Citation: Clin Transl Sci (2017) 10, 163–171; doi:10.1111/cts.12425

ARTICLE

Exploration of Biomarkers for Amoxicillin/Clavulanate-Induced Liver Injury: Multi-Omics Approaches

J Lee[1,**], SC Ji[1,**], B Kim[1], S Yi[1], KH Shin[3], JY Cho[1], KS Lim[4], SH Lee[1], SH Yoon[1], JY Chung[5], KS Yu[1], HS Park[2], SH Kim[2,*] and IJ Jang[1,*]

To explore potential biomarkers for amoxicillin/clavulanate-induced liver injury (AC-DILI), we conducted a clinical trial in 32 healthy subjects based on multi-omics approaches. Every subject was administered amoxicillin/clavulanate for 14 days. The liver-specific microRNA-122 (miR-122) level increased prior to and correlated well with the observed alanine aminotransferase (ALT) level increase. This result indicates its potential as a sensitive early marker for AC-DILI. We also identified urinary metabolites, such as azelaic acid and 7-methylxanthine, with levels that significantly differed among the groups classified by ALT elevation level on day 8 after drug administration ($P < 0.05$). 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. In conclusion, this study provides potential biomarkers for AC-DILI based on currently known mechanisms using comprehensive multi-omics approaches.

Clin Transl Sci (2017) **10**, 163–171; doi:10.1111/cts.12425; published online on 26 October 2016.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

✔Aminotransferases such as ALT and AST are used as gold standards for the evaluation of DILI. However, their elevation may not be specific to liver injury and may not reflect the mechanisms of DILI.

WHAT QUESTION DID THIS STUDY ADDRESS?

✔To identify sensitive biomarkers for AC-DILI, we evaluated changes in the levels of miR-122, urinary metabolites, and lymphocyte proliferation both in response to drug administration and with respect to risk genotypes.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

✔This study identifies potential biomarkers for AC-DILI based on currently known mechanisms and suggests that drug-induced hepatocellular injury, oxidative stress, and the adaptive immune response are underlying mechanisms of AC-DILI.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE

✔The biomarkers identified in this study by integrating omics data could enable more sensitive and earlier predictions of liver injury. These biomarkers could also provide mechanistic insight that is currently only available from liver biopsies.

Drug-induced liver injury (DILI) is a major challenge for both clinical care and drug development.[1] Monitoring the activity of the enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which reflect hepatic function, is the primary screening tool for DILI.[2] However, elevated levels of these enzymes may not be specific to liver disease and could represent an asymptomatic response.[3–5] 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.

Pharmacogenomic studies have demonstrated specific human leukocyte antigen (HLA) alleles that are associated with DILI that is caused by the following drugs:

[1]Department of Clinical Pharmacology and Therapeutics, Seoul National University College of Medicine and Hospital, Seoul, Korea; [2]Department of Allergy & Clinical Immunology, Ajou University School of Medicine, Suwon, Korea; [3]College of Pharmacy and Research Institute of Pharmaceutical Sciences, Kyungpook National University, Daegu, Korea; [4]Department of Clinical Pharmacology and Therapeutics, CHA University School of Medicine and CHA Bundang Medical Center, Seongnam, Korea; [5]Department of Clinical Pharmacology and Therapeutics, Seoul National University College of Medicine and Bundang Hospital, Seongnam, Korea. *Correspondence: In-Jin Jang (ijjang@snu.ac.kr) Seung-Hyun Kim (kimsh@ajou.ac.kr)

**These authors contributed equally to this work.

Received 14 July 2016; accepted 20 September 2016; published online on 26 October 2016. doi:10.1111/cts.12425

flucloxacillin (B*57:01), ximelagatran (DRB1*07:01 and HLA-DQA1*02), co-amoxiclav (DRB1*15:01), and antituberculosis drugs (HLA-DQB1*0502).[6–9] 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.[6,10]

Numerous studies have identified microRNAs, which are small, single-stranded noncoding regulatory RNA molecules, as possible sensitive biomarkers for DILI.[11–14] Notably, microRNA-122 (miR-122) is elevated earlier and demonstrates increased sensitivity in patients with acetaminophen-induced liver injury compared with ALT.[11,15] 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.[15] In a recent study, differences in the circulating serum levels of exosomal miR-122 were observed between hepatocyte injury and inflammation.[12]

Pharmacometabolomic approaches have been used to identify novel DILI biomarkers.[16,17] 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.[18] 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.[19]

Amoxicillin/clavulanate was selected as study drug in this study because it is the frequently prescribed antibiotic worldwide and is often associated with hepatotoxicity.[20,21] 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).[8,22,23] In a recent study, drug-responsive T cells that were specific for either amoxicillin or clavulanate were isolated from a patient with AC-DILI.[24] 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,[25] miR-122,[26] and endogenous metabolites,[27] well-controlled prospective clinical trials have not been conducted to validate these biomarkers. Based on the multidirectional approaches described above, we evaluated potential biomarkers of DILI in healthy subjects after multiple administrations of amoxicillin/clavulanate.

