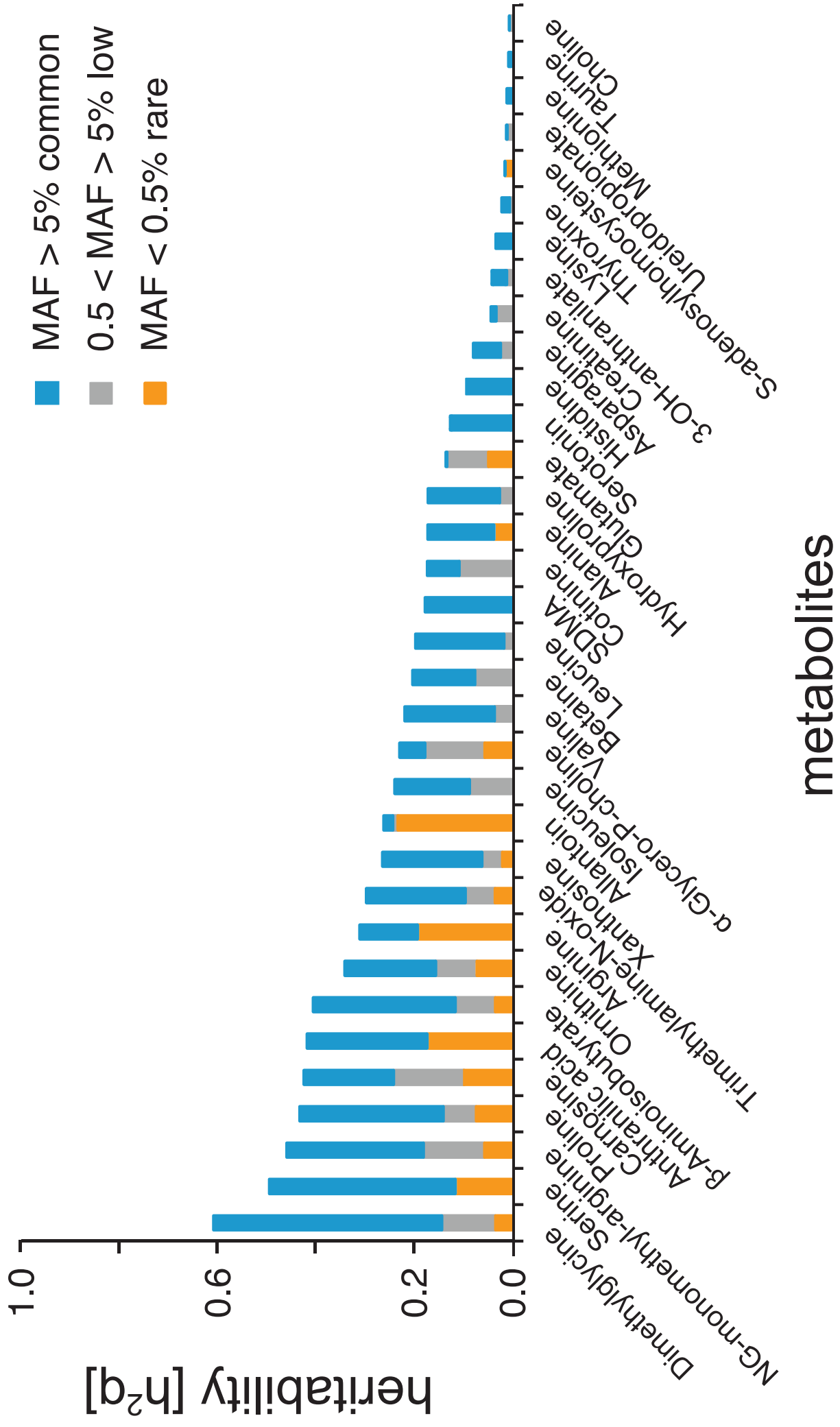
MAF > 5% common
0.5 < MAF > 5% low
MAF < 0.5% rare
heritability [h²g]
1.0
0.6
0.2
0.0
metabolites
Dimethylglycine
NG-monomethyl-arginine
Serine
Proline
Carnosine
Anthranilic acid
β-Aminoisobutyrate
Ornithine
Arginine
Trimethylamine-N-oxide
Xanthosine
Allantoin
Isoleucine
α-Glycero-P-choline
Valine
Betaine
Leucine
SDMA
Cotinine
Alanine
Hydroxyproline
Glutamate
Serotonin
Histidine
Asparagine
Creatinine
3-OH-anthranilate
Lysine
Thyroxine
S-adenosylhomocysteine
Ureidopropionate
Methionine
Taurine
Choline

