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ORIGINAL ARTICLE

Analysis of serum microRNA-122 in a randomized controlled trial of N-acetylcysteine for treatment of antituberculosis drug-induced liver injury

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Aim: Serum microRNA-122 (miR-122) is a novel biomarker for drug-induced liver injury, with good sensitivity in the early diagnosis of paracetamol-induced liver injury. We describe miR-122 concentrations in participants with antituberculosis drug-induced liver injury (AT-DILI). We explored the relationship between miR-122 and alanine aminotransferase (ALT) concentrations and the effect of N-acetylcysteine (NAC) on miR-122 concentrations.

Methods: We included participants from a randomized placebo-controlled trial of intravenous NAC in AT-DILI. ALT and miR-122 concentrations were quantified before and after infusion of NAC/placebo. We assessed correlations between ALT and miR-122 concentrations and described changes in ALT and miR-122 concentrations between sampling occasions.

Results: We included 45 participants; mean age (± standard deviation) 38 (±10) years, 58% female and 91% HIV positive. The median (interquartile range) time between pre- and post-infusion biomarker specimens was 68 h (47-77 h). The median pre-infusion ALT and miR-122 concentrations were 420 U/L (238-580) and 0.58 pM (0.18-1.47), respectively. Pre-infusion ALT and miR-122 concentrations were correlated (Spearman's $\rho = .54$, $P = .0001$). Median fold-changes in ALT and miR-122 concentrations between sampling were 0.56 (0.43-0.69) and 0.75 (0.23-1.53), respectively, and were similar in the NAC and placebo groups ($P = .40$ and $P = .68$ respectively).

Conclusions: miR-122 concentrations in our participants with AT-DILI were considerably higher than previously reported in healthy volunteers and in patients on antituberculosis therapy without liver injury. We did not detect an effect of NAC on miR-122 concentrations. Further research is needed to determine the utility of miR-122 in the diagnosis and management of AT-DILI.

Christopher Goldring and Karen Cohen are joint senior authors.

The authors confirm that the PI for this paper is Karen Cohen and that she had direct clinical responsibility for study participants.

KEYWORDS
antituberculosis drugs, biomarkers, liver injury, microRNA-122

1 | INTRODUCTION

Drug-induced liver injury (DILI) is the most frequent severe adverse effect of first-line antituberculosis therapy.[1] Antituberculosis drug-induced liver injury (AT-DILI) is primarily diagnosed by elevated serum alanine aminotransferase (ALT) concentrations, together with symptoms and signs of DILI.[2] However, ALT is not a specific biomarker of DILI because it may be released from tissues other than the liver (eg, skeletal muscle, kidney and heart).[3] More specific biomarkers of DILI are therefore potentially important.

MicroRNAs are small, single-stranded RNAs that regulate gene expression at a post-transcriptional level.[4] MicroRNAs can be detected in blood as stable complexes and quantified using quantitative real-time polymerase chain reaction (qRT-PCR).[5] Liver injury due to drug exposure, viral hepatitis or hepatocellular carcinoma has been shown to result in altered microRNA expression profiles.[6–9] MicroRNA-122 (miR-122) accounts for 70% of hepatic microRNA[10] and plays an essential role in lipid metabolism, anti-inflammatory and antitumorigenic mechanisms.[11] miR-122 has been explored as a novel biomarker of paracetamol-induced liver injury in pre-clinical[12,13] and clinical studies.[9,14–17] In paracetamol-induced liver injury, an increase in miR-122 concentrations occurred earlier than an increase in ALT concentrations and was a sensitive test for identifying those patients who went on to develop liver injury.[12,18] Peak increases in serum miR-122 concentrations correlated with later increases in serum ALT.[13,14,19] miR-122 concentrations have also been found to be elevated in patients with nonparacetamol DILI.[20,21] miR-122 is largely liver-specific and therefore concentrations are unaltered by impaired renal function[15] and muscle injury.[17]

There are limited data on miR-122 as a biomarker of AT-DILI. For this study, we quantified miR-122 concentrations in stored specimens from a randomized placebo-controlled trial of N-acetylcysteine (NAC) for treatment of AT-DILI. We found that NAC administration shortened the duration of hospitalization, but time for ALT to fall below 100 U/L was similar for NAC and placebo groups.[22]

Our aims for this analysis were to describe miR-122 concentrations in AT-DILI, to assess correlations between ALT and miR-122 concentrations, to describe changes in miR-122 concentrations over time and to determine if repeat miR-122 quantification would be more informative than ALT in detecting a biochemical response to NAC administration.