MATERIALS AND METHODS

Study design

A total of 32 healthy Korean male volunteers were enrolled and grouped according to four GSTT1/M1 genotypes (eight subjects per group): wild/wild, null/wild, wild/null, and null/null. 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. Subjects who had used any prescription drugs, including antibiotics such as amoxicillin/clavulanate or herbals agents, within 2 weeks before the drug administration of the study were excluded. (ClinicalTrials.gov registry number: NCT02143323).

This study had an open-label, single-sequence, three-period design. The workflow of the study design is shown in **Figure 1**. Each subject received three 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 Institutional Review Board of Seoul National University Hospital, Seoul, Korea, approved this clinical study, which was conducted in compliance with the ethical principles of the Declaration of Helsinki. All of the subjects provided written informed consent before being screened for eligibility.

PK analyses

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) coupled to an API 4000 mass spectrometer (Sciex, Toronto, Canada). Chromatographic separation of amoxicillin was conducted using a Synergi 4U HydroRP column (100 $\times$ 2.0 mm, 4 μm) (Phenomenex, Torrance, CA). A Luna column (100 $\times$ 2.0 mm, 5 μm) (Phenomenex) was used for the chromatographic separation of clavulanate. The lower limit of quantitation was 10 ng/mL for both amoxicillin and clavulanate. The intraday and interday precision data are described in **Supplementary Table S1**.

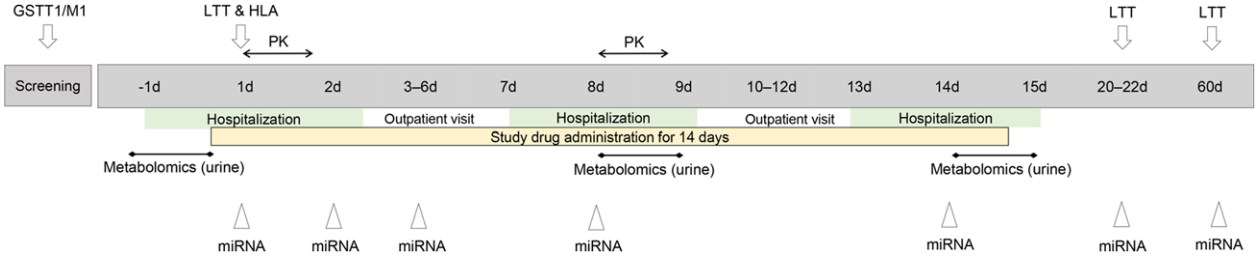

Figure 1 Study design. GSTT1/M1, blood collection for genotyping of glutathione s-transferase mu-1/theta-1; LTT, blood collection for lymphocyte transformation test; HLA, blood collection for genotyping human leukocyte antigen (HLA); PK, blood collection for pharmacokinetic analyses of amoxicillin and clavulanate; Metabolomics, 12-h interval urine collection for metabolomics analysis; miRNA, blood collection for microRNA analyses.

The PK parameters were derived using noncompartmental analyses in Phoenix WinNonlin (v. 6.3., Certara, St. Louis, MO). The C_{max} (maximum plasma concentration at steady state), $AUC_{0\text{-}12h}$ (area under the plasma concentration–time curves from zero to 12 h after a single administration), and $AUC_{ss,\tau}$ (area under the plasma concentration–time curves from zero to 12 h at steady state) were calculated using the linear-up and log-down method.

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, Seoul, Korea).[42] The primers and specific methods are available in the **Supplemental Methods**.

High-resolution sequence-based HLA typing was performed at the following loci: HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQB1, and HLA-DPB1 (Histogenetics, Ossining, NY). The HLA types of the study subjects are shown in **Supplementary Table S2**.

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$\times$ 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) under the following conditions: initial denaturation at 95ºC for 15 min followed by 40 cycles of denaturation for 15 s at 94ºC, annealing for 30 s at 55ºC, and elongation for 30 s at 70ºC. A total exosome isolation reagent (Invitrogen, Carlsbad, CA) was used for exosome isolation from serum according to the manufacturer's instructions.

Pharmacometabolomic analyses

Untargeted metabolomics profiling was performed using urine samples that were collected at 12-h intervals before and after the administration of the study drug. The collected urine samples were centrifuged at 14,000 rpm for 20 min at 4ºC 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 $\times$ 100 mm ACQUITY 1.8 μm HSS T3 column using an ACQUITY UPLC system (Waters, Milford, MA) coupled to a Waters Xevo Q-TOF. 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 data were collected in centroid mode over the range of 50–1000 m/z with a scan time of 0.4 s.

The multivariate data were analyzed using the EZinfo software (Waters). The stability and reproducibility of the unsupervised principal components analysis were checked using the pareto-scaled data by clustering quality controls (QCs).

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.[24] 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 h.

Statistical analysis was done with SPSS (v. 23.0; IBM, Armonk, NY)

The arithmetic means, standard deviations (SDs), 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 and 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 cutoff value of 3 for the stimulation index (SI).[43] 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.

RESULTS

Study subject demographics and group classification

A total of 31 subjects completed the study. The mean age was 30 years, and all of the subjects were male. The means $\pm$ standard deviations (SDs) of the weights and heights of the subjects were 71.9 $\pm$ 7.5 kg and 174.3 $\pm$ 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 events 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, biochemical variables, and lymphocyte transformation test (LTT) results are provided in **Table 1**.

