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Exploration of potential biomarkers for amoxicillin/clavulanate-induced liver injury in humans through multi-omics approaches

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의학과 임상약리학 전공

이 지 언

다중오믹스 분석법 기반

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간손상 생체지표 탐색

지도 교수 조 주 연

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이 지 언

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Exploration of potential biomarkers for amoxicillin/clavulanate-induced liver injury in humans through multi-omics approaches

By

Jieon Lee

A thesis submitted to the Department of Medicine in partial fulfillment of the requirement for the Degree of Doctor of Philosophy in Medical Science (Clinical Pharmacology) at Seoul National University College of Medicine

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Professor _____ **Chairman**

Professor _____ **Vice Chairman**

Professor _____

Professor _____

Professor _____

ABSTRACT

Introduction: Drug-induced liver injury (DILI) is a major challenge in the development and use of therapeutic drugs. The exploration for more sensitive biomarkers of DILI requires multidirectional approaches. However, a comprehensive clinical study has not been conducted in healthy volunteers. To explore potential biomarkers for and mechanisms of amoxicillin/clavulanate-induced liver injury (AC-DILI), we conducted a clinical trial based on multi-omics approaches.

Methods: Thirty-two healthy Korean male volunteers were enrolled and grouped according to 4 GSTT1/M1 genotypes (8 subjects per group). Blood and urine samples were collected before and after 14 days of amoxicillin/clavulanic acid administration for liver function tests and quantification of biomarkers. The approaches used throughout this study included a liver function test, pharmacokinetic analysis, microRNA quantification, pharmacometabolomics analysis, human leukocyte antigen (HLA) typing, and lymphocyte transformation test (LTT). We evaluated the correlations between liver function parameters and multi-omics biomarkers.

Results: Comparative analyses between Responder and Non-Responder groups classified by the alanine aminotransferase (ALT) elevation level revealed no statistically significant differences in primary pharmacokinetic (PK) parameters of amoxicillin or clavulanate. Liver-specific microRNA-122 (miR-122) was highly

correlated with ALT. Urinary metabolites, including 7-methylxanthine, 7-methyluric acid, 3-methylxanthine, and azelaic acid, showed significantly different levels between the two groups ($P<0.05$). Lymphocyte proliferation in response to the drug was also observed. These findings demonstrate sequential changes in the process of AC-DILI, including metabolic changes, increased miR-122 level, increased liver enzyme activity and enhanced lymphocyte proliferation after drug administration.

Conclusion: This is the first study to evaluate potential AC-DILI biomarkers in healthy volunteers. The results confirm miR-122 and four urinary metabolites as early and sensitive biomarkers for AC-DILI, suggesting hepatocellular injury, liver inflammation, and mitochondrial oxidative stress as mechanisms underlying AC-DILI. Proliferation of lymphocytes in response to the drug also suggests a role for the adaptive immune response in AC-DILI. The biomarkers evaluated in this study by integrating omics data could enable more sensitive and earlier prediction of liver injury in drug development and usage of therapeutics.

Keywords: Drug-induced liver injury; biomarker; pharmacogenomics; pharmacometabolomics; microRNA; clinical trials

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List of Abbreviations and Symbols

AC-DILI	Amoxicillin/clavulanate-induced drug induced liver injury
AC	Amoxicillin/clavulanate
AE	Adverse events
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
AT	Aminotransferase
$AUC_{0\text{-}12h}$	Area under the plasma concentration–time curves from zero to 12 hours at steady state
$AUC_{ss,\tau}$	Area under the plasma concentration–time curves from zero to 12 hours in the steady state on day 8
BMI	Body mass index
C_{max}	Maximum plasma concentration
CI	Confidence interval
CL	Clearance
CV	Coefficient of variation
DILI	Drug induced liver injury

ECG	12-lead electrocardiography
ESI-	Electrospray ionization in the negative ion mode
ESI+	Electrospray ionization in the positive ion mode
GCP	Good clinical practice
GST	Glutathione s-transferase
GSTM1	GST mu-1
GSTT1	GST theta-1
H	Hour(s)
HLA	Human leukocyte antigen
LC-MS/MS	Liquid chromatography-tandem mass spectrometer
LLOQ	Lower limit of quantitation
LTT	Lymphocyte transformation test
miRNA	MicroRNA
miR-122	MicroRNA-122
MR	Metabolic ratio
OPLS-DA	Orthogonal partial least squares discriminant analysis
PBMCs	Peripheral blood mononuclear cells
PCA	Principal component analysis
PHA	Phytohemagglutinin
PK	Pharmacokinetics

QC	Quality controls
ROC	Receiver operating curve
SD	Standard deviation
SI	Stimulation index
T.Bil	Total bilirubin
VIP	Variable influence on the projection
ULN	Upper limit of normal value
λz	Elimination rate constant

INTRODUCTION

Drug-induced liver injury (DILI) is a major challenge for both clinical care and drug development.[1] DILI is the most common cause of acute liver failure. Acetaminophen is the most frequent injury-inducing drug, but other drugs such as antibiotics and anti-tuberculosis drugs also cause DILI.[2] DILI is classified into predictable reactions and idiosyncratic reactions. Predictable reactions, called dose-related reactions, have a high incidence and occur with a short latency (e.g., within a few days). A classic example of these reactions is acetaminophen toxicity. By contrast, idiosyncratic reactions occur with variable latency (e.g., 1 week to 1 year or more) with a low incidence, and these reactions may not be dose related. The majority of hepatotoxic drugs including amoxicillin and clavulanate are reported to cause idiosyncratic reactions. DILI may also be classified as immune-mediated reactions and non-immune-mediated reactions.[3]

Monitoring the activities of the enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which reflect hepatic function, is the primary screening tool for DILI.[4] However, elevated levels of these enzymes may not be specific to liver disease and can represent an asymptomatic response.[5-7] Thus, there is a medical need for biomarkers that are more sensitive and specific for the early detection of DILI, and the rapidly evolving and high-throughput 'omics' technologies have been applied to identify prognostic

biomarkers of DILI.

Hy's law is a standard for diagnosing DILI.[8] This is referred to as Hy's Law after *Hy Zimmerman*, who reported that the mortality from drug-induced hepatocellular jaundice ranges from 10% to 50%. Hy's law has three criteria regarding drugs at high risk of causing DILI, as follows: (1) The drug causes 3-fold or greater elevations above the upper limits of normal (ULN) of ALT or AST compared with the (non-hepatotoxic) control agent or placebo; (2) Among subjects who exhibit such aminotransferase (AT) elevations, often with ATs much greater than 3×ULN, some subjects also show an elevation of serum total bilirubin (TBL) to >2×ULN, without initial findings of cholestasis (serum alkaline phosphatase (ALP) activity <2×ULN); and (3) No other reason can be found to explain the combination of increased AT and serum TBL, such as viral hepatitis, preexisting or acute liver disease, or another drug capable of causing the observed injury.[4,9]

Pharmacogenomics studies have demonstrated specific human leukocyte antigen (HLA) alleles that are associated with DILI that is caused by the following drugs: flucloxacillin (B*57:01), ximelagatran (DRB1*07:01 and HLA-DQA1*02), co-amoxiclav (DRB1*15:01), and anti-tuberculosis drugs (HLA-DQB1*0502).[10-13] Glutathione s-transferase (GST) is an essential phase II metabolic enzyme that is related to drug detoxification, and the mu-1 and theta-1 (GSTM1 and GSTT1) null genotypes have also been identified as genetic risk factors for DILI.[10,14]

Numerous studies have identified microRNAs, which are small, single-

stranded noncoding regulatory RNA molecules, as possible sensitive biomarkers for DILI.[15-18] Notably, microRNA-122 (miR-122) is elevated earlier and demonstrates increased sensitivity in patients with acetaminophen-induced liver injury compared to ALT.[15,19] Although the mechanism of miR-122 elevation is not yet known, hepatocyte damage may induce the release of cellular miR-122 into the circulation, which leads to increased miR-122 levels in the peripheral blood.[19] In a recent study, differences in the circulating serum levels of exosomal miR-122 were observed between hepatocyte injury and inflammation.[16]

Pharmacometabolomic approaches have been used to identify novel DILI biomarkers.[20,21] The results of a clinical trial that was performed to analyze the pharmacometabolomics patterns in urine samples from healthy subjects before and after the administration of acetaminophen indicated that N-acetyl-p-benzoquinone imine may be a useful biomarker of DILI.[22] Another clinical study demonstrated that acetaminophen-induced DILI was inversely correlated with *p*-cresol sulfate, which competes with acetaminophen entry into the portal circulation from the liver.[23]

Amoxicillin/clavulanate was selected for analysis in this study because it is the frequently prescribed antibiotic worldwide and is often associated with hepatotoxicity.[24,25] The delayed nature and HLA association of DILI indicate the involvement of the adaptive immune system, and immunological idiosyncrasies have been proposed as a possible mechanism for amoxicillin/clavulanate-induced DILI (AC-DILI).[12,26,27] In a recent study, drug-

responsive T cells that were specific for either amoxicillin or clavulanate were isolated from a patient with AC-DILI.[28] Furthermore, the type of liver damage most frequently associated has been cholestasis with signs of hypersensitivity. The mechanism that underlies AC-DILI has not yet been clearly identified; however, the activation of T cells by xenobiotics is likely to mediate the immune response that is involved in DILI.

Although several promising biomarkers have been identified in patients with DILI, including HLA,[29] miR-122[30] and endogenous metabolites,[31] well-controlled prospective clinical trials have not been conducted to validate these biomarkers. Based on the multidirectional approaches that were described in Figure 1, we evaluated potential biomarkers of DILI in healthy subjects after multiple administrations of amoxicillin/clavulanate.

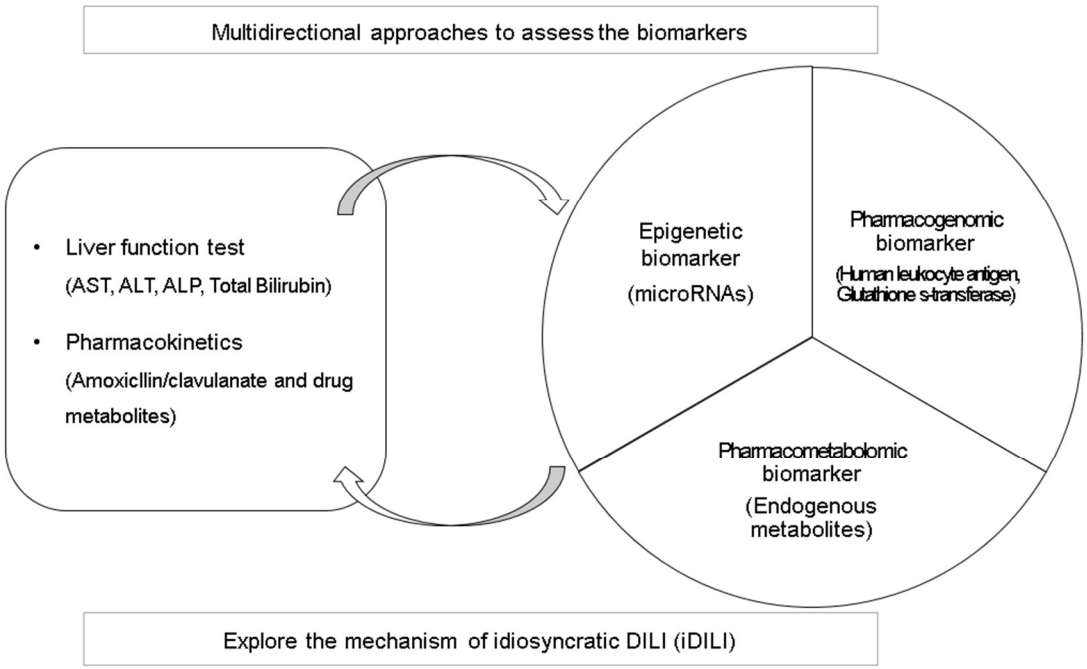


Figure 1. Overall concept of the study design

MATERIALS AND METHODS

Study Subjects

Thirty-two healthy Korean male volunteers were enrolled and grouped according to four GSTT1/M1 genotypes (8 subjects per group), such as wild/wild, null/wild, wild/null, and null/null types. Subjects were included in this study if they were in good health as indicated by their previous medical history, physical examination, vital signs, 12-lead electrocardiography (ECG), serology, and urinary drug screening.

