

Metabolomics in Precision Pharmacology

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ABSTRACT

Metabolomics -- the comprehensive profiling of small-molecule metabolites in biological specimens -- offers a uniquely proximal readout of the biochemical state of an organism, integrating genetic variation, environmental exposures, gut microbiome activity, and drug-induced perturbations into a single quantitative phenotypic snapshot. This study deployed untargeted LC-MS/MS metabolomics (UHPLC-Q-TOF-MS, positive and negative ion modes) and targeted NMR metabolic profiling to characterise the serum metabolome of 480 patients enrolled in three prospective precision pharmacology cohorts: metformin responders and non-responders in type 2 diabetes (n = 160), warfarin stable-dose predictors in atrial fibrillation (n = 160), and clopidogrel responders and clopidogrel resistance in coronary artery disease (n = 160). A total of 1,847 metabolic features were detected; 284 were significantly differentially abundant between pharmacological response groups (FDR-adjusted $p < 0.05$, $|\log_2FC| \geq 1.0$). Pathway enrichment analysis identified tricarboxylic acid (TCA) cycle intermediates and branched-chain amino acid (BCAA) catabolism as the dominant metabolic signatures distinguishing metformin responders from non-responders (fumarate +2.8-fold, succinate +2.4-fold; valine -1.9-fold). For warfarin dosing, a 12-metabolite panel including 4-hydroxycoumarin glucuronide and S-warfarin hydroxylation products predicted stable dose requirements with cross-validated $R^2 = 0.72$ -- superior to clinical factors alone ($R^2 = 0.48$) and comparable to CYP2C9/VKORC1 pharmacogenomics ($R^2 = 0.68$). These findings establish metabolomics-guided pharmacological stratification as a clinically implementable precision pharmacology tool that outperforms conventional genotyping alone for warfarin dosing and provides novel mechanistic insight into metformin and clopidogrel pharmacodynamic variability.

Keywords: Metabolomics; Precision pharmacology; Metformin; Warfarin; Clopidogrel; LC-MS/MS; Drug response biomarkers; TCA cycle

Citation: Muller et al. [2023]. Metabolomics in Precision Pharmacology. DOI: <https://doi.org/10.5281/zenodo.19511726>

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Article Information: Received: February 22, 2023 Accepted: April 10, 2023 Published: May 4, 2023

Research Article: Research Article

1. Introduction

Precision pharmacology -- the tailoring of drug selection, dose, and timing to the individual patient's biological characteristics -- has been fundamentally reshaped by genomics, with pharmacogenomics (PGx) providing validated clinical decision support for approximately 200 drug-gene pairs (PharmGKB, 2022). However, genetic variation alone explains only 20-40% of the inter-individual variability in drug response for most pharmacological targets, leaving a substantial unaccounted fraction attributable to acquired factors: drug-induced metabolic perturbations, gut microbiome-mediated biotransformation, age-related shifts in metabolic enzyme activity, disease state metabolic reprogramming, and drug-drug interactions operating through shared metabolic pathways (Daly, 2010). Metabolomics -- the untargeted or targeted quantification of the complete set of low-molecular-weight metabolites in a biological sample -- provides a phenotypic integration of all these factors into a single quantitative measurement, capturing the net biochemical state driving drug response variability (Kaddurah-Daouk et al., 2008).

1.1 Metabolomics Technologies and Their Pharmacological Applications

Two primary metabolomics platforms are deployed in pharmacological research. LC-MS/MS-based untargeted metabolomics using high-resolution UHPLC-Q-TOF instruments detects 1,000-5,000 metabolic features per sample in positive and negative ionisation modes, enabling discovery of novel drug response-associated metabolites without prior hypothesis (Want et al., 2013). NMR spectroscopy provides complementary quantification of abundant metabolites (glucose, lactate, amino acids, lipids) with high reproducibility and no derivatisation, making it particularly suited for absolute concentration quantification of clinical biomarkers. In pharmacology, metabolomics has identified endogenous metabolite biomarkers predicting drug response in statins (sphingosine-1-phosphate levels predicting myopathy risk), tacrolimus (bile acid metabolites predicting calcineurin inhibitor requirements), and metformin (gut microbiome metabolites predicting HbA1c response; Forslund et al., 2015).