Based on the extent of their ALT elevations relative to the baseline, the subjects were classified into three groups

Table 1 Study demographics of all subjects

	Demographics				Biochemical variables				LTT[a] results	
					Peak levels fold change					
ID	GSTT/GSTM	Age (yr)	Weight (kg)	Height (cm)	T. bilirubin	ALT	AST	ALP	Type	Drug
AN001	Wild/Wild	27	67.2	171.3	1.57	1.31	1.15	1.04	N[c]	
AN002		25	71.5	177.8	1.33	1.15	1.09	1.10	N	
AN003		32	71.9	167.5	1.25	1.43	1.42	1.24	N	
AN004		40	71.3	170.6	1.00	3.29	4.26	1.23	P[b]	C[e]
AN005		30	77.5	179.4	1.10	1.38	1.13	1.11	N	
AN006		30	73.3	180.4	0.90	1.73	1.73	1.14	R[d]	A
AN007		28	71.9	178.1	1.31	1.36	1.09	1.08	P	
AN008		26	69.0	174.8	1.71	2.25	2.21	1.07	R	A
AN101	Wild/Null	31	77.6	177.7	1.00	3.89	2.17	1.23	N	
AN102		30	80.0	178.5	1.11	1.42	1.22	1.05	R	A
AN103*		20	82.6	178.6	1.60	1.27	1.19	1.23	NA	
AN104		26	75.9	174.7	1.18	1.61	1.29	1.14	N	
AN105		30	73.8	171.3	1.10	1.68	1.16	1.10	N	
AN106		32	82.5	182.5	1.22	2.00	1.00	1.12	N	
AN107		29	59.9	175.7	1.20	1.54	1.82	1.14	N	
AN108		35	64.1	171.1	1.33	0.88	0.94	1.00	N	
AN201	Null/Wild	37	69.3	173.1	1.80	1.38	1.18	1.23	N	
AN202		23	88.4	183.4	1.00	2.33	1.38	1.29	N	
AN203		39	72.5	176.5	0.83	3.00	1.67	1.02	P	C, A[f]
AN204		36	71.4	167.4	1.25	1.08	1.09	1.18	N	
AN205		26	65.2	168.1	1.50	1.00	1.13	1.14	N	
AN206		30	59.5	168.4	1.00	3.00	1.86	1.03	N	
AN207		27	62.1	173.4	1.25	0.75	1.12	1.17	R	A
AN208		40	60.0	161.5	1.50	3.00	2.79	1.25	N	
AN301	Null/Null	38	62.9	168.2	1.33	0.91	1.50	1.10	N	
AN302		25	81.5	178.7	1.30	1.17	1.10	1.14	N	
AN303		30	77.3	172.6	1.60	2.92	1.64	1.13	N	
AN304		26	68.2	172.2	1.10	1.64	1.16	1.08	N	
AN305		32	65.3	171.7	1.11	1.23	1.38	1.08	N	
AN306		32	83.3	179.8	1.10	1.00	0.92	1.04	N	
AN307		23	71.8	174.8	1.70	2.00	4.00	1.11	N	
AN308		25	81.6	182.8	1.00	1.45	1.24	1.01	N	

[a]LTT, Lymphocyte transformation test;[b]P, positive type (stimulation index $>$ 3.0);[c]N, negative type (stimulation index $\leq$ 2.0);[d]R, recovery type (stimulation index $>$ 3.0 at day 20–22 but decreased below 3.0 at day 60);[e]C, clavulanate;[f]A, amoxicillin *Dropped out due to adverse events (headache, nausea). NA, not applicable.

(**Figure 2**): (1) *Responder* ($n = 6$), who had greater than a twofold change in ALT at more than two sampling points; (2) *Nonresponder* ($n = 17$), who had less than a 1.5-fold change in ALT at all of the sampling points; and (3) *Intermediate* ($n = 8$), who 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 twofold changes above the baseline values (i.e., ALT before drug administration on day 1) at two or more sampling points.

Correlation of PK parameters with ALT elevations

Comparative analyses between the *Responder* and *Nonresponder* 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} (**Supplementary Table S3**), 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. There were also no significant differences in the mean maximum ALT fold change among the groups that were classified by GSTT1/GSTM1 genotype (**Supplementary Figure S1**).

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 3** shows these results for both the *Responder* and *Nonresponder* groups. 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. We found significant correlations