Eligible subjects were Korean healthy male volunteers (ages 20 to 45 years) whose body mass index (BMI) was in the range of 18.5-27.5 kg/m^2. The subjects were in good health based on previous medical history, physical examination, resting blood pressure, 12-lead electrocardiography (ECG), serology (hepatitis B surface antigen, anti-hepatitis C virus and anti-human immunodeficiency virus antibody), and urinary drug screening (amphetamine, cocaine, barbiturate, benzodiazepines, and opioids) that were obtained within four weeks of the first administration of the study drug.

Subjects were excluded if they showed evidence or a history of any of the following: clinically significant hepatic, gastrointestinal, renal, neurologic, immunologic, hematologic, oncologic, psychiatric, or cardiovascular diseases; had taken any prescribed drugs, herbal agents or crude drugs within 2 weeks prior to the study's drug administration; had a history of drug allergies or drug

abuse; had used any prescriptive medication during the last 14 days before the first dosing; had participated in clinical trials of any drug within 60 days prior to this study; smoked 20 or more cigarettes per day within two months of the study; heavily used alcohol (over 21 units/week) or would not be able to stop drinking alcohol during the hospitalization; or had donated blood within one month prior to the start of the study. Subjects were also excluded if their sitting blood pressure fell within a certain range during the screening procedure. Subjects who had used any prescription drugs, including antibiotics such as amoxicillin/clavulanate, or who had a history of severe adverse reactions to the study drugs were also excluded.

The study was a clinical trial in healthy volunteers to explore the biomarkers of drug-induced liver injury; it was exploratory and descriptive but did not include statistical hypothesis testing. Thus, the present investigation was conducted with the minimum of subjects that empirically met the study objective.

Study Design

This study had an open-label, single-sequence, 3-period design. The workflow of the study design is shown in Figure 2. Each subject received 3 tablets of amoxicillin/clavulanate (375/125 mg) twice daily for 14 days. Blood and urine samples were obtained for laboratory tests, pharmacokinetic (PK) evaluations and multi-omics analyses such as miRNA-122, pharmacometabolites, HLA typing and LTT. The detailed design of the

clinical trial is depicted in Figure 3.

Blood samples were obtained at the following sampling points: pre-dose and up to 12 hours after the administration of the study drug on days 1 and 8 for pharmacokinetic (PK) analysis; pre-dose on days 1, 2, 5, 8, 14, 22, and 60 for miRNA quantification; pre-dose on days 1, 2, 5, 8, 9, 11, 14, 15, 21 and 60 for liver function tests; and pre-dose on day 14 for HLA typing (Table 1). Detailed sampling time points are described in Table 1. Urine samples were collected at 12-hour intervals prior to drug administration on day 1 and following drug administration on days 8 and 14 for pharmacometabolomics analyses. Breath alcohol tests were conducted on the first day of every period to exclude the effect of alcohol on liver function. Safety and tolerability were assessed for all subjects throughout the study.

The subjects were admitted on the evening of one day before the study drug administration (day -1). From day -1 evening (9 p.m.), urine samples were collected for 12 hours. From day 1, all subjects were administered the study drugs for 14 days. After the administration of the study drug, plasma samples for the quantification of study drug concentrations, pharmacometabolites and microRNA were collected. The subjects were discharged after all the procedures of day 2. From day 3 to day 7, each subject visited the clinical center daily and received the study drug for twice-daily administration. The subjects were admitted again on day 7 and day 13 and were discharged on day 9 and day 15; the study procedures were repeated as in period 1 (from day -1 to day 2).

Table 1. Detailed time point for blood sampling

Purpose	Time point
Laboratory test	Day 1, 2, 5, 8, 9, 11, 14, 15 0h, PSV
Pharmacokinetic test	Day 1, 8: 0h, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12h
microRNA analysis	Day 1, 8: 0h, post-dose (2, 4, 6, 8, 12h) Day 2, 5, 14 pre-dose (0h), PSV
HLA locus full sequencing and Lymphocyte transformation test	Day 1 pre-dose(=0h), PSV

*PSV, post-study visit

Ethical Consideration

The Institutional Review Board of Seoul National University Hospital (Seoul, Korea) approved this clinical study (H-1309-041-519) which was conducted in compliance with the ethical principles of the Declaration of Helsinki (6^{th} revised) [32], the International Conference on Harmonization Good Clinical Practice Guidelines, and local laws and regulations.

All subjects provided written informed consent before being screened for eligibility. Before the enrollment of the study subjects, this study was registered and disclosed in the US National Institutes of Health clinical database (the ClinicalTrials.gov registry number is NCT02143323.).

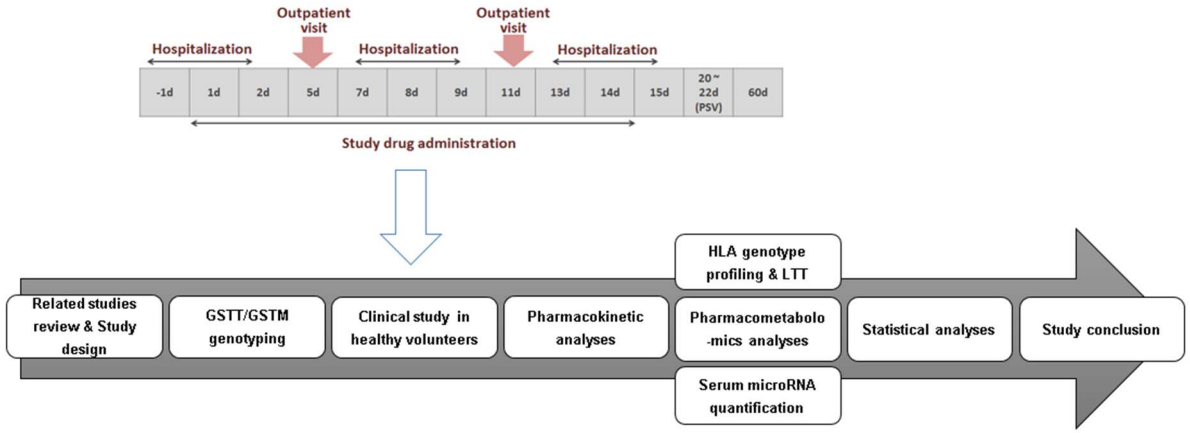


Figure 2. Overall study flow; PSV, post-study visit; GST, glutathione s-transferase; LTT, lymphocyte transformation test.

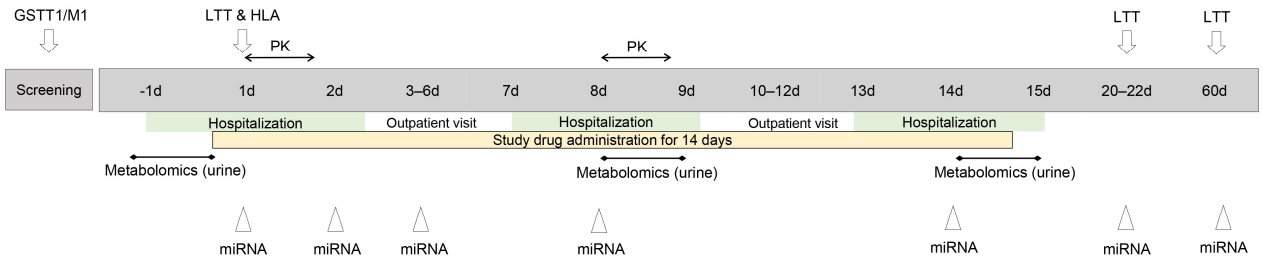


Figure 3. Detailed schedule of the clinical study. GSTT1/M1, blood collection for genotyping of glutathione s-transferase mu-1/theta-1; LTT, blood collection for lymphocyte transformation test; HLA: blood collection for genotyping human leukocyte antigen (HLA); PK, blood collection for pharmacokinetic evaluation

Quantification of the amoxicillin/clavulanate concentrations

The plasma concentrations of amoxicillin and clavulanate were determined using validated high-performance liquid chromatography-tandem mass spectrometry with an Agilent 1260 series chromatography system (Agilent, Santa Clara, CA, USA) coupled to an API 4000 mass spectrometer (Sciex, Toronto, Canada). Chromatographic separation of amoxicillin was conducted using a Synergi 4U HydroRP column (100 × 2.0 mm, 4 μm) (Phenomenex Inc., Torrance, CA, USA). A Luna column (100 × 2.0 mm, 5 μm) (Phenomenex Inc.) was used for the chromatographic separation of clavulanate. The lower limit of quantitation was 10 ng/mL for both amoxicillin and clavulanate. The intra-day and inter-day precision data are described in Table 2 for amoxicillin and clavulanate.

Table 2. Accuracy and precision of the (a) amoxicillin and (b) clavulanate concentration measurements using liquid chromatography-tandem mass spectrometry (n=5).

(a)

Concentration (ng/mL)	Accuracy (%)		Precision (% Coefficient of variance)	
	Intra-day (*n*=5, day 1)	Inter-day (*n*=5)	Intra-day (*n*=5, day 1)	Inter-day (*n*=5)
10	102.1	95.48	2.76	7.67
30	94.57	90.81	4.49	5.20
1500	102.4	99.14	2.99	5.80
6400	97.23	94.73	2.59	4.08
10000	98.72	-	2.71	-

(b)

Concentration (ng/mL)	Accuracy (%)		Precision (% Coefficient of variance)	
	Intra-day (*n*=5, day 1)	Inter-day (*n*=5)	Intra-day (*n*=5, day 1)	Inter-day (*n*=5)
10	98.83	102.2	7.488	6.018
30	99.44	100.3	3.332	5.798
400	97.72	99.67	0.993	4.916
4000	99.77	100.9	1.676	4.071
8000	95.50	-	1.055	-

PK Analyses

The PK parameters were derived using non-compartmental analyses in Phoenix WinNonlin (Version 6.3., Certara, St. Louis, MO, USA). Non-compartmental methods were used to determine the individual PK parameters of amoxicillin and clavulanic acid.

The C_{max} (maximum plasma concentration at steady state), $AUC_{0\text{-}12h}$ (area under the plasma concentration–time curves from zero to 12 hours after a single administration) and $AUC_{ss,\tau}$ (area under the plasma concentration–time curves from zero to 12 hours at steady state) were calculated using the linear-up and log-down method. The area under the curve from time zero to infinity (AUC_{inf}) was calculated by adding $C_{last}/\lambda z$ to AUC_{last}, where C_{last} is the last measurable concentration. Clearance was determined as administered dose/AUC_{inf}. The terminal elimination rate constant (λz) was estimated by linear regression of the log-linear decline in the individual plasma concentration-time data.

The individual half-life ($t_{1/2}$) was calculated as $\ln(2)/\lambda z$, where ln is the natural logarithm. The volume of distribution (V_d) was calculated by dose/$\lambda z \bullet AU_{Cinf}$. The C_{max} and AUC_{last} were log-transformed, and the mean differences and a 90% confidence interval (CI) were back-transformed to obtain the geometric mean ratios along with a CI for the ratios.