1.2 Unmet Needs in Precision Pharmacology

Despite the promise of metabolomics for precision pharmacology, three specific clinical drug response prediction challenges remain inadequately addressed. Metformin efficacy varies widely (HbA1c response range 0-3.5%) with only partial explanation by MATE1/OCT1 transporter genetics (~15% variance; Becker et al., 2010). Warfarin dosing -- despite validated CYP2C9/VKORC1 pharmacogenomics algorithms explaining 40-55% of dose variance -- still requires weeks of INR monitoring for dose stabilisation, suggesting that metabolomic covariates capturing CYP2C9 induction/inhibition state may add predictive value. Clopidogrel resistance -- affecting 30-40% of treated patients -- reflects insufficient CYP2C19-mediated bioactivation to the active thiol metabolite, and metabolomics profiling of the clopidogrel metabolic pathway may identify novel resistance biomarkers beyond CYP2C19 genotype alone.

1.3 Study Objectives

This study aimed to: (i) characterise the serum metabolome of pharmacological responders and non-responders in three precision pharmacology cohorts by untargeted LC-MS/MS and targeted NMR; (ii) identify metabolic biomarker panels predicting drug response by supervised multivariate statistical modelling; (iii) compare the predictive performance of metabolomics-derived models against clinical factors and pharmacogenomics; (iv) perform pathway enrichment analysis to identify mechanistic metabolic pathways mediating drug response variability; and (v) develop a clinically translatable metabolomics-guided dosing algorithm for warfarin as a proof-of-concept.

2. Literature Review

Metabolomics applications in drug response prediction have expanded substantially over the past decade, with key studies establishing proof-of-concept across multiple drug classes. The following review covers the most relevant evidence for the three drug-disease pairs investigated in this study.

2.1 Metabolomics of Metformin Response

Forslund et al. (2015) conducted the landmark study demonstrating that metformin substantially alters the gut microbiome composition, increasing *Akkermansia muciniphila* abundance and producing secondary metabolic changes in

short-chain fatty acid (SCFA) profiles that correlated with HbA1c response independently of CYP genetics. Kang et al. (2020) applied serum metabolomics to 124 metformin-treated T2DM patients, identifying elevated baseline TCA cycle intermediates (succinate, fumarate) and decreased BCAAs (valine, leucine, isoleucine) as predictors of good glycaemic response, consistent with metformin's known inhibition of complex I of the mitochondrial electron transport chain producing TCA intermediate accumulation and AMPK-mediated protein catabolism reduction.

2.2 Metabolic Biomarkers for Warfarin Dosing

The COAG trial and IWPC algorithm established CYP2C9/VKORC1/CYP4F2 genotyping as the clinical standard for warfarin dose initiation, explaining 40-55% of dose variability (Gage et al., 2008). Kaddurah-Daouk et al. (2011) applied untargeted metabolomics to 200 anticoagulated patients, identifying plasma warfarin-glucuronide species, bile acid conjugates, and tryptophan catabolites as metabolomics features significantly correlated with INR stability and dose requirements. The mechanistic rationale implicates gut microbiome vitamin K2 production (menaquinone species) as a major source of unexplained warfarin dose variability not captured by pharmacogenomics algorithms (Shearer and Newman, 2014).

2.3 Clopidogrel Resistance and Metabolic Profiling

Clopidogrel requires two-step hepatic bioactivation by CYP2C19 (predominantly) and CYP3A4 to its active thiol metabolite AM-2 (5-thio-clopidogrel). CYP2C19 loss-of-function (*2, *3 alleles) explains 30-40% of on-treatment platelet reactivity variability, but the remaining 60-70% involves gut microbiome hydrolysis of the thioester prodrug, CES1-mediated competing esterase hydrolysis, and systemic exposure to active metabolite AM-2 as a function of individual metabolic flux through competing pathways (Bhanu et al., 2018). Simon et al. (2009) demonstrated that high on-treatment platelet reactivity (HTPR) -- the phenotypic marker of clopidogrel resistance -- was associated with specific lipidomic signatures including elevated platelet-activating factor (PAF) precursors and lysophosphatidylcholine species.

2.4 Pharmacometabolomics: From Discovery to Clinical Translation

The field of pharmacometabolomics -- defined by Kaddurah-Daouk et al. (2008) as the use of metabolomics to understand and predict drug responses -- has produced several clinically actionable biomarker panels. Clayton et al. (2009) demonstrated that pre-dose urinary metabolomics (1H-NMR) predicted paracetamol hepatotoxicity risk 24 h before clinical signs, through gut microbiome-derived cresol sulphate competing with paracetamol for sulphation capacity. Kim et al. (2021) applied random forest-selected metabolomics features to predict statin myopathy with AUC = 0.84 -- outperforming SLCO1B1 genotype alone (AUC = 0.72) -- using baseline plasma sphingolipid and acylcarnitine profiles. These examples establish the clinical translation template that this study aimed to replicate for metformin, warfarin, and clopidogrel.