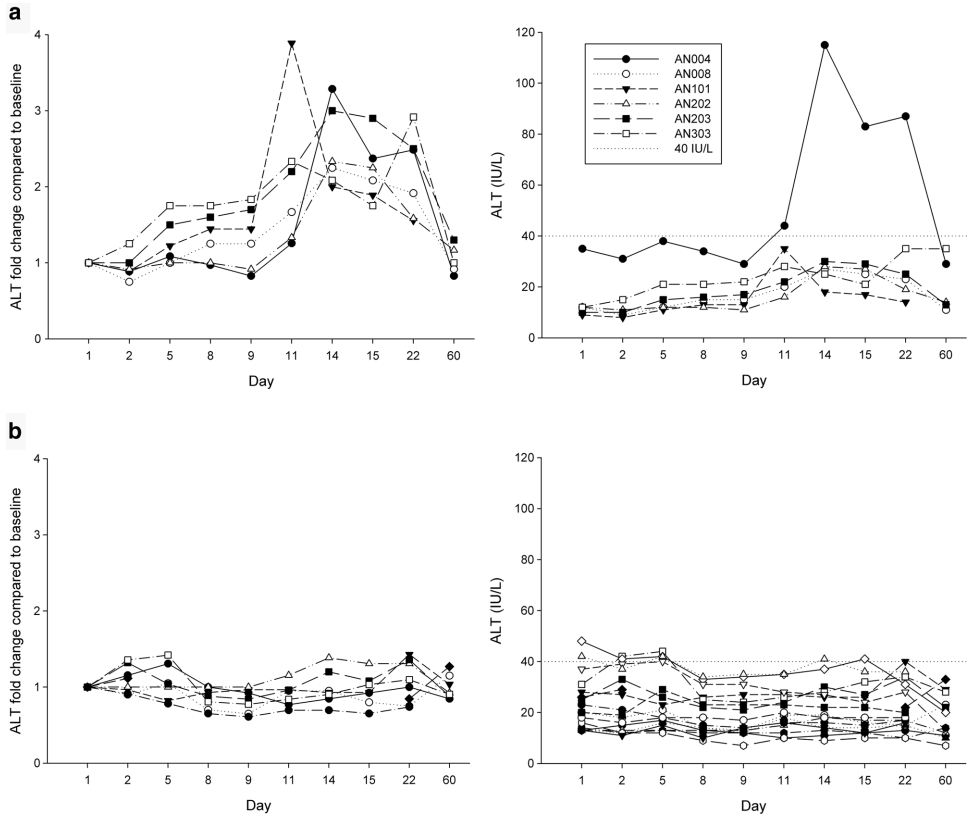

Figure 2 ALT fold changes relative to baseline levels (IU/L) in the subjects over the course of the 60-day study in the (**a**) *Responder* group (left: ALT fold change, right: ALT levels) and the (**b**) *Nonresponder* group (left: ALT fold change, right: ALT levels). The baseline ALT levels were defined as the value of the first day predose sample.

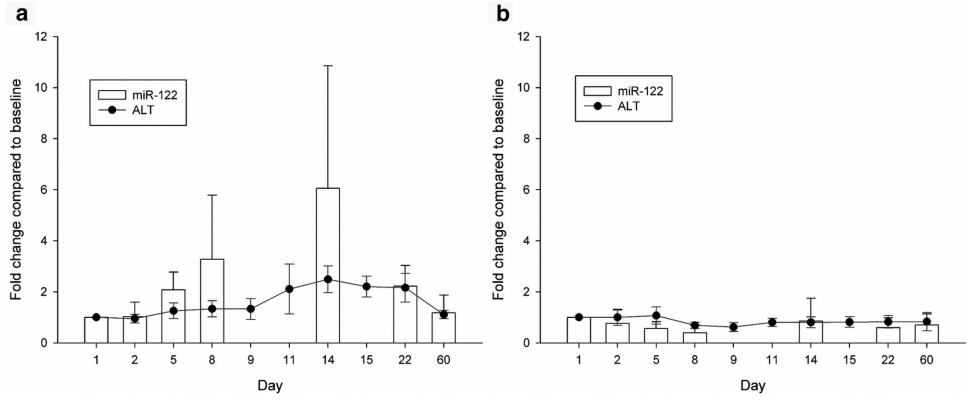

Figure 3 Time courses depicting the serum miR-122 and ALT fold changes in the (**a**) *Responder* and (**b**) *Nonresponder* groups. The data represent the mean ± standard deviation.

between the serum miR-122 and ALT fold changes (Pearson's r of 0.613–0.923, $P < 0.05$, **Supplementary Table S4**).

Pharmacometabolomic analyses

A total of 5,169 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 (**Supplementary Figure S2**), the concentrations of 5 urinary metabolites (variable influence on the projection > 5) were significantly different in the *Responder* and *Nonresponder* groups (**Table 2**). The identified urinary markers 7-methylxanthine (7MX), 7-methyluric acid (7MU), 3-methylxanthine (3MX), acetylcarnitine (ACar), and azelaic acid (AzA) were quantified using QuanLynx (Waters) 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 *Nonresponder* group (**Figure 4**). Although the urinary metabolite

Table 2 Urinary metabolites that were significantly different between the *Responder* and *Nonresponder* groups at day 8 after amoxicillin/clavulanate administration