GSTT1/GSTM1 and HLA typing

A multiplex polymerase chain reaction method was used to simultaneously analyze the GSTM1 and GSTT1 genotypes using blood samples that were obtained during screening (DNA Link, Inc., Seoul, Korea).[33] The following primers were used to amplify the exons and promoters of the GSTT1/M1 genes: 5' GAA CTC CCT GAA AAG CTA AAG C 3' (forward primer) and 3' GTT GGG CTC AAA TAT ACG GTG G 5' (reverse primer) for GSTM1; and 5' TTC CTT ACT GGT CCT CAC ATC TC 3' (forward primer) and 5' TCA CCG GAT CAT GGC CAG CA 3′ (reverse primer) for GSTT1.

β-Globin was amplified as an internal control with the following primers: 5' GAA GAG CCA AGG ACA GGT AC 3' (forward primer) and 3' CAA CTT CAT CCA CGT TCA CC 5' (reverse primer). The following steps were used for amplification: an initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 55°C for 1 min, and an additional cycle at 72°C for 10 min. Agarose gel electrophoresis (3%) resolved the amplified DNA fragments for GSTT1 (459 bp), β-globin (268 bp), and GSTM1 (219 bp). The absence of an appropriate PCR product despite the presence of β-actin indicated a null genotype.

DNA template sequences were produced for HLA typing using locus- and group-specific amplifications that included exons 2 and 3 for class I (A, B, C) and exon 2 for class II (DRB1, DRB3/4/5, DQB1, DQA1, DPA1, and DPB1).

The results are reported as antigen recognition site alleles as per the recommendations of the American Society for Histocompatibility and Immunogenetics[34]. High-resolution sequence-based HLA typing was performed by the Histogenetics laboratory (Histogenetics, New York, USA) at the following loci: HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1.

Measurement of serum miRNAs

Total RNA was extracted for quantitative analyses of serum microRNAs using a miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. A total volume of 100 µL of serum was used for the RNA extraction. A synthetic miRNA from *Caenorhabditis elegans* was included in the sample after homogenization in QIAzol Lysis Reagent (Qiagen) to validate the RNA extraction efficiency. Complementary DNA was prepared using a miScript Reverse Transcription Kit II (Qiagen). The 20 µL PCR reaction contained 10 µL of miScript SYBR Green PCR Master Mix (Qiagen), 4 µL of nuclease-free water, 2 µL of 10× miScript primer assay, 2 µL of 10X miScript Universal Primer and 2 µL of cDNA template. Amplification was performed using a CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA) under the following conditions: initial denaturation at 95°C for 15 min followed by 40 cycles of denaturation for 15 sec at 94°C, annealing for 30 sec at 55°C, and elongation for 30 sec at 70°C. A total exosome isolation reagent (Invitrogen, Carlsbad, CA, USA) was used

for exosome isolation from serum according to the manufacturer's instructions. To measure microRNAs in the exosome-rich and protein-rich fractions, RNA extraction, reverse transcription and real-time PCR were performed using the same procedures described above. Quantitation of the microRNA was performed by Dr. Sang Chun Ji. The overall procedure for the microRNA quantification is described in Figure 4.

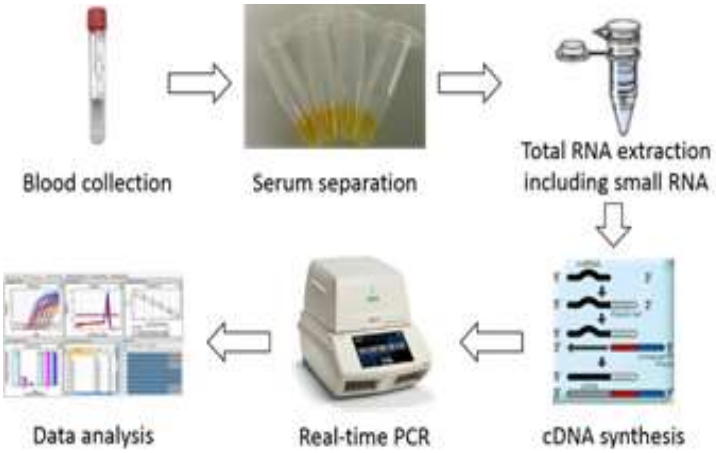


Figure 4. Workflow of the serum microRNAs analysis

Pharmacometabolomic analyses

Untargeted metabolomics profiling was performed using urine samples that were collected at 12-hour intervals before and after the administration of the study drug. The collected urine samples were centrifuged at 14,000 rpm for 20 min at 4°C to remove any solid debris, and the supernatant was diluted with distilled water. A 4 μL aliquot of the prepared urine sample was injected into a 2.1 × 100 mm ACQUITY 1.8 μm HSS T3 column using an ACQUITY UPLC system (Waters Corp., Milford, MA, USA) coupled to a Waters Xevo Q-TOF (Waters Corp.).

The gradient mobile phase condition consisted of phase A (water with 0.1% formic acid) and phase B (methanol containing 0.1% formic acid). Each sample was resolved for 20 min at a flow rate of 0.4 mL/min under the following gradient conditions: 0-1 min, 5% B; 1-4 min, 5-20% B; 4-7.5 min, 20-60% B; 7.5-11.5 min, 60-95% B; 11.5-15.5 min, 95% B; 15.5-16.2 min, 95-5% B; and 16.2-20 min, 5% B. The optimal capillary and cone voltages were set at 3 kV and 40 V, respectively. The source and desolvation temperature were maintained at 120 and 350°C, respectively. The data were collected in centroid mode over the range of 50-1000 m/z with a scan time of 0.4 sec. The data were monitored in real-time using leucine enkephalin as the reference lock mass. This process for quantification of metabolites was performed by Bora Kim.

Using 2 The multivariate data were analyzed using the EZinfo software (Waters Corp.). The stability and reproducibility of the unsupervised

principal components analysis were checked using the Pareto-scaled data by clustering quality controls (QCs). Orthogonal partial least squares discriminant analysis (OPLS-DA) and s-plotting were performed to select the biomarker candidates

Lymphocyte transformation test (LTT)

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation from blood that was collected on day 1 before the study drug administration, day 20-22, and day 60. The proliferation of PBMCs (0.15×10^6 cells) in response to amoxicillin (0.25-2 mM), clavulanate (0.0625-0.5 mM) and PHA (5 μg/mL; positive control) was measured using the LTT as previously described.[28] The proliferative response was calculated as a stimulation index (SI; cpm in drug-treated cells/cpm in mock-treated cells). PBMCs secreting interferon-gamma were visualized using ELISpot (MabTech, Nacka Strand, Sweden) by culturing the PBMCs with each drug for 48 hours.

Statistical analysis

The arithmetic means, standard deviations (SD), medians, and maximum and minimum values of the continuous data were calculated. The subjects were classified into subgroups based on the extent of the ALT elevations, HLA and GSTT1/M1 genotypes and LTT results to evaluate the associations between the biomarkers. The genotypic frequencies of the GSTM1 and T1 polymorphic variants the groups that were classified by ALT elevation were compared. The LTT results were also used to classify subjects as positive type, recovery type or negative type using a cut-off value of 3 for the SI.[35] An analysis of variance was conducted to compare the maximum ALT change among the groups.

A t-test was conducted to compare the changes in ALT and endogenous metabolites between the groups. The correlations between the potential biomarkers and liver function parameters were evaluated using Pearson's correlation test. All statistical analyses were performed using SPSS Version 21.0 (IBM Corp., NY, USA). A receiver operating characteristic (ROC) curve was also used to compare the diagnostic performance of the markers to predict liver function change.

RESULTS

Demographics

Thirty-one subjects completed the study. The mean age was 30 years, and all of the subjects were male. The means ± standard deviations (SDs) of the weights and heights of the subjects were 71.9 ± 7.5 kg and 174.3 ± 5.2 cm, respectively. One subject (AN103) dropped out because of adverse events, including headache and vomiting on day 4. A total of 48 adverse events were reported in 24 subjects during the entire study period. No serious adverse event occurred, and the most frequent adverse events were nausea, dyspepsia, and diarrhea, which have commonly been associated with the use of amoxicillin and clavulanate. The demographics and biochemical variables of the baseline results are provided in Tables 3-4. As shown in Table 4, the baseline liver function parameters were within the normal range in every subject.

Table 3. Demographic characteristics of the study subjects

Demographics	Mean ± SD	Range [min-max]	CV (%)
Age (years)	30.0 ± 5.2	20.0 - 40.0	17.3
Weight (kg)	72.2 ± 7.6	59.5 - 88.4	10.6
Height (cm)	174.5 ± 5.2	161.5 183.4	3.0

Table 4. Baseline laboratory test results of the liver function parameters

Parameters	Mean ± SD [range]
ALT (IU/L)	21.0 ± 10.5 [9.0-48.0]
AST (IU/L)	19.4 ± 5.4 [12.0-35.0]
ALP (IU/L)	58.3 ± 13.7 [35.0-85.0]
Total bilirubin	1.0 ± 0.3 [0.5-1.8]
Albumin	4.3 ± 0.2 [4.0-5.0]

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase.

Group classification

Based on the extent of their ALT elevations relative to the baseline, the subjects were classified into 3 groups (Figure 5): (1) Responder (n=6), which had greater than a 2-fold change in ALT at more than 2 sampling points; (2) Non-Responder (n=17), which had less than a 1.5-fold change in ALT at all of the sampling points; and (3) Intermediate (n=8), which had a 1.5- to 2.0-fold change in ALT at all of the sampling points.

Most of the ALT levels were within the normal range (0–40 IU/L). However, in 19.35% (6/31) of the subjects, the ALT levels after drug administration showed more than 2-fold changes above the baseline values (i.e., ALT before drug administration on day 1) at two or more sampling points. Detailed trends of ALT and AST changes are described in Tables 6-9. As described in Tables 7 and 9, the ALT and ALT fold changes showed significant differences between the two groups 8-22 days after the drug administration. We set ALT as the primary standards, and for the analyses of the effects of the factors, we classified the subgroups by HLA genotype (Table 5).

We also classified the groups by the change in microRNA-122 based on the level of microRNA-122 expression relative to baseline. The subjects were classified into 2 groups: Group M1 (the maximum fold change in microRNA122 was >2.0 compared to baseline) and Group M2 (the fold change in microRNA122 was <1.5).

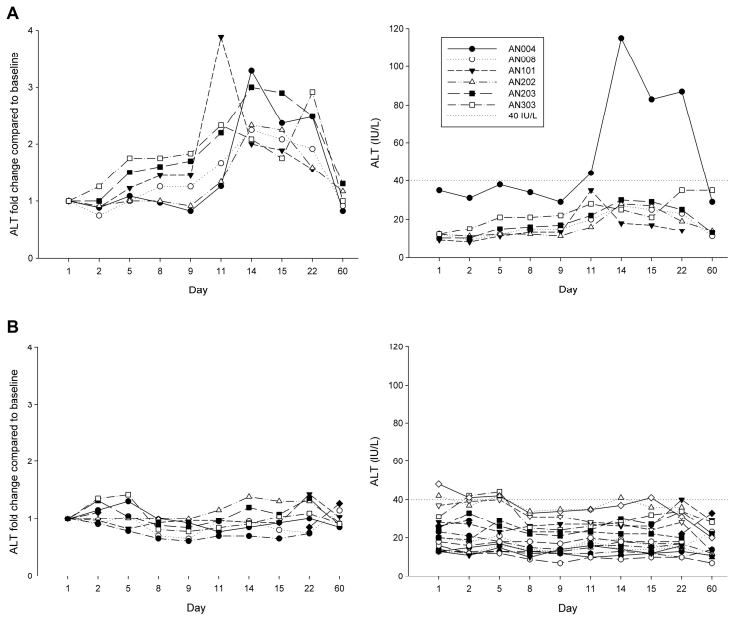


Figure 5. ALT fold changes relative to baseline levels (IU/L) in the subjects over the course of the 60-day study in the (A) Responder group (left: ALT fold change, right: ALT levels) and the (B) Non-Responder group (left: ALT fold change, right: ALT levels).