What is already know about this subject?

- Serum microRNA (miR)-122 is a sensitive biomarker for early detection of paracetamol-induced liver injury.
- Serum miR-122 concentrations increase slightly in participants taking antituberculosis treatment without liver injury.
- Serum miR-122 concentrations increased markedly in participants with antituberculosis drug-induced liver injury (AT-DILI) in one study but decreased in another.
- In a randomized controlled trial of intravenous N-acetylcysteine (NAC) in participants with AT-DILI, NAC did not hasten the decline of alanine aminotransferase (ALT) concentrations. The effect of N-acetylcysteine on miR-122 concentrations in this setting has not previously been studied.

What this study adds?

- In our cohort drawn from a randomized controlled trial of intravenous NAC in participants with AT-DILI, miR-122 concentrations were markedly higher than those seen in healthy volunteers and in patients on antituberculosis treatment without liver injury.
- We did not detect an effect of NAC on the change in miR-122 concentrations observed during the first week after infusion of NAC/placebo.
- We found high intra-individual and inter-individual variability in serum microRNA-122 concentrations following AT-DILI.

2 | METHODS

2.1 | Study participants

Participants were drawn from a previously reported[22] randomized placebo-controlled trial (RCT) of NAC in the management of AT-DILI conducted in Cape Town, South Africa. NAC was dosed intravenously according to the regimen for paracetamol overdose: 150 mg/kg over 1 h, 50 mg/kg over 4 h and 100 mg/kg over 16 h. The study was a pragmatic randomized trial nested within routine clinical care at the participating hospitals. The study protocol specified that participants' ALTs be tested before the study infusion and twice weekly during hospital follow-up. These ALT samples were generally taken by the hospital clinical care team. In addition to the monitoring of ALT concentrations for the clinical study, investigators collected blood samples for storage for future biomarker research. For this analysis, we included the subset of participants from the RCT with paired specimens stored for biomarker quantification: the first specimen taken between 24 h before and 30 min after

commencement of the study infusion and the second specimen taken within 7 days after initiation of the study infusion. Blood was centrifuged within 8 h of collection, and serum was stored at −80°C. We compared baseline characteristics of the participants with paired samples, and study participants not included in this analysis to confirm that they were similar.

2.2 | ALT quantification

ALT was quantified in stored serum using an International Federation of Clinical Chemistry recommended method. ALT and miR-122 concentrations were determined from the same sample in each case, except for two pre-infusion samples which were unsuitable for ALT determination; in those two cases, we used the ALT concentration from clinical records from an assay performed on the same day of the infusion. Both of these ALTs were from the samples taken within 3 h of the sample used for miR-122 determination.

2.3 | MicroRNA isolation from serum

For recovery of miRNA-enriched fractions from serum, the miRNeasy Mini Kit and the RNeasy MinElute Cleanup Kit (Qiagen, Venlo, Netherlands) were used on the QIAcube automated platform (Qiagen), following manufacturer's instructions with an addition of 200 pM cel-lin-4 (cat. 4464066, ID MC10768; Ambion, Thermo Fisher Scientific, Waltham, MA, USA) as a spiked-in exogenous nonhuman miRNA to monitor for the efficiency of the miRNA extraction process. Freshly isolated miRNA-containing eluate (15 µL) was stored at −80°C.

2.4 | Reverse transcriptase and RT-qPCR reactions

TaqMan miRNA Assays for miR-122 (Assay ID 002245) and cel-lin-4 (Assay ID 000258; Thermo Fisher Scientific) were used to perform real-time quantitative PCR (RT-qPCR). miRNA-containing eluate was reverse-transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) and a custom Multiplex RT Primer pool in a GeneAmp 9700 PCR System (Applied Biosystems, Foster City, CA, USA). A fixed volume of 2 µL miRNA-containing eluate was added to an RT Master Mix containing dNTPs, RNase Inhibitor, Reverse Transcription Buffer, 75 units per reaction of MultiScribe Reverse Transcriptase and a multiplex RT primer pool consisting of primers for miR-122 and cel-lin-4 (Thermo Fisher Scientific). A 1 nM synthetic miR-122 (mirVana miRNA mimic, Assay ID MC11012; Ambion) was reverse-transcribed together with the samples to allow the generation of a standard curve in each plate. No-template samples were included as negative controls. Reaction conditions followed the manufacturer's instructions: annealing for 30 min at 16°C, followed by cDNA synthesis for 30 min at 42°C and denaturation for 5 min at 85°C. The resulting cDNA was diluted 1:3 with RNase-free water and stored at −20°C until further processing.