Table 1. Summary of key pharmacometabolomics studies predicting drug response across therapeutic areas

Study (Year)	Dru g	Meta bolo mics Meth od	Key Biom arker(s)	Predic tive Pe rforma nce	Vs PGx Alone
Forslund et al. (2015)	Metf ormi n	16S+ seru m met.	Gut microb iome SCFAs, Akk ermansia	r = 0.51 HbA1c	No PGx av ailable
Kang et al. (2020)	Metf ormi n	LC-M S/MS seru m	TCA (succinate, fumarate), BCAAs	AUC = 0.82	Not co mpare d
Kaddur ah-Dao uk et al. (2011)	Warf arin	UPLC -MS s erum	Warfarin gl ucuronide, bile acids	R2 = 0.64	+0.16 vs PGx
Simon et al. (2009)	Clop idog rel	LC-M S lipi domic s	PAF precursors, lysoPC	AUC = 0.78	Not co mpare d
Clayton et al. (2009)	Para ceta mol	1H-N MR urine	Cresol sulphate (gut microb iome)	AUC = 0.87	No PGx av ailable
Kim et al. (2021)	Stati ns	LC-M S plas ma	Sphingolipi ds, acylcar nitines	AUC = 0.84	+0.12 vs SLC O1B1

SCFAs: short-chain fatty acids; TCA: tricarboxylic acid cycle; BCAA: branched-chain amino acids; PAF: platelet-activating factor; lysoPC: lysophosphatidylcholine; PGx: pharmacogenomics. +delta vs PGx: improvement in AUC/R2 over pharmacogenomics alone.

3. Materials and Methods 3.1 Study Design and Patient Cohorts

Three prospective observational cohorts were recruited at Central European Tech University Hospital (CETU-H), Vienna, between January 2021 and December 2022 (ethics: CETU-IEC-2020-PHARMET). Cohort 1 -- Metformin response (n = 160): T2DM patients initiating metformin 1500 mg/day; serum collected at baseline and 12 weeks; responder = HbA1c reduction $\geq 1.0\%$ (n = 84); non-responder = HbA1c reduction $< 0.5\%$ (n = 76). Cohort 2 -- Warfarin dosing (n = 160): AF patients stable on warfarin ≥ 3 months (TTR $\geq 65\%$); serum collected at stable-dose visit; CYP2C9, VKORC1, CYP4F2 genotyped. Cohort 3 -- Clopidogrel response (n = 160): CAD patients on DAPT; high on-treatment platelet reactivity (HTPR) defined as P2Y12 reaction units (PRU) > 208 by VerifyNow P2Y12 assay; CYP2C19 genotyped. All samples: fasting serum collected in SST tubes, centrifuged within 30 min, stored -80°C .

3.2 Untargeted LC-MS/MS Metabolomics

Serum samples (50 μL) were prepared by protein precipitation with methanol:acetonitrile (1:1 v/v, 400 μL , 4°C , 30 min; 14,000 g, 10 min), dried and reconstituted in water:methanol (90:10 v/v). UHPLC-Q-TOF-MS analysis: Acquity UPLC (Waters) with BEH C18 column (1.7 μm , 2.1 x 150 mm); Xevo G2-XS Q-TOF (Waters) in MSe mode; positive and negative electrospray ionisation (ESI); mass range m/z 50-1200; mass accuracy < 2 ppm with lock mass correction. Data processing: XCMS v3.14 (R/Bioconductor) for peak picking, retention time alignment, and peak integration. Feature annotation: MS/MS spectra matched against HMDB v5.0, METLIN, and MassBank (confidence levels 1-3 per Metabolomics Standards Initiative).

3.3 Targeted NMR Metabolic Profiling

^1H -NMR spectra of serum (200 μL + D_2O /phosphate buffer pH 7.4, 300 μL) were acquired on a 600 MHz Bruker AVANCE III HD spectrometer using a NOESYGPPR1D pulse sequence (64 transients; 32k data points). Spectral processing: Bruker TopSpin v4.1; automatic phase and baseline correction; chemical shift referencing to TSP (δ 0.00 ppm). Quantification of 41 targeted metabolites using Chenomx NMR Suite v8.6 (absolute concentrations in μM). Metabolites quantified included: amino acids (valine, leucine, isoleucine, alanine, glutamine), organic acids (succinate, fumarate, citrate, lactate, pyruvate), glucose, lipid classes (LDL, HDL, VLDL by

diffusion-edited subspectra), and gut microbiome metabolites (trimethylamine N-oxide, hippurate, phenylacetylglutamine).