		Responder			*Nonresponder*			
Metabolite	**VIP**	**Baseline**	**Day 8**	**Day 14**	**Baseline**	**Day 8**	**Day 14**	**Ratio of metabolites**
Azelaic acid	5.68	1,227.9 ± 468.4	530.3 ± 409.3	971.5 ± 762.1	1,415.2 ± 1,298.1	1,191.7 ± 706.2	1,084.9 ± 983.6	0.4*
7-Methylxanthine	11.78	5,020.6 ± 7,165.8	12,159.2 ± 6,520.7	8,352.7 ± 6,863.8	2,441.2 ± 2,203.0	3,703.3 ± 4,612.0	4,277.7 ± 5,458.8	3.3*
3-Methylxanthine	7.67	1,631.5 ± 955.3	2,802.6 ± 1,096.9	2,343.3 ± 1,691.5	968.0 ± 876.2	1,137.1 ± 1,226.6	1,494.5 ± 2,132.8	2.5*
7-Methyluric acid	8.71	944.5 ± 076.4	2,759.1 ± 1,799.7	2,489.4 ± 2,122.3	650.3 ± 681.0	712.3 ± 905.9	897.0 ± 1,176.2	3.9*
Acetylcarnitine	5.93	5,490.0 ± 221.6	2,570.5 ± 1,654.6	6,299.0 ± 4,098.6	5,593.9 ± 4,417.5	7,724.3 ± 9,887.3	6,502.1 ± 10,705.5	0.3

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

levels showed large interindividual variations, the levels of 7MX, 7MU, and 3MX in the *Responder* group on day 8 were significantly higher (3.3, 3.9, and 2.5 times, respectively) than in the *Nonresponder* group, whereas the AzA level was significantly lower (0.4 times; **Figure 4**).

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 stimulation index 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 5**), with a very strong proliferative response to clavulanate and enhanced interferon gamma (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.

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,[11] 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[28] or with specific HLA types that are known risk factors for DILI.[28] 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 interindividual variations, we identified four urinary markers with the potential to predict ALT elevation: AzA was significantly downregulated, whereas 7MX, 3MX, and 7MU were significantly upregulated.

AzA is known to scavenge radicals and modulate the inflammatory response in human keratinocytes.[29] A recent

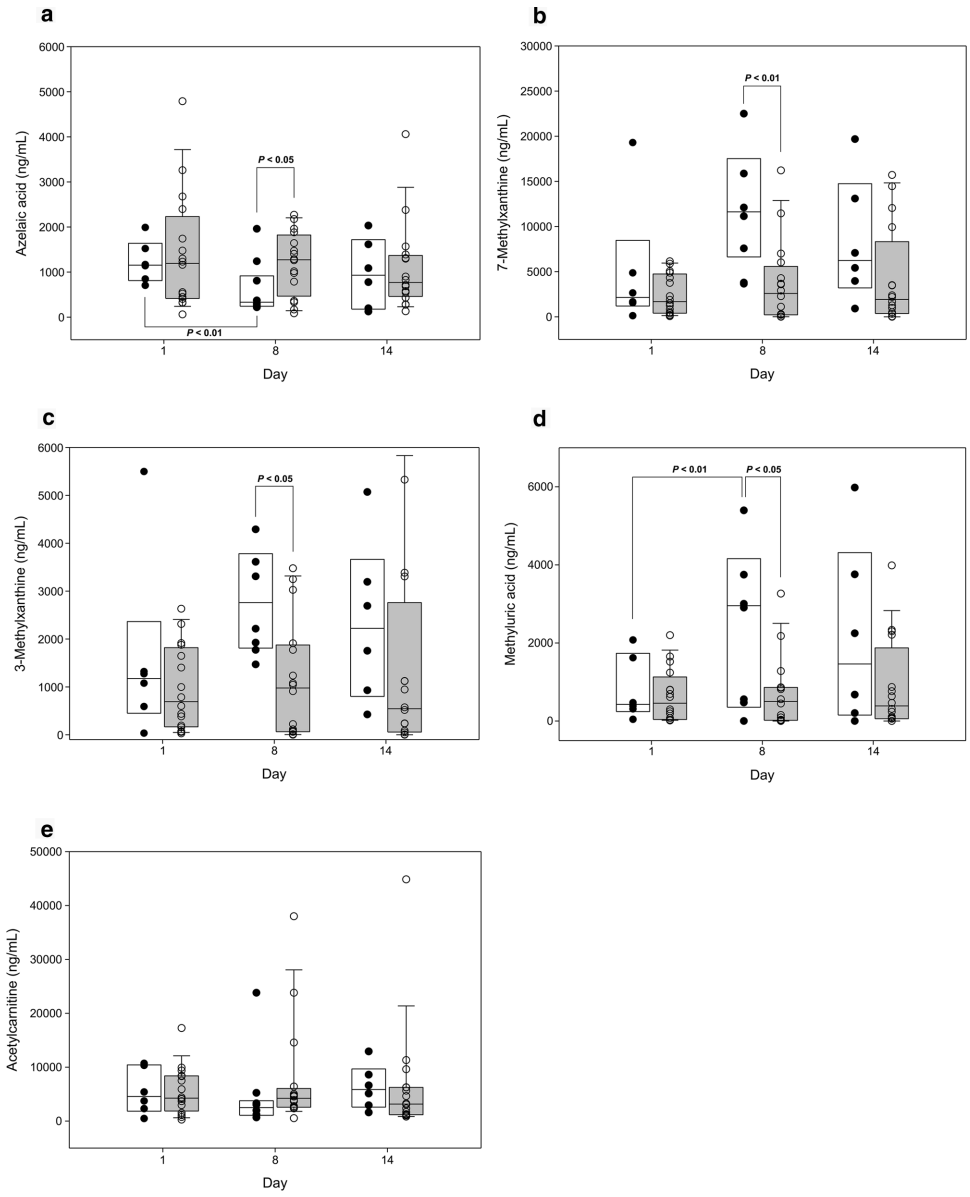

Figure 4 Box-whisker plots for significantly regulated endogenous metabolites. (**a**) Azelaic acid, (**b**) 7-methyluric acid, (**c**) 3-methylxanthine, (**d**) 7-methylxanthine, and (**e**) acetylcarnitine on days 1, 8, and 14 (white box: *Responder*; gray box: *Nonresponder*). The box plot shows the median (line) and 25–75% interquartile range for endogenous metabolites. Circles represent outliers.