Table 5. HLA types of the study subjects.

ID	HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DQB1	HLA-DPB1
001	02:01/26:01	40:02/51:01	03:04/14:02	15:01/12:01	03:01/06:02	05:01/09:01
002	33:03/33:03	58:01/58:01	03:02/03:02	03:01/13:02	02:01/06:09	02:01/05:01
003	Assay fail	Assay fail	Assay fail	Assay fail	Assay fail	Assay fail
004	11:01/24:02	51:01/51:01	14:02/15:02	11:01/09:01	03:01/03:03	05:01/05:01
005	02:01/33:03	40:01/67:01	03:03/07:02	16:02:01/12	03:01/05:02	02:02/02:01
006	02:01/11:01	40:01/51:01	07:02/15:02	14:01/08:03	05:02/06:01	02:01/05:01
007	02:01/02:01	44:02/52:01	05:01/12:02	15:02/13:01	06:01/06:03	02:01/09:01
008	02:06/33:03	58:01/59:01	01:02/03:02	13:02/04:05	04:01/06:09	04:02/14:01
101	24:02/31:01	40:01/54:01	01:02/15:02	14:05/04:05	04:01/05:03	05:01/05:01
102	11:01/30:01	13:02/35:01	06:02/08:01	04:03/07:01	02:01/03:02	02:01/05:01
103	02:01/24:02	39:01/52:01	07:02/12:02	15:02/11:06	03:01/06:01	04:02/09:01
104	24:02/24:02	52:01/54:01	01:02/12:02	15:02/04:05	04:01/06:01	04:02/09:01
105	02:06/11:01	44:03/51:01	14:03/14:02	13:02/08:03	06:04/06:01	02:02/04:01
106	26:03/33:03	44:03/51:01	07:01/14:02	14:01/07:01	02:01/05:02	02:01/05:01
107	24:02/33:03	07:02/58:01	03:02/07:02	01:01/08:03	05:01/06:01	04:02/05:01
108	02:01/33:03	40:01/48:01	03:28/08:01	11:01/14:01	03:01/05:03	04:02/05:01
201	02:01/24:02	13:01/27:05	01:02/03:04	01:01/15:01	05:01/06:02	04:01/05:01
202	32:01/33:03	44:02/58:01	03:02/05:01	03:01/12:02	02:01/03:01	02:01/04:01
203	02:01/33:03	44:03:02/48	07:01/08:03	14:05/07:01	02:01/05:03	02:01/13:01
204	11:01/24:02	40:06/56:03	01:02/08:01	04:01/09:01	03:01/03:03	02:01/05:01
205	24:02/33:03	07:02/44:03	07:02/07:01	01:01/07:01	02:01/05:01	04:02/13:01
206	26:01/30:01	13:02/35:01	06:02/08:01	07:01/12:01	02:01/03:03	05:01/17:01
207	26:02:01/31	51:02:01/59	01:02/15:02	04:05/08:02	04:02/04:01	04:02/05:01
208	30:04/31:01	14:01:01/15	04:01/08:02	04:04/04:06	03:02/04:02	05:01/13:01
301	02:06/02:01	40:01/44:02	05:01/15:02	04:05/12:01	03:01/04:01	04:01/05:01
302	02:06/02:01	13:02/15:01	04:01/06:02	04:0/04:06	03:02/04:01	05:01/05:01
303	11:01/26:02	15:01/40:02	03:04/04:01	04:06/09:01	03:02/03:03	02:01/02:01
304	24:02/33:03	55:02/58:01	03:02/12:03	13:02/14:05	05:03/06:09	02:02/05:01
305	02:01/02:01	27:05/39:01	01:02/07:02	01:01/11:01	03:01/05:01	02:01/05:01
306	01:01/33:03	15:18/58:01	03:02/07:04	01:01/07:01	02:01/05:01	02:01/13:01
307	24:02/33:03	44:03:02/51	07:01/14:02	11:01/07:01	02:01/03:01	04:02/13:01
308	02:06/33:03	40:02/44:03	03:04/07:01	14:01/07:01	02:01/05:02	04:02/13:01

Table 6. Difference in the ALT fold change after drug administration between the Non-Responder group and the Responder group.

Day	Non-Responder(n=17)		Responder(n=6)		$t^{2)}$
	M ± SD	$t^{1)}$	M ± SD	$t^{1)}$	
1	1.0 ± 0.0		1.0 ± 0.0		
2	1.0 ± 0.2	0.423	1.0 ± 0.2	0.730	0.417
5	1.1 ± 0.2	-1.726	1.3 ± 0.3	-4.519**	-1.790
8	0.9 ± 0.2	4.567**	1.3 ± 0.3	-1.289	-4.683**
9	0.8 ± 0.2	0.521	1.3 ± 0.4	0.178	-3.944**
11	0.9 ± 0.2	-2.513*	2.1 ± 1.0	-2.355	-4.710**
14	0.9 ± 0.2	-0.237	2.5 ± 0.5	-0.696	-10.191**
15	0.9 ± 0.2	1.343	2.2 ± 0.4	2.154	-10.391**
22	1.0 ± 0.3	-1.423	2.2 ± 0.6	0.169	-7.101**
60	0.9 ± 0.3	1.838	1.1 ± 0.2	4.624*	-1.857

$t^{1)}$: t-test statistics on paired samples t-test, $t^{2)}$: t-test statistics on independent two-sample t-test, $p<0.01$:**, $p<0.05$:*

Table 7. Difference in the AST fold change after drug administration between the Non-Responder group and the Responder group.

Day	Non-Responder(n=17)		Responder(n=6)		$t^{2)}$
	M ± SD	$t^{1)}$	M ± SD	$t^{1)}$	
1	1.0 ± 0.0		1.0 ± 0.0		
2	1.0 ± 0.2	0.111	1.0 ± 0.2	-0.449	-0.440
5	1.0 ± 0.1	-1.255	1.1 ± 0.1	-1.021	-0.570
8	1.0 ± 0.1	2.882*	1.1 ± 0.2	-0.196	-1.600
9	0.9 ± 0.2	3.406**	1.1 ± 0.2	0.127	-2.305*
11	1.0 ± 0.2	-3.286**	1.4 ± 0.4	-2.291	-3.096**
14	1.0 ± 0.2	-0.853	2.0 ± 1.1	-1.116	-3.508**
15	0.9 ± 0.2	3.257**	1.4 ± 0.2	1.430	-5.682**
22	1.0 ± 0.2	-2.540*	1.4 ± 0.3	0.653	-3.172**
60	0.9 ± 0.2	0.893	1.0 ± 0.2	1.976	-0.808

$t^{1)}$: t-test statistics on paired samples t-test

$t^{2)}$: t-test statistics on independent two-sample t-test

$p<0.01$:**, $p<0.05$:*

Table 8. Difference in the ALT after drug administration between the Non-Responder group and the Responder group.

Day	Non-Responder(n=17)		Responder(n=6)		$t^{2)}$
	M ± SD	$t^{1)}$	M ± SD	$t^{1)}$	
1	23.5 ± 10.8		15.0 ± 9.9		1.702
2	23.4 ± 11.3	0.164	14.0 ± 8.7	1.000	1.832
5	24.3 ± 11.5	-1.348	18.2 ± 10.4	-4.580**	1.141
8	19.5 ± 8.3	3.773**	18.5 ± 8.2	-0.337	0.252
9	19.3 ± 8.8	0.510	17.8 ± 6.6	0.725	0.373
11	20.9 ± 7.9	-2.552*	27.5 ± 10.5	-3.288*	-1.606
14	21.4 ± 9.3	-0.816	40.5 ± 36.7	-1.052	-1.986
15	20.9 ± 9.4	0.929	33.7 ± 24.5	1.352	-1.816
22	22.1 ± 9.7	-1.005	33.8 ± 27.0	-0.052	-1.578
60	18.8 ± 8.0	2.001	18.8 ± 12.2	1.933	0.013

$t^{1)}$: t-test statistics on paired samples t-test

$t^{2)}$: t-test statistics on independent two-sample t-test

$p<0.01$:**, $p<0.05$:*

Table 9. Difference in AST after drug administration between the Non-Responder group and the Responder group.

Day	Non-Responder(n=17)		Responder(n=9)		$t^{2)}$
	M ± SD	$t^{1)}$	M ± SD	$t^{1)}$	
1	20.6 ± 5.8		16.0 ± 3.1		2.197
2	20.3 ± 6.2	0.329	17.0 ± 4.2	-1.200	1.430
5	21.4 ± 4.7	-0.996	17.7 ± 3.9	-0.667	2.033
8	20.0 ± 4.7	2.785*	17.6 ± 3.8	0.130	1.329
9	18.3 ± 4.4	3.656**	16.3 ± 2.0	1.417	1.224
11	20.1 ± 3.9	-3.478**	21.3 ± 3.7	-3.354**	-0.752
14	20.8 ± 4.2	-1.000	28.9 ± 21.2	-1.072	-1.495
15	19.3 ± 4.1	3.236**	20.6 ± 6.8	1.571	-0.606
22	20.4 ± 4.2	-2.227*	29.9 ± 21.2	-1.253	-1.805
60	19.1 ± 3.8	1.145	17.2 ± 4.5	1.529	1.019

$t^{1)}$: t-test statistics on paired samples t-test

$t^{2)}$: t-test statistics on independent two-sample t-test

$p<0.01$:**, $p<0.05$:*

Correlation of PK parameters with ALT elevations

Comparative analyses between the *Responder* and *Non-Responder* groups revealed no statistically significant differences in the primary PK parameters of amoxicillin or clavulanate, including $AUC_{0\text{-}12h}$, $AUC_{last,ss}$ and C_{max} (Figure 6, Table 10), which indicates that the nature of idiosyncratic DILI that is induced by amoxicillin and clavulanate may not be related to the dose or the drug pharmacokinetics.

In addition, there were also no significant differences in the mean maximum ALT fold change among the groups that were classified by GSTT1/GSTM1 genotype (Figure 7).

Table 10. Pharmacokinetic parameters of amoxicillin/clavulanate after a single dose and in the steady state on day 8 (n=31).