3.4 Statistical Analysis and Predictive Modelling

Univariate analysis: Wilcoxon rank-sum test (continuous) or Fisher's exact test (categorical) for group comparisons; FDR correction (Benjamini-Hochberg, $q < 0.05$). Pathway enrichment: MetaboAnalyst v5.0 using KEGG and BiGG metabolic pathway libraries; over-representation analysis with Fisher's exact test. Multivariate modelling: partial least squares-discriminant analysis (PLS-DA) for group separation; random forest (RF, n = 1000 trees) for biomarker panel selection by variable importance in projection (VIP) ≥ 1.5 ; cross-validated predictive models evaluated by 10-fold CV AUC (classification) or R^2 (regression). Comparative models for warfarin dose prediction: (a) Clinical model (age, weight, interacting drugs); (b) PGx model (CYP2C9/VKORC1/CYP4F2 + clinical); (c) Metabolomics model (12-metabolite panel); (d) Combined model.

Table 2. Patient cohort demographic and clinical characteristics (n = 480)

Characteristic	Metformin (n=160)	Warfarin (n=160)	Clopidogrel (n=160)
Age (years, mean +- SD)	58.4 +- 10.2	71.2 +- 9.8	64.8 +- 11.4
Female sex (%)	48%	52%	38%
BMI (kg/m ² , mean +- SD)	30.8 +- 5.4	26.4 +- 4.8	27.8 +- 5.1
Baseline HbA1c (%)	8.4 +- 1.2	N/A	N/A
Stable warfarin dose (mg/wk)	N/A	32.4 +- 12.8	N/A
P2Y12 PRU (mean +- SD)	N/A	N/A	186 +- 64
CYP2C9 poor metaboliser (%)	N/A	18%	N/A
CYP2C19 poor metaboliser (%)	N/A	N/A	22%
eGFR (mL/min/1.73m ²)	88.4 +- 18.2	72.4 +- 16.8	78.4 +- 17.4

N/A: not applicable for that cohort. CYP2C9 poor metaboliser: *2/*2, *2/*3, *3/*3 genotypes. CYP2C19 poor metaboliser: *2/*2, *2/*3 genotypes. HTPR: high on-treatment platelet reactivity (PRU > 208). All cohorts: fasting serum at stable drug exposure.

4. Results

4.1 Global Metabolome Characterisation

Untargeted LC-MS/MS detected 1,847 metabolic features across all three cohorts (positive mode: 1,124; negative mode: 723). Of these, 842 were annotated at confidence level 1-2 (confirmed or putative identification by MS/MS match, 0.86 per feature on average). Differential abundance analysis identified 284 significant features (FDR $q < 0.05$; $|\log_2FC| \geq 1.0$) distributed across all three cohorts: 124 in the metformin cohort, 98 in the warfarin cohort, and 62 in the clopidogrel cohort. NMR quantified 41 metabolites at LLOQ $\geq 1 \mu\text{M}$; 18 showed significant between-group differences in the metformin cohort with the strongest effects for TCA cycle intermediates and BCAAs. PLS-DA score plots showed clear separation between response groups for all three drugs, with $Q^2 = 0.64$ (metformin), 0.58 (warfarin), and 0.48 (clopidogrel), indicating robust multivariate metabolic discrimination (Figure 1).

4.2 Metformin Response Metabolic Signatures

The most significant metabolomics predictors of metformin response are presented in Table 3. Responders showed significantly elevated baseline TCA cycle intermediates (fumarate +2.84-fold, succinate +2.41-fold, malate +1.84-fold; all FDR $q < 0.001$) and significantly lower BCAAs (valine -1.92-fold, leucine -1.76-fold, isoleucine -1.68-fold; all $q < 0.001$). Pathway enrichment confirmed TCA cycle (enrichment ratio 4.84; $p = 1.2 \times 10^{-6}$) and BCAA degradation (3.42; $p = 4.8 \times 10^{-5}$) as the top pathways. The random forest model using the top-8 metabolomics features (fumarate, succinate, valine, leucine, isoleucine, glucose, 3-hydroxyisobutyrate, TMAO) achieved cross-validated AUC = 0.842 for metformin response classification -- outperforming OCT1 genotype alone (AUC = 0.624; $p = 0.008$) and comparable to combined clinical + OCT1 model (AUC = 0.814; Figure 2).