study in mice demonstrated that exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced oxidative stress in the liver and decreased AzA levels.[30] The AzA levels changed even with low doses of TCDD, which did not significantly increase the serum ALT or AST levels. Therefore, AzA was suggested to be an early indicator of liver damage that is related to mitochondrial oxidative stress. Interestingly, 7MX also appears to be related to oxidative stress. It is metabolized in the liver by xanthine oxidase to yield 7MU, which generates reactive oxygen species (ROS) in the liver,[31] which indicates that these metabolites are involved in drug-induced oxidative stress in the liver.[32,33] The metabolomics results thus support the conclusion that mitochondrial oxidative stress could be another mechanism that underlies AC-DILI.

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.[23,24] The technologically demanding LTT assay was used here as an *in vitro* testing method for detecting drug-responsive T cells.[34] 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

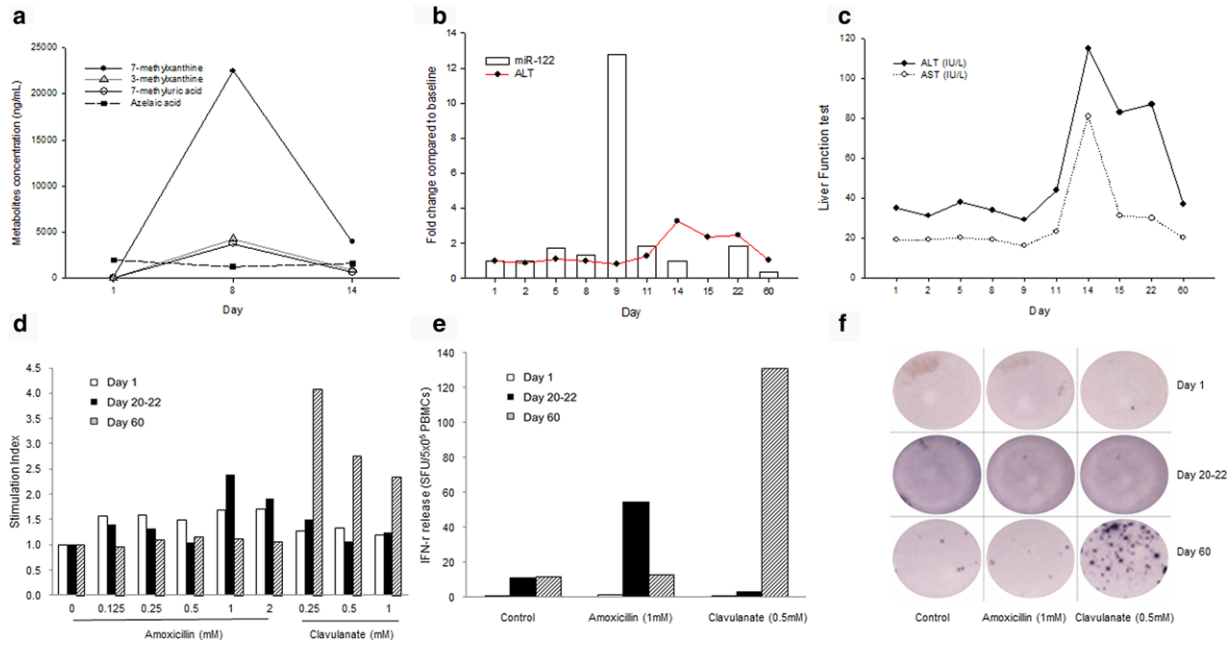

Figure 5 Time courses depicting changes in (**a**) urinary metabolites, (**b**) miR-122, (**c**) ALT and AST, (**d**) lymphocyte proliferation, and (**e**) INF-γ release in subject AN004.

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.[35,36] A recent study reported that circulating miRNAs are associated with either the exosome-rich or protein-rich fraction, depending on the type of liver injury.[12,37] Circulating miR-122 in the exosome-rich fraction is more related to inflammation. On the other hand, miR-122 in protein-rich fraction is known to be associated with hepatocyte injury induced by necrosis.[12] Our results showed that miR-122 increased by 7.2- and 5.2-fold in the exosome-rich and protein-rich fractions, respectively (**Supplementary Figure S3**), which indicates that amoxicillin and clavulanate induce a mixed-type liver injury.

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,[20] 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.[38] 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.[39,40] 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 have typically been retrospective studies on DILI patients. Considering this, 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.[41] However, a control group of either the same subjects or different subjects not taking the antibiotic would have added validity to changes in transaminases.