Criteria		N	Amoxicillin				Clavulanate		
			$C_{max,ss}$ (ng/mL)	$AUC_{0\text{-}12h}$ (h·ng/mL)	$AUC_{ss,\tau}$ (h·ng/mL)	Metabolic ratio	$C_{max,ss}$ (ng/mL)	$AUC_{0\text{-}12h}$ (h·ng/mL)	$AUC_{ss,\tau}$ (h·ng/mL)
Total	Total	31	5218.3 ± 1418.7	21453.6 ± 4380.9	18190.8 ± 5154.3	0.07	3387.2 ± 1389.8	3009.2 ± 4654.4	8457.7 ± 3855.2
GSTT1/M1	Wild/wild	8	5395.9 ± 1372.3	23949.6 ± 4082.2	20248.4 ± 3891.4	0.07	3411.4[ab] ± 1491.5	7904.7 ± 6023.3	9300.8 ± 4348.6
	Wild/null	7	4622.9 ± 734.5	19272.5 ± 3992.3	18842.5 ± 3925.8	0.08	3007.0[b] ± 1105.9	6363.7 ± 3635.1	9291.7 ± 4907.2
	Null/wild	8	4581.0 ± 1073.3	20503.0 ± 4757.4	17693.1 ± 4179.7	0.06	2502.1[b] ± 806.9	6106.6 ± 2898.7	6536.2 ± 2112.4
	Null/null	8	6199.1 ± 1781.7	21552.6 ± 4283.3	18229.8 ± 3191.3	0.07	4530.5[a] ± 1439.5	11782.4 ± 3978.5	8363.6 ± 3778.4
ALT elevation	*Group 1*	6	5180.2 ± 851.3	22801.4 ± 3424.5	18670.6 ± 2278.4	0.07	2893.2 ± 900.4	7497.5 ± 3285.6	7256.9 ± 2157.9
	Group 2	17	5217.5 ± 1260.7	20154.2 ± 4549.6	17274.5 ± 6556.6	0.08	3686.9 ± 1457.8	8803.8 ± 4990.7	8990.2 ± 4160.1
	Group 3	8	5289.6 ± 1265.4	20211.3 ± 4692.5	18354.2 ± 4971.7	0.07	3828.6 ± 3450.6	8921.9 ± 5129.8	9552.1 ± 3568.7

All data are presented as arithmetic means ± standard deviation. *P* values were calculated from analysis of variance (ANOVA).
Metabolic ratio (Amoxicillin diketopiperazine/amoxicillin); $C_{max,\ ss}$, maximum plasma concentration at steady state; $AUC_{0–12h}$, area under the plasma concentration-time curve from 0 h to 12 h on day 1; $AUC_{ss,\tau}$, area under the plasma concentration-time curve over the dosing interval at steady state; GSTT1/M1, glutathione s-transferase theta 1/mu 1; ALT, aminoalanine transferase. [ab] Global comparisons estimated with ANOVA and *post hoc* comparisons analyzed using Bonferroni's test

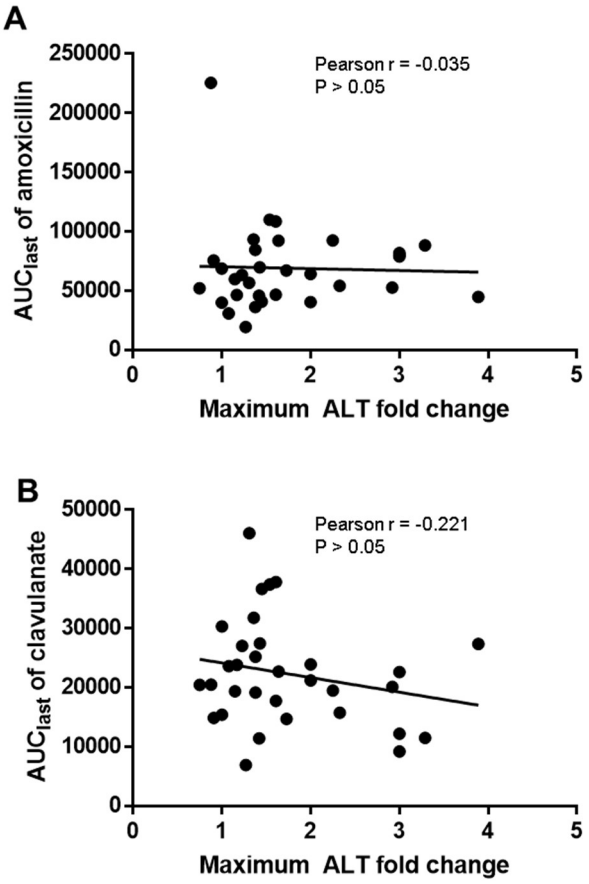


Figure 6. Correlation between the area under the time-concentration curve from zero to the last time points (AUC) of (a) amoxicillin and (b) clavulanate and ALT change

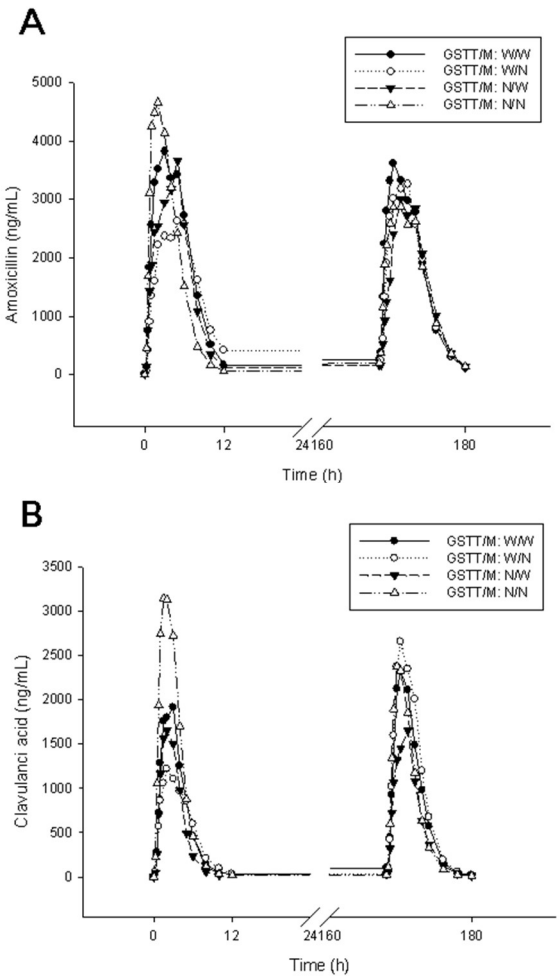


Figure 7. Mean plasma concentration-time profiles of (a) amoxicillin and (b) clavulanic acid by GSTT1/M1 genotype. GSTT, glutathione S-transferase T; GSTM, glutathione s-transferase mu; W/W, wild/wild; W/N, wild/null; N/W, null/wild; N/N, null/null.

Correlation between miR-122 and ALT elevation

We measured the serum levels of miR-122, which has been proposed as an alternative biomarker of DILI. After drug administration, the miR-122 levels exhibited a similar increasing trend to that of the ALT levels (Pearson's *r* of 0.613–0.923, $P<0.05$) but to a larger extent. Figure 8 shows these results for both the *Responder* and *Non-Responder* groups. As shown in Figure 9, the correlation analysis of pooled data for the miR-122 fold-change and ALT fold-change also showed statistical significance after amoxicillin/clavulanate administration (Pearson's r value = 0.624, $P=0.003$).

The miR-122 levels tended to increase on day 8, which was earlier than the increase in the ALT levels that were first noted on day 14 (Table 11). We found significant correlations between the serum miR-122 and ALT fold changes (Pearson's *r* of 0.613–0.923, $P<0.05$, Table 12). As described in Table 13-14, the between group tests indicated that there the variable group was significant ($P<0.05$, RM-ANOVA). On the other hand, the within subject test indicated that there was no significant time effect.

Table 11 Descriptive statistics of miR-122 fold changes throughout the study

Day	Group	Mean	SD
1	Responder	1.00	0.00
	Non-Responder	1.00	0.00
	total	1.00	0.00
2	Responder	1.06	0.62
	Non-Responder	0.77	0.53
	total	0.93	0.56
5	Responder	1.66	0.30
	Non-Responder	0.56	0.27
	total	1.19	0.64
8	Responder	1.96	1.14
	Non-Responder	0.38	0.21
	total	1.28	1.17
9	Responder	2.17	1.64
	Non-Responder	0.39	0.29
	total	1.41	1.51
14	Responder	7.33	5.45
	Non-Responder	0.86	0.88
	total	4.56	5.20
22	Responder	2.01	0.95
	Non-Responder	0.60	0.38
	total	1.40	1.03
60	Responder	1.18	0.70
	Non-Responder	0.70	0.44
	total	0.97	0.61

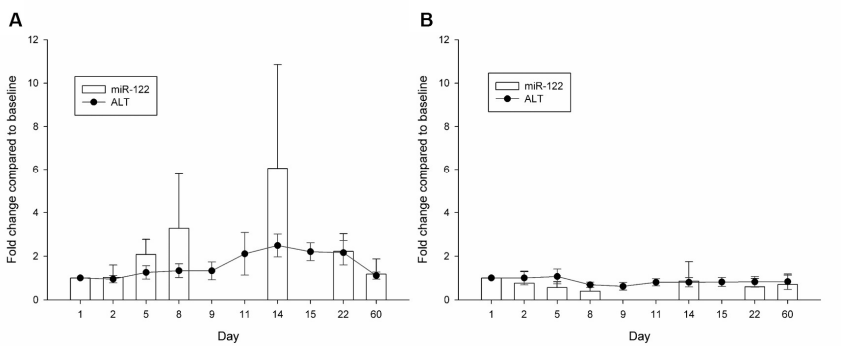


Figure 8. Time courses depicting the serum miR-122 and ALT fold changes in the (A) Responder and (B) Non-Responder groups. The data represent the mean ± standard deviation.

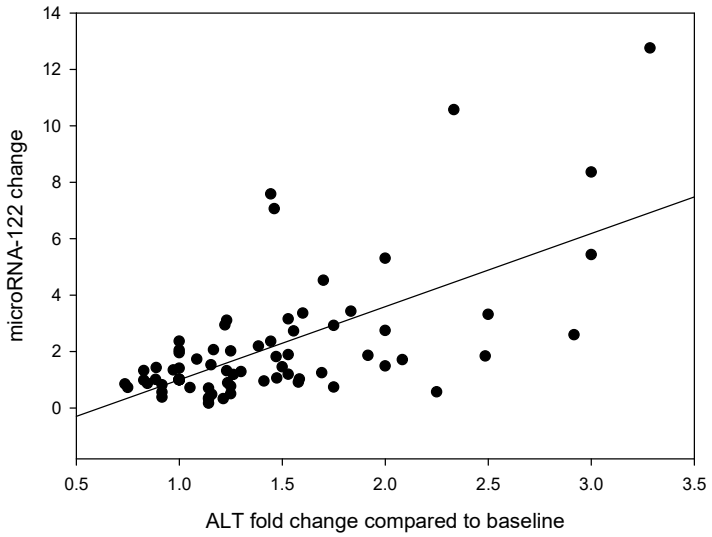


Figure 9. Correlations between serum miR-122 fold-change and ALT fold-change. Pearson's r value was 0.624 (P=0.003) after amoxicillin/clavulanate administration

Table 12. Correlations between ALT changes and microRNA-122 (miR-122) changes throughout the study.

ALT change	miR-122 change					
	Day 2	Day 5	Day 8	Day 14	Day 22	Day 60
Day 2	-0.024	-0.203	-0.142	-0.163	0.051	-0.326
Day 5	-0.117	0.401	0.384	-0.062	0.350	0.264
Day 8	-0.018	0.491	0.705*	0.123	0.319	0.234
Day 9	0.024	0.397	0.713*	0.068	0.249	0.138
Day 11	0.145	0.707*	0.923**	0.189	0.284	0.257
Day 14	0.133	0.241	0.434	0.743**	0.251	0.098
Day 15	0.190	0.255	0.520	0.688**	0.234	0.067
Day 22	0.106	0.005	0.205	0.336	0.624**	0.082
Day 60	-0.030	0.341	0.543	0.343	0.171	0.606**

**$P<0.01$, *$P<0.05$; Pearson correlation test.

Table 13. Tests results of between-subject effects

Source	Type III Sun of Squares	Degree of freedom	Mean Square	F	Sig.
intercept	119.516	1	119.516	34.430	.002
Group	36.681	1	36.681	10.567	.023
Error	17.357	5	3.471		

Table 14. Test results of within-subjects effects

Source		Type III Sun of Squares	Degree of freedom	Mean Square	F	Sig.
Time	Sphericity Assumed	54.811	7	7.830	2.955	0.015
	Greenhouse-Geisser	54.811	1.189	46.106	2.955	0.136
	Huynh-Feldt	54.811	1.659	33.045	2.955	0.112
	Lower-bound	54.811	1.000	54.811	2.955	0.146
Time*group	Sphericity Assumed	50.662	7	7.237	2.732	0.023
	Greenhouse-Geisser	50.662	1.189	42.616	2.732	0.150
	Huynh-Feldt	50.662	1.659	30.543	2.732	0.127
	Lower-bound	50.662	1.000	50.662	2.732	0.159
Error (time)	Sphericity Assumed	92.736	35	2.650		
	Greenhouse-Geisser	92.736	5.944	15.602		
	Huynh-Feldt	92.736	8.293	11.182		
	Lower-bound	92.736	5.000	18.547		

Localization of miR-122

We next performed serum exosomal microRNA analysis to determine whether amoxicillin/clavulanate-induced ALT elevation and miR-122 changes were associated with hepatocellular liver damage or liver inflammation. The results showed that the levels of miR-122 in both exosome-rich and protein-rich fractions were substantially increased, as described in Figure 10.