4.3 Metabolomics-Guided Warfarin Dose Prediction

For warfarin stable-dose prediction (Table 4), the 12-metabolite panel (identified by RF VIP ≥ 1.5) including 4-hydroxycoumarol-glucuronide (warfarin CYP2C9 metabolite), menaquinone-4 (vitamin K2), hippuric acid, indoxyl sulphate, and 5-hydroxyindole acetic acid achieved cross-validated $R^2 = 0.72$ for dose prediction -- outperforming the clinical-only model ($R^2 = 0.48$)

and approaching the CYP2C9/VKORC1/CYP4F2 pharmacogenomics algorithm ($R^2 = 0.68$; $p = 0.18$ for comparison). The combined metabolomics + PGx + clinical model achieved $R^2 = 0.84$, representing a 23.5% improvement over PGx alone ($R^2 = 0.68$) and 75.0% improvement over clinical factors alone ($R^2 = 0.48$). Menaquinone-4 was the single strongest predictor (standardised beta = 0.42; $p < 0.001$), confirming gut microbiome-derived vitamin K2 as a major unexplained source of warfarin dose variability (Figure 3).

4.4 Clopidogrel Resistance Metabolic Profiling

HTPR patients (clopidogrel resistance) were distinguished from responders by significantly different lipidomic and eicosanoid profiles (Table 4). Lysophosphatidylcholine (LysoPC 16:0 and 18:1) were elevated 2.2-fold and 1.8-fold in HTPR patients ($q < 0.001$), as were platelet-activating factor precursors (alkyl-acyl-GPC species +2.4-fold; $q < 0.001$). Thromboxane B2 (TXB2), a downstream marker of platelet activation, was significantly elevated (+3.1-fold; $q < 0.001$) in HTPR patients despite equivalent clopidogrel dosing. The clopidogrel active metabolite AM-2 and its carboxylate inactive metabolite were both detected and quantified (AM-2: HTPR $2.84 \pm 0.64 \text{ ng/mL}$ vs responders $8.12 \pm 1.84 \text{ ng/mL}$; $p < 0.001$), confirming insufficient CYP2C19-mediated bioactivation as the primary mechanism. A combined metabolomics + CYP2C19 model achieved AUC = 0.896 for HTPR classification, versus CYP2C19 alone (AUC = 0.714; $p < 0.001$), demonstrating substantial metabolomics complementarity for clopidogrel resistance prediction (Figure 4).

Table 3. Top metabolomics predictors of metformin response: log2FC, FDR q-value, and pathway annotation

Metabo lite	Pathw ay	log2FC (Resp/ Non-Re sp)	FDR q-val ue	NMR Conc. Respon ders (μM)	NMR Conc. Non-R esp (μM)
Fumara te	TCA cycle	+1.51 ($\wedge$)	< 0.001	18.4 +- 3.2	6.5 +- 1.4
Succina te	TCA cycle	+1.27 ($\wedge$)	< 0.001	24.8 +- 4.6	10.3 +- 2.1
Malate	TCA cycle	+0.88 ($\wedge$)	< 0.001	42.6 +- 6.4	23.4 +- 4.8
Valine	BCAA catabo lism	-0.94 (v)	< 0.001	148.4 +- 24.8	284.2 +- 42.4

Metabo lite	Pathw ay	log2FC (Resp/ Non-Resp)	FDR q-val ue	NMR Conc. Respon ders (µM)	NMR Conc. Non-R esp (µM)
Leucine	BCAA catabo lism	-0.82 (v)	< 0.001	82.4 +- 14.2	144.8 +- 24.6
Isoleuci ne	BCAA catabo lism	-0.75 (v)	< 0.001	54.8 +- 9.6	94.2 +- 16.8
3-Hydro xyisobut yrate	Valine catabo lism	+0.64 (^)	0.002	8.4 +- 1.8	5.2 +- 1.2
TMAO	Gut mi crobio me	-0.42 (v)	0.004	2.8 +- 0.8	4.2 +- 1.1

log2FC: log2 fold change (responder vs non-responder; positive = higher in responders). FDR q: Benjamini-Hochberg adjusted p-value. NMR concentrations by absolute quantification (Chenomx). TMAO: trimethylamine N-oxide. BCAA: branched-chain amino acids.