In conclusion, we evaluated and identified potential DILI biomarkers based on currently known AC-DILI mechanisms. Our results demonstrate that drug-induced hepatocellular injury, oxidative stress, and adaptive immune response may represent the main mechanisms that underlie DILI. Further confirmatory studies that demonstrate and validate the clinical benefits of these markers for use in bedside applications should be performed.

Acknowledgments. We thank Jingshun Li and Soyoung Kim for coordinating the clinical trials. We acknowledge Min Chang Kim and

Jaejin Lee for the quantitative analyses of the study drug. ClinicalTrials.gov registry number:NCT02143323

Author Contributions. I-J.J., J.L., S.Y., J-Y.C., and S-H.K. wrote the manuscript; I-J.J., J.L., S.C.J., K-H.S., J-Y.C., K.S.L., S.L., J-Y.C., and K-S.Y. designed the research; I-J.J., J.L., S.C.J., B.K., S.Y., K.S.L., and H-S.P. performed the research; J.L., S.C.J., B.K., K-H.S., S.L., S.H.Y., K-S.Y., and H-S.P. analyzed the data; S.C.J., J-Y.C., S.H.Y., and S-H.K. contributed new reagents/analytical tools.

Conflict of Interest. The authors declared no conflict of interest.

Financial Support. This research was supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute, which is funded by the Ministry of Health & Welfare, Republic of Korea (HI14C0065 and HI14C2770).