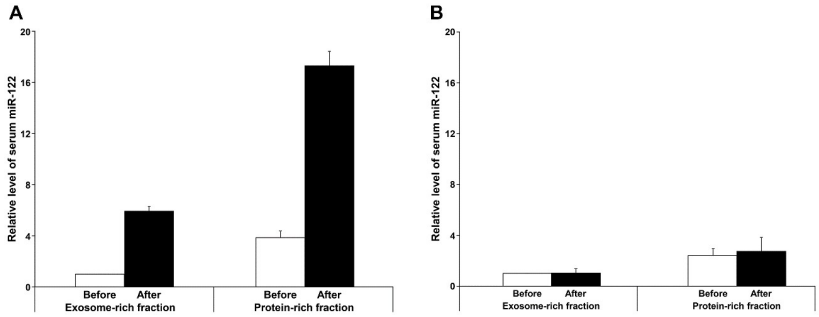


Figure 10. Changes in miR-122 levels in the exosome-rich and non-exosome rich fraction (protein-rich fraction) separated from the Non-Responder serum (a) and Responder serum (b). In the Responder group, the miR-122 levels massively increased in both fractions at peak ALT time points.

Pharmacometabolomic analyses

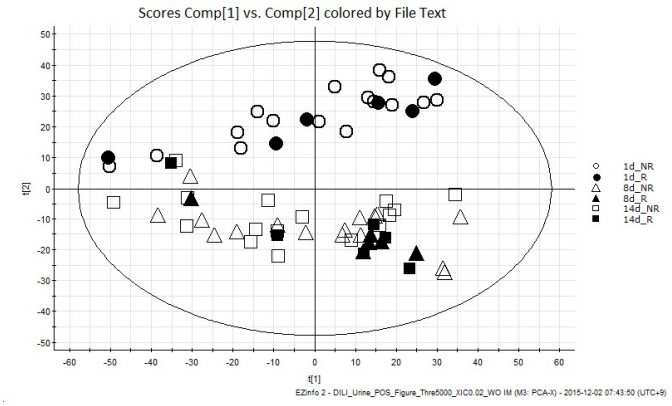
A total of 5169 peaks in electrospray ionization in the positive ion mode (ESI+) and 4261 peaks in the negative ion mode (ESI-) were detected after peak alignment and were subsequently imported into the EZinfo software. According to the OPLS-DA model (Figure 11-13), the concentrations of 5 urinary metabolites (variable influence on the projection > 5) were significantly different in the *Responder* and *Non-Responder* groups (Table 15). The identified urinary markers 7-methylxanthine, 7-methyluric acid, 3-methylxanthine, acetylcarnitine, and azelaic acid were quantified using QuanLynx (Waters Corp.) and normalized to the creatinine concentration.

Four of the urinary metabolites showed significant changes between days 1 and 8 after amoxicillin/clavulanate administration in the *Responder* group but not in the *Non-Responder group* (Figure 14). Although the urinary metabolite levels showed large inter-individual variations, the levels of 7-methylxanthine, 7-methyluric acid and 3-methylxanthine in the *Responder* group on day 8 were significantly higher (3.3, 3.9 and 2.5 times, respectively) than in the *Non-Responder* group, whereas the azelaic acid level was significantly lower (0.4 times; Figure 4).

We also used ROC curves to compare the diagnostic performance of 5 pharmacometabolites (Table 16, Figure 15). This curve and the corresponding AUC show that 3-methylxantine as an early biomarker has predictive ability to discriminate possibility of liver function change. (the AUC was 0.8)

In addition, we also evaluated the metabolites based on the miR-122 group classification (Group M1, Group M2). From our evaluation, the R2 and Q2 of every marker were lower than 0.5 and 0.1, respectively, indicating there was no significant marker.

(a)



(b)

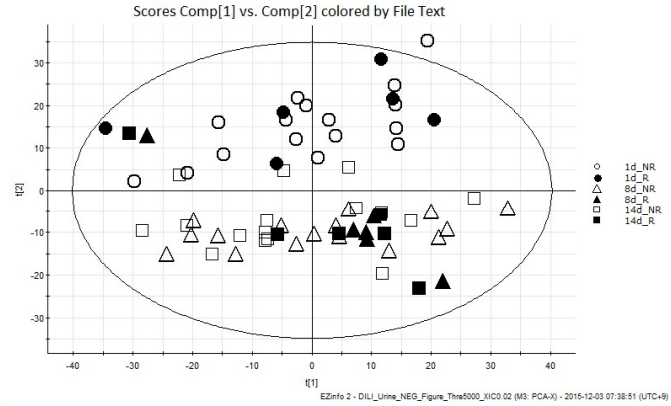
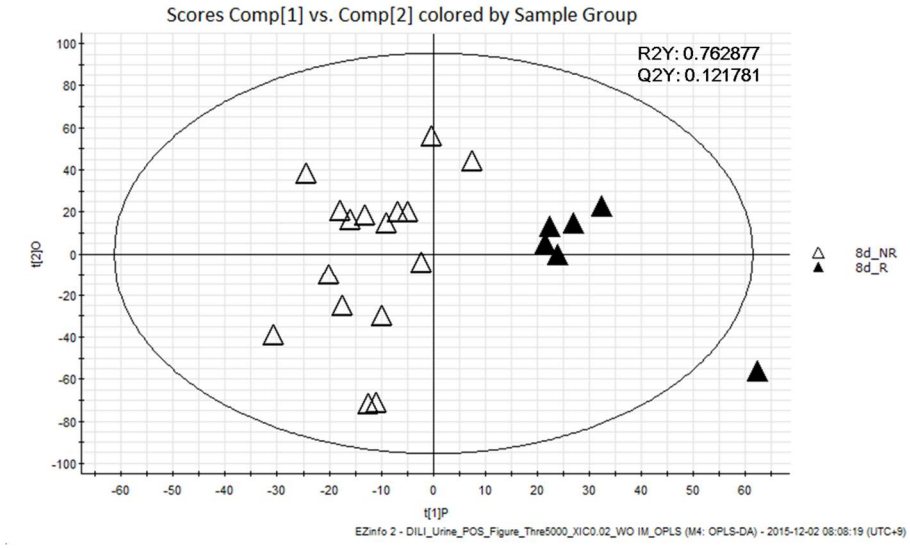


Figure 11. Principal component analysis (PCA) plot of individual samples of 12-hour interval urine in three periods in the (a) positive ion mode and (b) negative ion mode.

(a)



(b)

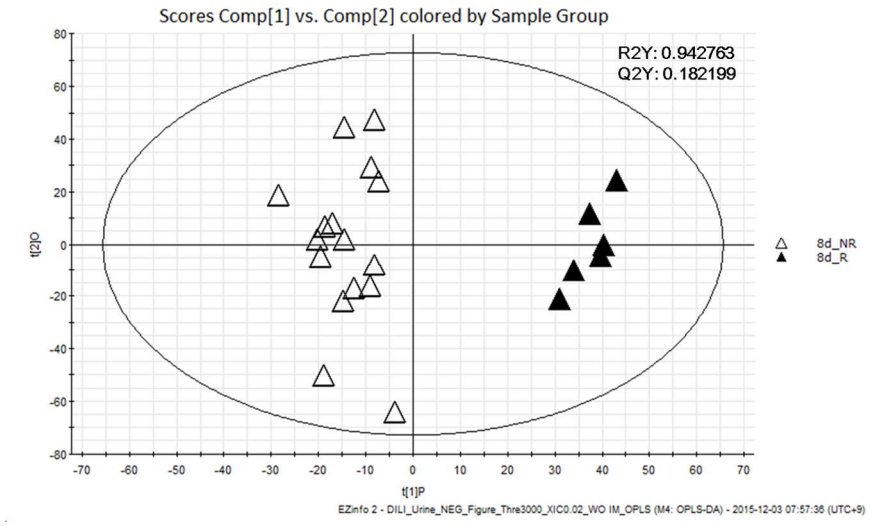
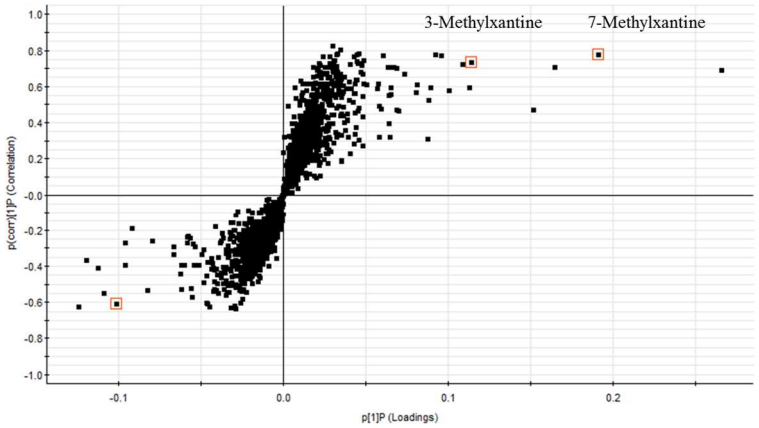


Figure 12. Orthogonal PLS (OPLS-DA) plot of individual samples of 12-hour interval urine in three periods in the (a) positive ion mode and (b) negative ion mode.