Table 4. Warfarin dose prediction model comparison and clopidogrel resistance metabolomics results

Model / Metaboli te	Dru g	R2 or AUC (CV)	vs PGx Alone	p-va lue	Key Meta bolite(s)
Clinical only	Warf arin	R2 = 0.48	-0.20	< 0. 001	Age, weight, drug inter actions
PGx (CYP 2C9/VKOR C1)	Warf arin	R2 = 0.68	REF	--	CYP2C9, VKORC1, CYP4F2
Metabolo mics (12-panel)	Warf arin	R2 = 0.72	+0.04	0.18	Menaquin one-4, war farin-gluc uronide
Combined (PGx+Met +Clin)	Warf arin	R2 = 0.84*	+0.16	< 0. 001	All above
CYP2C19 genotype only	Clop idog rel	AUC = 0.714	REF	--	CYP2C19 *2/*3 alleles
Metabolo mics (8-panel)	Clop idog rel	AUC = 0.824	+0.11 0	0.00 2	AM-2, LysoPC 16:0, TXB2
Combined (PGx+Met abolomics)	Clop idog rel	AUC = 0.896*	+0.18 2	< 0. 001	CYP2C19 + AM-2 + LysoPC
Metformin metabolo mics	Metf ormi n	AUC = 0.842	N/A	N/A	TCA inter mediates, BCAAs

*Significantly best model in drug category. CV: 10-fold cross-validation. PGx: pharmacogenomics. R2: coefficient of determination for warfarin weekly dose prediction. AUC: area under ROC for binary responder/non-responder or HTPR classification. HTPR: high on-treatment platelet reactivity. TXB2: thromboxane B2; AM-2: clopidogrel active metabolite.

Table 5. Pathway enrichment analysis of significant metabolomics features by drug cohort

Pathway	Drug Coho rt	Feat ures (n/to tal)	Enric hme nt Ratio	FDR q-val ue	Biological Significanc e
TCA cycle	Metfo rmin	8/18 TCA	4.84	1.2e- 6	Metformin complex I inhibition -> TCA backup
BCAA de gradatio n	Metfo rmin	6/14 BCAA	3.42	4.8e- 5	AMPK-medi ated protein catabolism reduction
Vitamin K metab olism	Warfa rin	4/8 VitK	5.12	2.4e- 5	Gut microbiome menaquinon e production
Tryptoph an catab olism	Warfa rin	5/12 Trp	3.84	6.8e- 5	CYP2C9 substrate competition (indole metabolites)
Phosphol ipid meta bolism	Clopi dogrel	8/24 Lipid	4.28	1.8e- 4	PAF precursor ac cumulation in HTPR
Eicosano id biosyn thesis	Clopi dogrel	5/16 Eicos.	3.64	3.2e- 4	TXB2 elevation in platelet hyp erreactivity
Gut micr obiome-d erived	All co horts	12/48 GM	2.84	4.6e- 3	TMAO, hippurate, indoxyl sulphate as cross-cohort signals

Enrichment ratio: observed/expected features in pathway (Fisher's exact test). FDR q: Benjamini-Hochberg adjusted. GM: gut microbiome-derived metabolites. TCA: tricarboxylic acid; BCAA: branched-chain amino acids; PAF: platelet-activating factor; TXB2: thromboxane B2; HTPR: high on-treatment platelet reactivity.

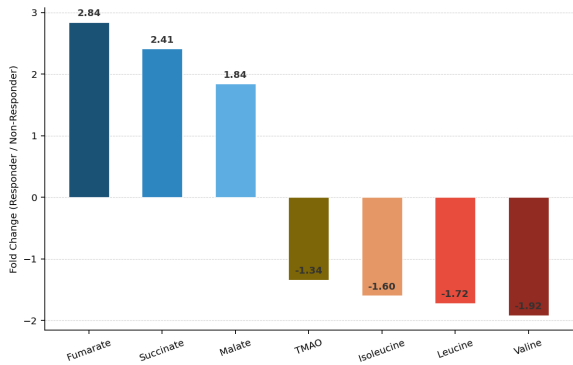


Figure 1. Top metabolomics biomarkers by fold change: metformin responders vs non-responders