Figure 5

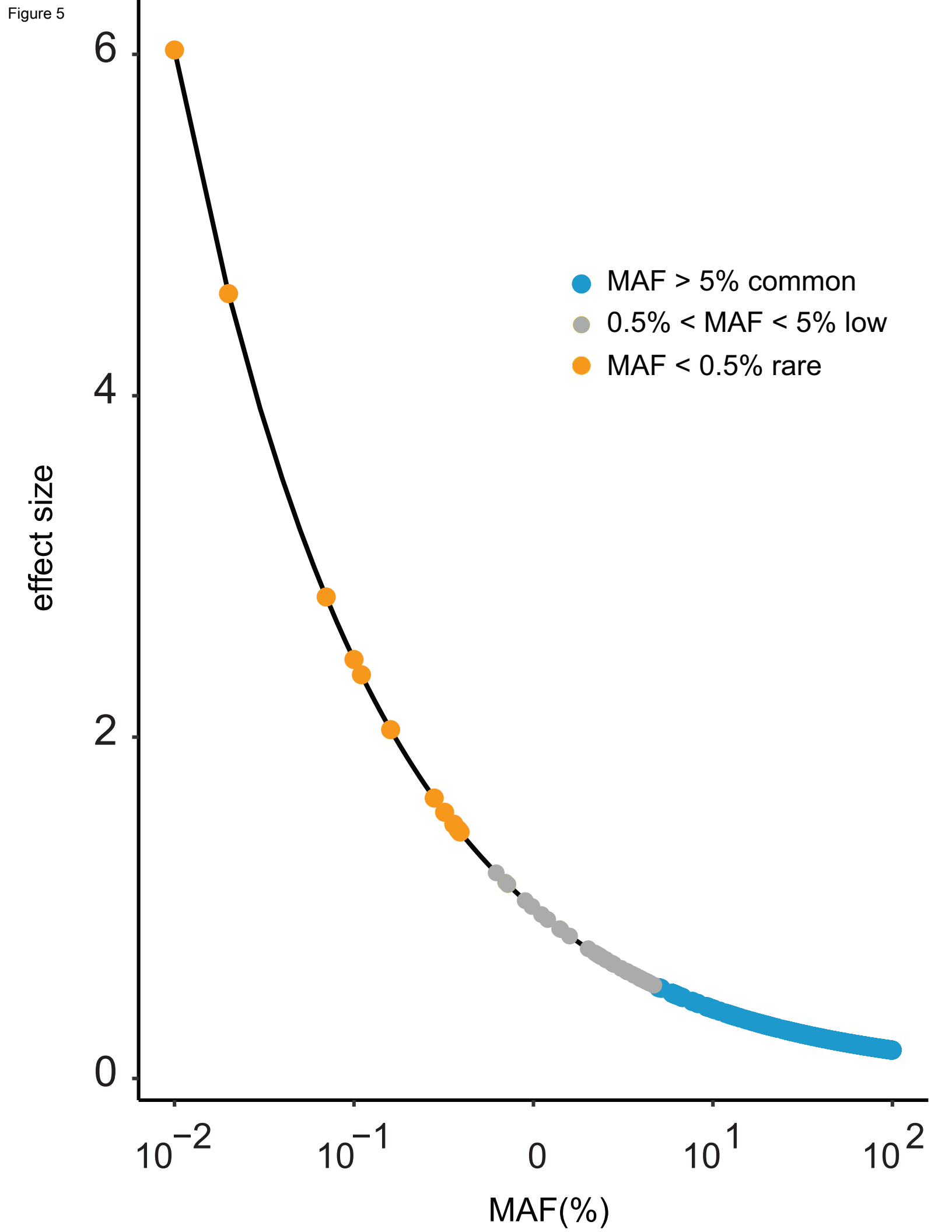
6
4
2
0
effect size
MAF > 5% common
0.5% < MAF < 5% low
MAF < 0.5% rare
10^{-2}
10^{-1}
0
10^{1}
10^{2}
MAF(%)

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Figure_5.ai