(a)



(b)

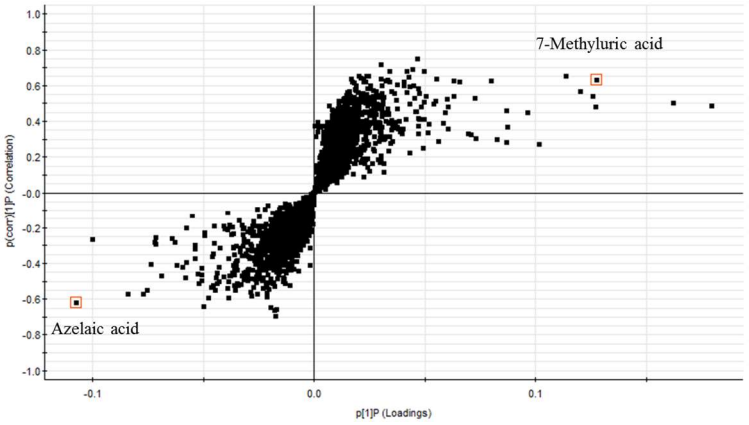


Figure 13. S-plot corresponding to the OPLS-DA analysis for the new model built on samples in the (A) positive ion mode and (B) negative ion mode

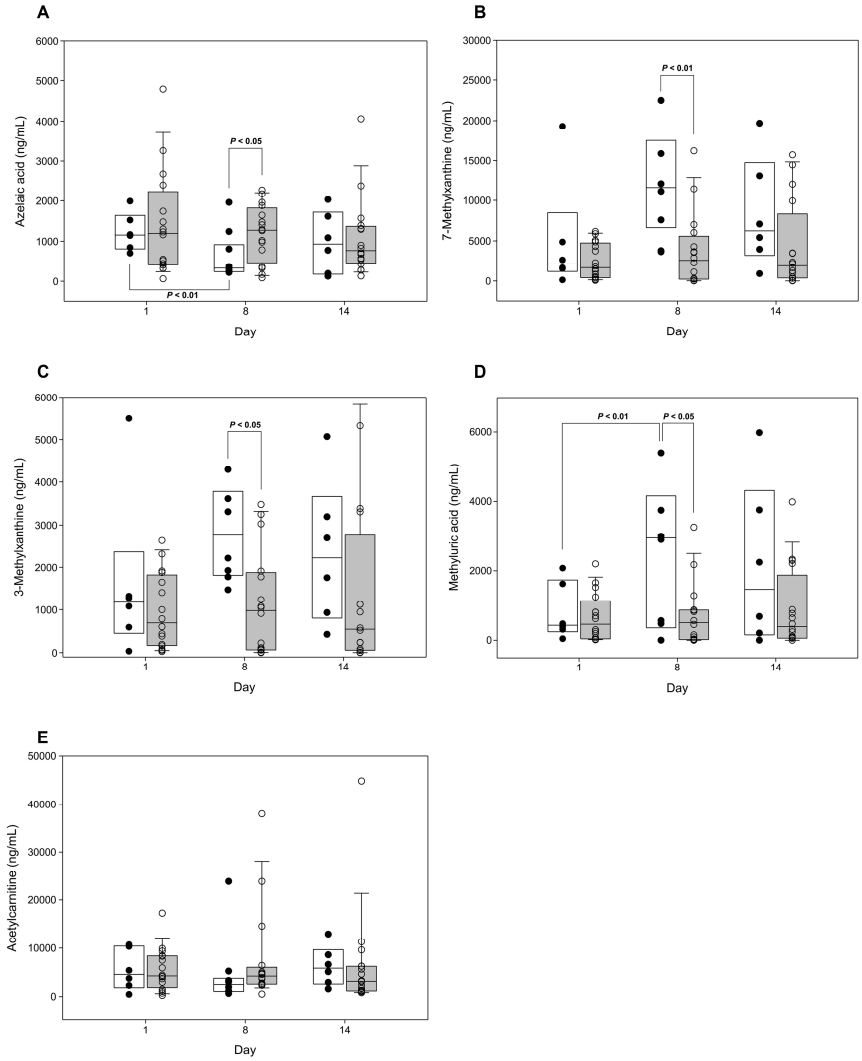


Figure 14. Box-whisker plots for significantly regulated endogenous metabolites. (A) azelaic acid, (B) 7-methyluric acid, (C) 3-methylxanthine, (D) 7-methylxanthine and (E) acetylcarnitine on days 1, 8, and 14 (white box: Responder; gray box: Non-Responder).

Table 15. Urinary metabolites that were significantly different between the Responder and Non-Responder groups at day 8 after amoxicillin/clavulanate administration

Metabolite	VIP	*Responder*			*Non-Responder*			Ratio of Metabolites
		Baseline	Day 8	Day 14	Baseline	Day 8	Day 14	
Azelaic acid	5.68	1227.9 ± 468.4	530.3 ± 409.3	971.5 ± 762.1	1415.2 ± 1298.1	1191.7 ± 706.2	1084.9 ± 983.6	0.4*
7-Methylxanthine	11.78	5020.6 ± 7165.8	12159.2 ± 6520.7	8352.7 ± 6863.8	2441.2 ± 2203.0	3703.3 ± 4612.0	4277.7 ± 5458.8	3.3*
3-Methylxanthine	7.67	1631.5 ± 955.3	2802.6 ± 1096.9	2343.3 ± 1691.5	968.0 ± 876.2	1137.1 ± 1226.6	1494.5 ± 2132.8	2.5*
7-Methyluric acid	8.71	944.5 ± 076.4	2759.1 ± 1799.7	2489.4 ± 2122.3	650.3 ± 681.0	712.3 ± 905.9	897.0 ± 1176.2	3.9*
Acetylcarnitine	5.93	5490.0 ± 221.6	2570.5 ± 1654.6	6299.0 ± 4098.6	5593.9 ± 4417.5	7724.3 ± 9887.3	6502.1 ± 10705.5	0.3

*$P<0.05$ by t-test statistics using an independent two-sample t-test between the *Responder* and *Non-Responder* averages on day 8; Ratio of endogenous metabolites, ratio for a given metabolite (*Responder*/*Non-Responder*) assayed on day 8; VIP, variable influence on the projection.

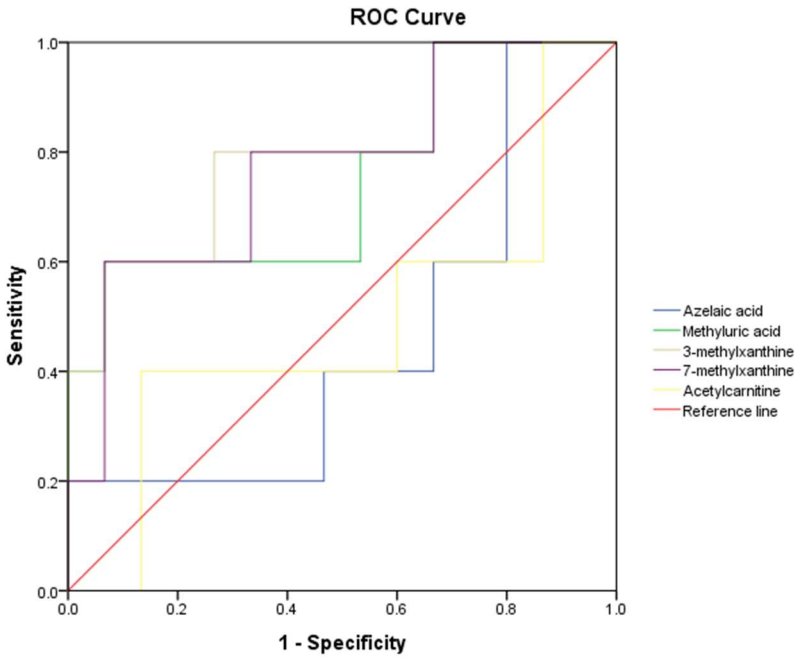


Figure 15. Receiver operating characteristic (ROC) curve of 5 urinary metabolites for predicting liver injury

Table 16. Results of the ROC analysis

Variables	AUC	Standard error[a]	Significant probability	95% confidence interval	
				Upper	Lower
Azelaic acid	0.453	0.158	0.760	0.143	0.763
MU	0.747	0.142	0.106	0.469	1.000
3-MX	0.800	0.126	0.050	0.554	1.000
7-MX	0.773	0.128	0.074	0.523	1.000
Acetylcarnitine	0.480	0.166	0.896	0.154	0.806

MU, methyluric acid; MX, methylxanthine

Lymphocyte proliferation against amoxicillin and clavulanate

The LTT was performed on three different visits (day 1, days 20-22, and day 60) to determine the lymphocyte response against amoxicillin and clavulanate. Based on the LTT results, for which SI values above 3 were considered positive, amoxicillin appeared to transiently induce lymphocyte proliferation at 20-22 days after administration in four subjects (AN006, AN008, AN102, AN207), although the proliferation levels had returned to a normal range on day 60. After clavulanate stimulation, a strong proliferative response was observed in two subjects (AN004 and AN203) on day 60. The lymphocyte response of one subject (AN004) appeared to follow a similar process to that of a patient with AC-DILI (Figure 16) with a very strong proliferative response to clavulanate and enhanced IFN-γ release from lymphocytes after clavulanate stimulation. The biomarkers showed that there were metabolic changes on day 8, increased miR-122 levels on day 9, increased enzyme activity, and then an enhanced proliferative response to clavulanate.

HLA genotyping

We hypothesized that there would be a higher incidence of ALT change in subjects with HLA types that are known risk factors for DILI compared to subjects with HLA types that are known protective factors. We classified HLA as risky and protective type based on previous studies (Table 17) [27,36,37]. However, we could not find an association between ALT changes and these genotypes in our genotypes as described in Table 18. This result may have been related to the limited sample size.

Table 17. Classification of the HLA genotype

Group	Genotype
Risky	HLA-DRB1*1505, DRB1*0301, DRB1*0401, DRB2*1301, DRB2*1501
Protective	HLA-DRB1*1701 and DRB2*0701

Table 18. Study subjects grouped by ALT fold change and HLA group

ALT group	Responder (n=10)	Non-responder (n=17)	Intermediate (n=5)
Susceptible (n=6)	1	5	0
Protective (n=8)	4	4	0
NA (n=18)	5	8	5

NA, not applicable; HLA, human leukocyte antigen

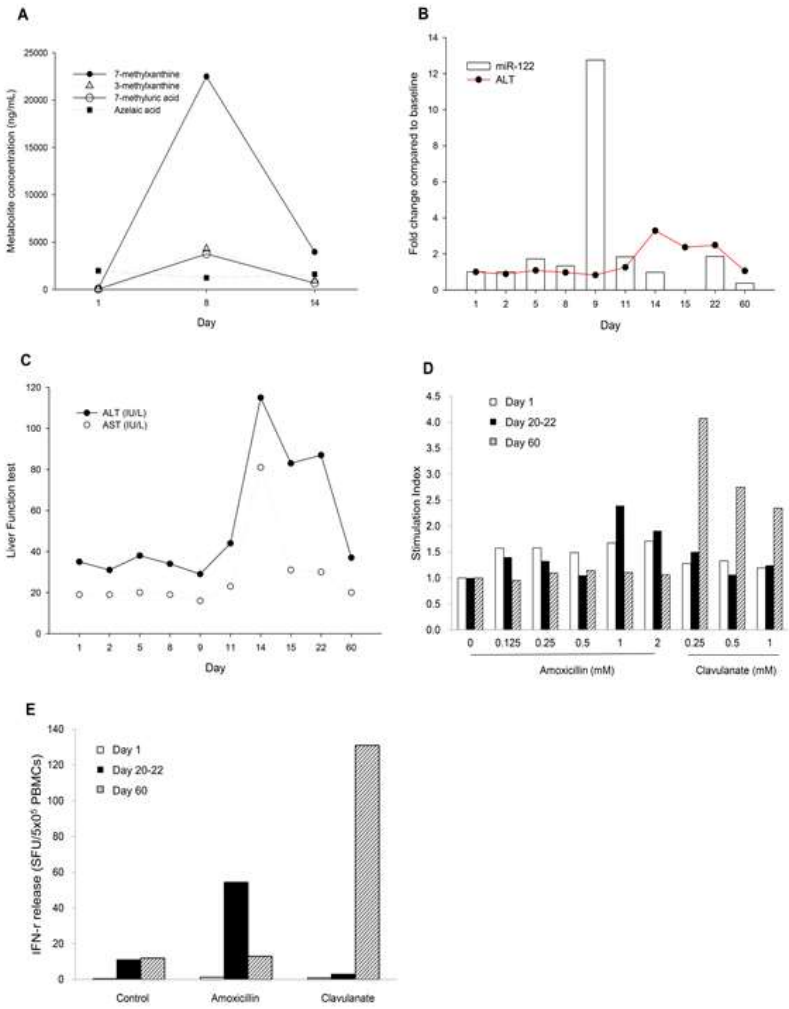


Figure 16. Time courses depicting changes in (A) urinary metabolites, (B) miR-122, (C) ALT and AST, (D) lymphocyte proliferation, and (E) IFN-γ release in subject AN004.

DISCUSSION

Given the heterogeneous and complex nature of DILI, we used multidirectional approaches to identify and evaluate biomarkers in a well-controlled clinical trial in healthy subjects.