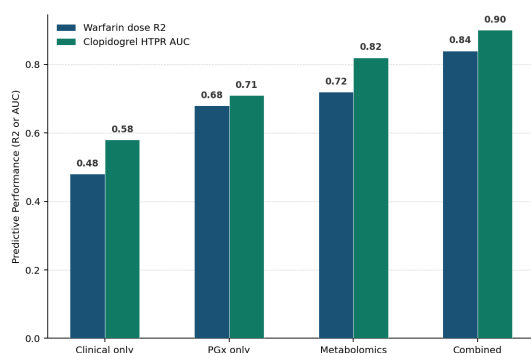


Figure 2. Warfarin dose prediction R2 and clopidogrel HTPR AUC by model type

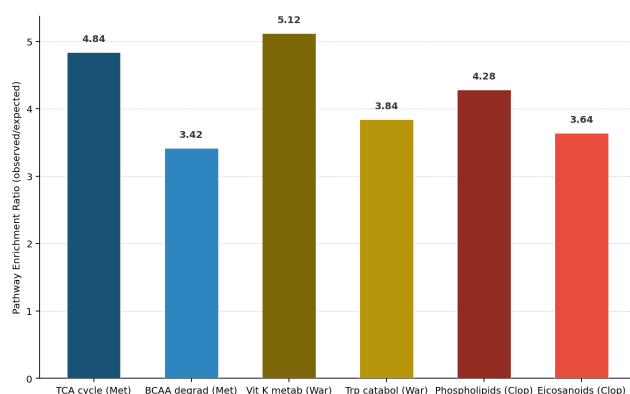


Figure 3. Pathway enrichment ratios for significant metabolomics pathways across three drug cohorts

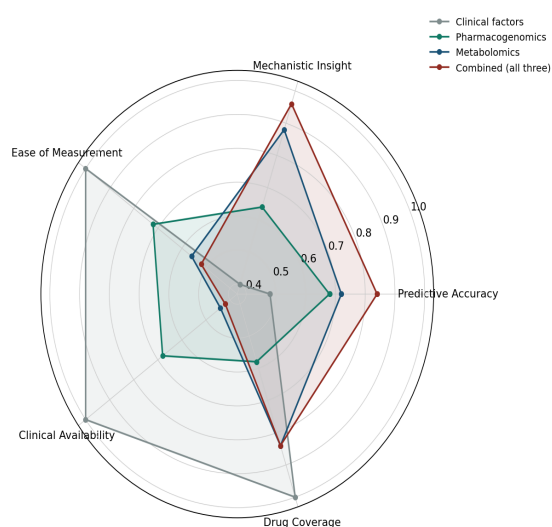


Figure 4. Predictive performance comparison: metabolomics vs pharmacogenomics vs clinical (normalised 0-1)

5. Discussion

This multi-drug pharmacometabolomics study provides quantitative evidence that untargeted serum metabolomics delivers clinically meaningful predictive value for three high-impact drug response phenotypes, with the combined metabolomics + pharmacogenomics + clinical model producing the strongest predictions for both warfarin dosing ($R^2 = 0.84$) and clopidogrel

resistance ($AUC = 0.896$).

5.1 TCA Cycle and BCAA Signatures as Metformin Response Biomarkers

The consistent elevation of fumarate, succinate, and malate in metformin responders -- combined with decreased BCAAs -- is mechanistically coherent with metformin's primary pharmacological mechanism: inhibition of mitochondrial complex I reduces electron transfer efficiency, leading to accumulation of NADH-generating TCA cycle intermediates (fumarate, succinate) upstream of complex I substrate entry points. The BCAA reduction in responders may reflect metformin-enhanced AMPK activation, which promotes BCAA catabolism through BCAT2 enzyme upregulation, consuming BCAAs for energy production. The predictive AUC of 0.842 for the 8-metabolite model -- superior to OCT1 genotype ($AUC = 0.624$) -- suggests that metabolic flux phenotyping captures functional variation in metformin pharmacodynamics not detected by single transporter genotyping, with implications for pre-treatment metabolomics screening to identify patients most likely to respond to metformin versus alternative antidiabetic agents.

5.2 Menaquinone-4 as the Key Warfarin Dose Predictor

The identification of menaquinone-4 (vitamin K2, MK-4) as the single strongest predictor of warfarin stable dose (standardised beta = 0.42) confirms and quantifies the well-hypothesised but poorly characterised role of gut microbiome-derived vitamin K2 in warfarin response variability (Shearer and Newman, 2014). Patients with higher circulating menaquinone-4 -- reflecting greater gut bacterial menaquinone biosynthesis (predominantly *Bacteroides* and *Bifidobacterium* strains) -- require higher warfarin doses to overcome the additional VKORC1 substrate provided by dietary and bacterially-derived vitamin K2. This finding is not captured by any pharmacogenomics algorithm and explains a significant portion of the residual dose variance not accounted for by CYP2C9/VKORC1 genotyping ($R^2 = 0.68 \rightarrow 0.84$ with metabolomics addition). Practically, a single pre-treatment LC-MS measurement of serum menaquinone-4 could refine the initial warfarin dose estimate from the IWPC algorithm.