1. Qureshi, Z.P., Seoane-Vazquez, E., Rodriguez-Monguio, R., Stevenson, K.B. & Szeinbach, S.L. Market withdrawal of new molecular entities approved in the United States from 1980 to 2009. *Pharmacoepidemiol. Drug Saf.* **20**, 772–777 (2011).
2. Regev, A. & Bjornsson, E.S. Drug-induced liver injury: morbidity, mortality, and Hy's law. *Gastroenterology* **147**, 20–24 (2014).
3. Kong, A.P. *et al.* Independent associations of alanine aminotransferase (ALT) levels with cardiovascular risk factor clustering in Chinese adolescents. *J. Hepatol.* **49**, 115–122 (2008).
4. Lee, J.K. *et al.* Estimation of the healthy upper limits for serum alanine aminotransferase in Asian populations with normal liver histology. *Hepatology* **51**, 1577–1583 (2010).
5. Dufour, D.R. Alanine aminotransferase: is it healthy to be "normal"? *Hepatology* **50**, 1699–1701 (2009).
6. Daly, A.K. *et al.* HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. *Nat. Genet.* **41**, 816–819 (2009).
7. Kindmark, A. *et al.* Genome-wide pharmacogenetic investigation of a hepatic adverse event without clinical signs of immunopathology suggests an underlying immune pathogenesis. *Pharmacogenomics J.* **8**, 186–195 (2008).
8. Lucena, M.I. *et al.* Susceptibility to amoxicillin-clavulanate-induced liver injury is influenced by multiple HLA class I and II alleles. *Gastroenterology* **141**, 338–347 (2011).
9. Chen, R. *et al.* The association between HLA-DQB1 polymorphism and antituberculosis drug-induced liver injury: a case-control study. *J. Clin. Pharm. Ther.* **40**, 110–115 (2015).
10. Huang, Y.-S. *et al.* Genetic polymorphisms of manganese superoxide dismutase, NAD (P) H: quinone oxidoreductase, glutathione S-transferase M1 and T1, and the susceptibility to drug-induced liver injury. *J. Hepatol.* **47**, 128–134 (2007).
11. Starkey Lewis, P.J. *et al.* Circulating microRNAs as potential markers of human drug-induced liver injury. *Hepatology* **54**, 1767–1776 (2011).
12. Bala, S. *et al.* Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases. *Hepatology* **56**, 1946–1957 (2012).
13. Nagano, T. *et al.* Liver-specific microRNAs as biomarkers of nanomaterial-induced liver damage. *Nanotechnology* **24**, 405102 (2013).
14. Shah, N., Nelson, J.E. & Kowdley, K.V. MicroRNAs in liver disease: bench to bedside. *J. Clin. Exp. Hepatol.* **3**, 231–242 (2013).
15. Starkey Lewis, P.J. *et al.* Serum microRNA biomarkers for drug-induced liver injury. *Clin. Pharmacol. Ther.* **92**, 291–293 (2012).
16. O'Connell, T.M. & Watkins, P.B. The application of metabonomics to predict drug-induced liver injury. *Clin. Pharmacol. Ther.* **88**, 394–399 (2010).
17. Mattes, W. *et al.* Detection of hepatotoxicity potential with metabolite profiling (metabolomics) of rat plasma. *Toxicol. Lett.* **230**, 467–478 (2014).
18. Winnike, J.H., Li, Z., Wright, F.A., Macdonald, J.M., O'Connell, T.M. & Watkins, P.B. Use of pharmaco-metabonomics for early prediction of acetaminophen-induced hepatotoxicity in humans. *Clin. Pharmacol. Ther.* **88**, 45–51 (2010).
19. Clayton, T.A., Baker, D., Lindon, J.C., Everett, J.R. & Nicholson, J.K. Pharmacometabonomic identification of a significant host-microbiome metabolic interaction affecting human drug metabolism. *Proc. Natl. Acad. Sci. U S A* **106**, 14728–14733 (2009).
20. Robles, M., Toscano, E., Cotta, J., Lucena, M.I. & Andrade, R.J. Antibiotic-induced liver toxicity: mechanisms, clinical features and causality assessment. *Curr. Drug Saf.* **5**, 212–222 (2010).
21. Andrade, R.J. *et al.* Drug-induced liver injury: an analysis of 461 incidences submitted to the Spanish registry over a 10-year period. *Gastroenterology* **129**, 512–521 (2005).
22. Invernizzi, P. Drug-induced liver injury: is it time for genetics to change our clinical practice? *J. Hepatol.* **53**, 993–994 (2010).
23. Stephens, C. *et al.* HLA alleles influence the clinical signature of amoxicillin-clavulanate hepatotoxicity. *PLoS One* **8**, e68111 (2013).
24. Kim, S.H. *et al.* Characterization of amoxicillin- and clavulanic acid-specific T-cells in patients with amoxicillin-clavulanate-induced liver injury. *Hepatology* **62**, 887–899 (2015).
25. Tujios, S. & Fontana, R.J. Mechanisms of drug-induced liver injury: from bedside to bench. *Nat. Rev. Gastroenterol. Hepatol.* **8**, 202–211 (2011).
26. Hornby, R.J., Starkey Lewis, P., Dear, J., Goldring, C. & Park, B.K. MicroRNAs as potential circulating biomarkers of drug-induced liver injury: key current and future issues for translation to humans. *Expert Rev. Clin. Pharmacol.* **7**, 349–362 (2014).
27. Zhang, A., Sun, H., Wang, P., Han, Y. & Wang, X. Metabonomics for discovering biomarkers of hepatotoxicity and nephrotoxicity. *Pharmazie* **67**, 99–105 (2012).
28. Lucena, M.I. *et al.* Glutathione S-transferase m1 and t1 null genotypes increase susceptibility to idiosyncratic drug-induced liver injury. *Hepatology* **48**, 588–596 (2008).
29. Mastrofrancesco, A. *et al.* Azelaic acid modulates the inflammatory response in normal human keratinocytes through PPARgamma activation. *Exp. Dermatol.* **19**, 813–820 (2010).
30. Matsubara, T. *et al.* Metabolomics identifies an inflammatory cascade involved in dioxin- and diet-induced steatohepatitis. *Cell Metab.* **16**, 634–644 (2012).
31. Frederiks, W.M. & Bosch, K.S. The proportion of xanthine oxidase activity of total xanthine oxidoreductase activity in situ remains constant in rat liver under various (patho)physiological conditions. *Hepatology* **24**, 1179–1184 (1996).
32. Kellogg, E.W. 3rd & Fridovich, I. Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by a xanthine oxidase system. *J. Biol. Chem.* **250**, 8812–8817 (1975).
33. Chowdhury, A. *et al.* Induction of oxidative stress in antitubercular drug-induced hepatotoxicity. *Indian J. Gastroenterol.* **20**, 97–100 (2001).
34. Pichler, W.J. & Tilch, J. The lymphocyte transformation test in the diagnosis of drug hypersensitivity. *Allergy* **59**, 809–820 (2004).
35. Arroyo, J.D. *et al.* Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc. Natl. Acad. Sci. U S A* **108**, 5003–5008 (2011).
36. Vickers, K.C., Palmisano, B.T., Shoucri, B.M., Shamburek, R.D. & Remaley, A.T. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat. Cell Biol.* **13**, 423–433 (2011).
37. Hunter, M.P. *et al.* Detection of microRNA expression in human peripheral blood microvesicles. *PLoS One* **3**, e3694 (2008).
38. Lucena, M.I. *et al.* Determinants of the clinical expression of amoxicillin-clavulanate hepatotoxicity: a prospective series from Spain. *Hepatology* **44**, 850–856 (2006).
39. Kechagias, S. *et al.* Fast-food-based hyper-alimentation can induce rapid and profound elevation of serum alanine aminotransferase in healthy subjects. *Gut* **57**, 649–654 (2008).
40. Johnston, D.E. Special considerations in interpreting liver function tests. *Am. Fam. Physician* **59**, 2223–2230 (1999).
41. Giboney, P.T. Mildly elevated liver transaminase levels in the asymptomatic patient. *Am. Fam. Physician* **71**, 1105–1110 (2005).
42. Abdel-Rahman, S.Z., el-Zein, R.A., Anwar, W.A. & Au, W.W. A multiplex PCR procedure for polymorphic analysis of GSTM1 and GSTT1 genes in population studies. *Cancer Lett.* **107**, 229–233 (1996).
43. Schreiber, J., Zissel, G., Greinert, U., Schlaak, M. & Muller-Quernheim, J. Lymphocyte transformation test for the evaluation of adverse effects of antituberculous drugs. *Eur. J. Med. Res.* **4**, 67–71 (1999).

Supplementary information accompanies this paper on the *Clinical and Translational Science* website. (http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1752-8062)