Consistent with previous findings,[15] the liver-specific miR-122 response was highly correlated with ALT changes. Our results demonstrated that the miR-122 levels changed more dramatically than ALT, which indicates that miR-122 is more sensitive to liver injury. The miR-122 also began to increase earlier than ALT, which demonstrates the potential of miR-122 as an early marker of AC-DILI.

We used a pharmacogenomics approach combined with immunologic concepts to evaluate GSTT1/M1 genotypes and HLA types as risk factors for DILI. We hypothesized that there would be a higher incidence of ALT elevation in subjects with the GSTT1/M1 null/null genotype[38] or with specific HLA types that are known risk factors for DILI[38]. However, we did not find an association between ALT changes and these genotypes. This result may have been related to the limited sample size or to slight elevations in ALT.

We also used a metabolomics platform to investigate the metabolomic signatures of amoxicillin/clavulanate exposure in healthy volunteers as predictors of AC-DILI. Although the levels of urinary metabolites showed large inter-individual variations, we identified 4 urinary markers with the

potential to predict ALT elevation: azelaic acid was significantly down-regulated, whereas 7-methylxanthine, 3-methylxanthine and 7-methyluric acids were significantly up-regulated.

Azelaic acid is known to scavenge radicals and modulate the inflammatory response in human keratinocytes.[39] A recent study in mice demonstrated that exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced oxidative stress in the liver and decreased azelaic acid levels.[40] The azelaic acid levels changed even with low doses of TCDD, which did not significantly increase the serum ALT or AST levels. Therefore, azelaic acid was suggested to be an early indicator of liver damage that is related to mitochondrial oxidative stress. Interestingly, 7-methylxanthine also appears to be related to oxidative stress. It is metabolized in the liver by xanthine oxidase to yield 7-methyluric acid, which generates reactive oxygen species (ROS) in the liver,[41] which indicates that these metabolites are involved in drug-induced oxidative stress in the liver.[42,43] The metabolomics results thus support the conclusion that mitochondrial oxidative stress could be another mechanism that underlies AC-DILI and it could be also supported by the change of microRNA in exosome fraction.

The immune basis of AC-DILI has been supported by the delayed onset, individual susceptibility, HLA association and presence of drug-specific T cells in patients with AC-DILI.[27,28] The technologically demanding LTT assay was used here as an in vitro testing method for detecting drug-responsive T cells.[44] As expected because of the low incidence of DILI, we

observed that a small number of subjects showed a positive response to the drug. The lymphocyte response to amoxicillin appears to be transient and unrelated to changes in ALT, miR-122 or other metabolic markers, which indicates that it may be an asymptomatic response. However, the positive lymphocyte response to clavulanate in one subject (AN004) appears to be related to AC-DILI because we found a similar sequence of metabolic, biochemical and biological changes with early metabolic changes detected on day 8 and increased miR-122 levels on day 9 followed by increased enzyme activity (ALT/AST) and finally by an enhanced proliferative response to clavulanate. Overall, this finding indicates that one biomarker alone cannot distinguish between the various etiologies of DILI, which supports the need for multi-omics approaches to identify a prognostic biomarker for DILI.

We also attempted to define the DILI mechanism by examining the levels of circulating miR-122. MicroRNAs can circulate in the blood either bound to proteins (e.g., Argonaute, lipoprotein) or packaged in extracellular vesicles such as exosomes and microvesicles.[45,46] A recent study reported that circulating miRNAs are associated with either the exosome-rich or the protein-rich fraction depending on the type of liver injury.[16,47] Circulating miR-122 in the exosome-rich fraction is more related to inflammation. On the other hand, the miR-122 in the protein-rich fraction is known to be associated with hepatocyte injury induced by necrosis.[16] Our results showed that miR-122 increased by 7.2- and 5.2-fold in the exosome-rich and protein-rich fractions, respectively (Supplemental Figure S3), which indicates that amoxicillin and

clavulanate induce a mixed-type liver injury. From these results of the localization of microRNA-122 and metabolites, we could identify the potential role of inflammation in DILI.

A major limitation of this study is the relatively small number of subjects, which decreases the statistical power. Another limitation is in the study subjects themselves. In general, DILI occurs more frequently in elders and females,[24] while this study was performed with healthy male volunteers, not with DILI patients. Realistically, applying amoxicillin/clavulanate to relatively vulnerable groups, such as elders and females, may be problematic because of subject safety and ethical issues. It has also been reported that while the frequency of DILI differs depending on gender and age, the manifestations of DILI do not differ significantly.[48] Considering these factors, we assumed that potential biomarkers that are identified in males could represent those present in all populations. Finally, our study was limited by the fact that the levels of transaminases such as ALT, which we used as an evaluation marker, may change depending on diet or alcohol intake.[49,50] However, we limited alcohol consumption during the study period by performing alcohol air breathing tests upon hospitalization to exclude the possibility of ALT elevation by alcohol. We also attempted to minimize interfering factors other than drugs that could affect liver function by limiting the intake of any prescribed drugs and high-carbonate or high-lipid diets beginning 2 weeks before the test.

Nevertheless, to the best of our knowledge, this study is the first

prospective clinical trial that has been performed on healthy subjects with antibiotic-induced DILI that is based on multi-omics tools. Previously, DILI biomarker explorations typically were retrospective studies on DILI patients. Consequently, the significance of this study is the detection of altered biomarker levels upon slight changes in liver function under well-controlled normal conditions or subclinical conditions.[51] However, a control group of either the same subjects or different subjects not taking the antibiotic would have added validity to the changes in transaminases.

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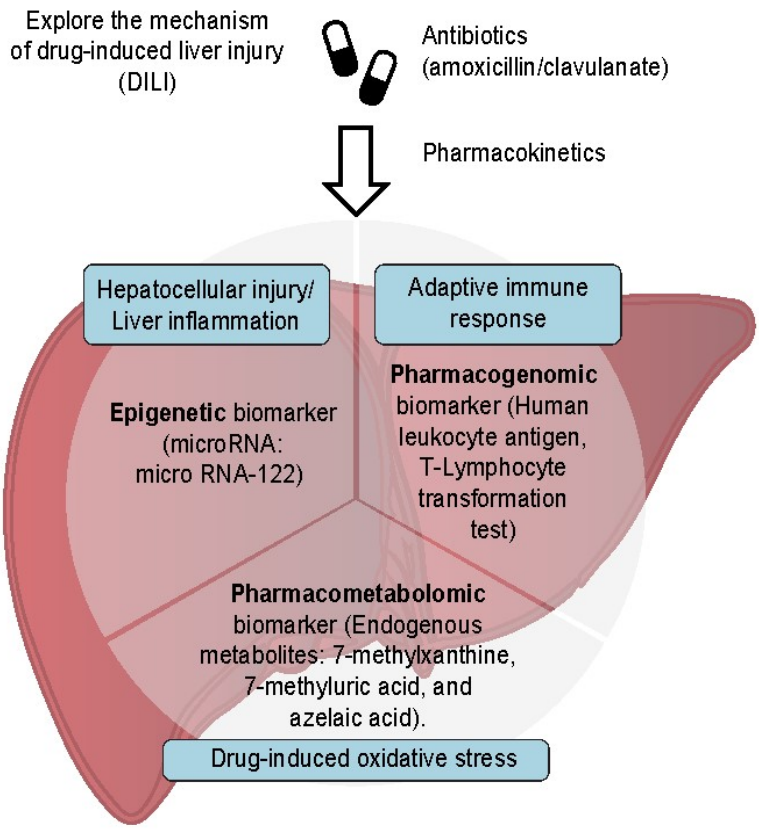

Figure 17. Study Summary

CONCLUSION

We evaluated and identified potential DILI biomarkers based on currently known AC-DILI mechanisms. Our study identifies miR-122 and four urinary metabolites as early and sensitive biomarkers for AC-DILI and the potential role for an adaptive immune response to the drug as a possible mechanism underlying AC-DILI. Further confirmatory studies that demonstrate and validate the clinical benefits of these markers for use in bedside applications should be performed.

Furthermore, we concluded that comprehensive multi-omics approaches can be employed to evaluate prognostic biomarkers by integrating omics data with clinical investigations. This study provides potential biomarkers for AC-DILI based on currently known AC-DILI mechanisms.

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국문 초록

서론: 약물유발간손상은 약물 개발 및 약제 사용에 있어 주요한 걸림돌 중 하나이다. 약물유발간손상이 여러 기전에 의해서 발생함을 고려하여, 이에 대한 보다 더 민감한 생체지표에 대한 탐색은 다각적인 접근이 필요하다. 그러나, 현재까지는 건강 자원자에서 체계적인 임상 연구가 이루어져 있지 않은 상황이다. 본 연구에서는 아목시실린/클라불란산 유발 간 손상 (AC-DILI)에 대한 가능성 있는 바이오마커 및 기전을 찾고자, 멀티-오믹스 방법론에 기반한 임상시험을 수행하였다.

방법: 전체 32명의 건강한 한국인 남성 자원자가 4개의 GSTT1/M1 유전형 (군당 각각 8명)에 따라 임상시험에 참여하였다. 시험대상자에게 14일간 아목시실린/클라불란산을 투여한 다음 수집된 혈액과 뇨 시료에서, 간기능 검사 및 바이오마커 정량을 진행하였다. 이 때, 본 연구에서는 간 기능 지표 검사, microRNA 정량, 약리대사체학 분석, 사람백혈구항원 (HLA 항원) 유전형 검사, 림프구 변이 검사 (LTT) 및 약동학 분석 등의 다양한 오믹스 분석 방법이 사용되었다.

결과: 알라닌아미노전달효소 (ALT)에 상승 정도에 따라 나누어진 군인 반응군과 비반응군으로 나누어 비교 분석이 진행되었으며, 해당 군간에는 아목시실린이나 클라불란산의 약동학적 지표에는 통계적인 차이를 보이지 않았다. 간 특이 microRNA-122 (miR-122)의 경우 ALT와 매우 상관성 있는 변화 추이를 보였다. 투약 후 7-methyxanthine, 7-methyluric acid, 3-methylxanthine 및 azelaic acid 등의 뇨 대사체의 경우 두 군간에 유의한 차이를 보였다 (P <0.05). 약물 투약 후 림프구 반응도 함께 관찰되었다. 이로부터, 아목시실린/클라불란산 유발 간 손상의 경과로서, 대사체 변화, miR-122의 증가, 간효소 활성 증가 및 림프구 활성능 증가 추이 순의 단계적인 변화를 설명할 수 있다.

결론: 이는 건강 자원자에서 AC-DILI 바이오마커를 평가하기 위한 첫 번째 연구이다. 본 연구에서의 결과로부터, miR-122및 4개의 뇨대사체가 AC-DILI에 있어서 조기에 측정가능하며 민감한 바이오마커로서의 가능성을 확인할 수 있다. 더불어, 확인된 마커들로부터 간세포 손상, 간 염증 및 미토콘드리아 산화 스트레스를 AC-DILI 기전으로 들 수 있다. 또한, 약물에 반응한 림프구 활성의 경우도 AC-DILI에 있어서 면역반응의 역할을 제안하여 준다. 종합하여 보았을 때, 본 연구에서는 현재 알려져 있는 AC-DILI 기전에 기반하여 가능성 있는 바이오마커들을 제안하였다.

본 연구에서 오믹스 데이터를 종합적으로 분석하여 평가된 바이오마커는 향후 약물 개발 및 사용에서 보다 민감하고 빠른 간독성 예측을 하는데 활용할 수 있을 것으로 기대된다.

주요어 : 약물유발간손상; 바이오마커; 약물유전체; 약리대사체; microRNA; 임상시험

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