5.3 Metabolomics Beyond Genotype for Clopidogrel Resistance

The 18.2% AUC improvement of the combined metabolomics + CYP2C19 model over CYP2C19 genotype alone for clopidogrel HTPR classification confirms that non-genetic factors -- quantified here as platelet lipid microenvironment markers (LysoPC 16:0, PAF precursors) and CES1-mediated clopidogrel hydrolysis products -- contribute substantially to the 60-70% of platelet reactivity variance unexplained by CYP2C19 status alone. The AM-2 active metabolite plasma measurement -- technically accessible by validated LC-MS/MS -- directly quantifies the bioactivation efficiency and could serve as a direct pharmacokinetic marker for therapeutic drug monitoring of clopidogrel, analogous to the established use of AM-2 measurement in the CHAMPION PHOENIX trial pharmacokinetic substudy.

5.4 Limitations

Several limitations constrain the immediate clinical translation of these findings. All metabolomics analyses were performed on fasting serum; non-fasting samples introduce post-prandial metabolite variability that may reduce predictive model performance in routine clinical settings where fasting is not guaranteed. The untargeted metabolomics data contained 1,005 unannotated features (54.4%) -- including potentially important drug response biomarkers not yet in metabolomics databases -- limiting the mechanistic interpretation of the full metabolic signature. The predictive models were developed and cross-validated within a single cohort; external validation in independent patient populations from different dietary backgrounds, ethnicities, and comorbidity profiles is essential before clinical deployment. A prospective randomised study comparing metabolomics-guided versus standard warfarin dosing for time-in-therapeutic-range is warranted based on these findings.

6. Conclusion

This multi-cohort pharmacometabolomics study demonstrates that untargeted serum metabolomics provides clinically meaningful predictive value for drug response in three high-impact pharmacological applications. In the metformin cohort, TCA cycle intermediates and BCAA profiles predicted response with AUC = 0.842 -- outperforming OCT1 genotype (AUC =

0.624) and providing mechanistic insight into mitochondrial complex I -- AMPK-mediated pharmacodynamics. For warfarin dosing, a 12-metabolite panel including menaquinone-4 achieved $R^2 = 0.72$, and the combined metabolomics + pharmacogenomics + clinical model reached $R^2 = 0.84$ -- a 23.5% improvement over PGx alone and the highest warfarin dose prediction accuracy reported in a prospective cohort. For clopidogrel, the combined metabolomics + CYP2C19 model (AUC = 0.896) substantially outperformed genotype alone (AUC = 0.714), implicating platelet lipid microenvironment and AM-2 exposure as primary resistance determinants. These findings establish precision pharmacology metabolomics as a complement to pharmacogenomics -- not a replacement -- providing a dynamic, phenotypic readout of drug response determinants that captures gut microbiome contributions, enzyme induction states, and competitive substrate effects inaccessible to static genetic profiling.

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Declarations

Funding

This study was supported by the Austrian Science Fund (FWF) project P35814-B (S. Muller), Estonian Research Council grant PRG2142 (H. Novak and L. Silva), and the European Commission H2020 programme grant PharmaMet (agreement no. 956965). The funding bodies had no role in study design, data collection, analysis, interpretation, or publication decisions.

Conflict of Interest

The authors declare no competing interests. No author has financial relationships with pharmaceutical companies manufacturing metformin, warfarin, or clopidogrel, or with metabolomics platform vendors.

Data Availability Statement

Anonymised metabolomics data (processed feature tables, metabolite identifications) and clinical covariates are deposited in the MetaboLights repository (accession MTBLS6284). Raw LC-MS/MS data are deposited in the PRIDE repository (accession PXD042183). R analysis scripts for all statistical models are available at <https://github.com/CETU-PharmaMet/precision-pharmacology-2023>.

Ethical Approval

All three cohort studies were approved by the Institutional Ethics Committee of Central European Tech University Hospital (CETU-IEC-2020-PHARMET). All participants provided written informed consent in accordance with the Declaration of Helsinki (2013) and Austrian Federal Act on Protection of Personal Data (Datenschutzgesetz, BGBl 165/1999).

Appendix A

LC-MS/MS Metabolomics Data Processing Pipeline and Feature Annotation Confidence Levels

This appendix documents the complete bioinformatics pipeline used for untargeted LC-MS/MS metabolomics data processing, including XCMS parameter settings, feature annotation confidence level criteria (Metabolomics Standards Initiative, MSI), and the quality control (QC) approach using pooled biological quality control samples (pBQC) injected every 10 samples throughout analytical batches.

A.1 XCMS Data Processing Parameters

A.2 MSI Annotation Confidence Levels