Pre-amplification of cDNA was run on a GeneAmp 9700 PCR System (Applied Biosystems). Negative controls containing RNase-free water instead of cDNA were included. Reaction conditions followed the manufacturer's instructions: 95°C for 10 min, 55°C for 2 min, 72°C for 2 min, followed by 12 cycles at 95°C for 15 s and 60°C for 4 min followed by enzyme inactivation at 99.9°C for 10 min. The pre-amplified synthetic miR-122 was serially diluted 1:10 (ranging from 1 nM to 0.1 fM) and stored at −20°C.

Two microlitres of the diluted pre-amplification product was combined with a qPCR Master Mix containing 5 µL of 2x TaqMan Universal MasterMix II without Uracil-N glycosylase (Thermo Fisher Scientific) in a total reaction volume of 10 µL. RT-qPCR was then performed in duplicates on an ABI ViiA 7 Thermocycler (Applied Biosystems) using a two-step thermal cycling protocol of 95°C for 10 min followed by 40 cycles of 95°C (15 s) and 60°C (60 s) for both miR-122 and cel-lin-4. Data were analysed using QuantStudio Real-Time PCR Software v1.3. The number of copies of miR-122 in each sample was quantified using the absolute quantification method with the standard curve generated with synthetic miR-122 in each plate. miR-122 levels were normalized to the level of cel-lin-4 to account for technical variations between samples. Then, 10% duplicates (including samples run in a separate plate) were added to each run to account for intra- and inter-plate variability, and final miR-122 copy numbers were averaged across experiments (intra- and inter-assay coefficient of variation (CV) less than 5% and 10%, respectively).

2.5 | Statistical analysis

Data were analysed using Stata SE Version 13.1 (Statacorp, College Station, TX, USA) and Microsoft Excel. We compared numerical data with a normal distribution using the Student's *t*-test, non-normally distributed data using the Wilcoxon rank sum test and categorical data using the Fisher's exact test. We calculated the Spearman's rank correlation coefficient (ρ) to assess correlation between ALT and miR-122 concentrations. We calculated the CV for ALT and miR-122 concentrations pre- and post-study infusion. We log transformed ALT and miR-122 concentrations for parametric comparative analyses. To explore change in miR-122 and ALT concentration between samples, we calculated the ratio of post-infusion to pre-infusion concentration and performed a Student's *t*-test on logged ratios to look for differences between groups. To determine the average change in ALT and miR-122 concentrations per day, we calculated the slope between the concentration before and the concentration after study infusion. A P value of <.05 was considered to be statistically significant throughout.

2.6 | Ethics

The study protocol was approved by University of Cape Town Human Research Ethics Committee (HREC 087/2012 and HREC 421/2017) and the Western Cape Department of Health. Participants provided written informed consent. The trial was registered with the South African National Clinical Trials Registry (SANCTR: DOH-27-0414-4719) and clinicaltrials.gov (NCT02182167).

3 | RESULTS

3.1 | Participant characteristics

We included 45 of the 102 NAC randomized controlled trial participants from whom paired biomarker specimens had been collected within the specified time windows, 26 of these were from the NAC and 19 were from the placebo group (see Supporting Information Figure S1). Baseline characteristics of the 45 included participants did not differ significantly from the 57 participants without paired specimens (see Supporting Information Table S1). Four of the 45 participants with paired specimens died during study follow-up (two in the NAC group and two in the placebo group) *vs* 10/57 participants without paired specimens (Fisher's exact $P = .255$).

Participant characteristics were similar between the NAC and placebo groups in those participants with biomarker samples (Table 1). The mean age was 38 years ± standard deviation (SD) 10, 58% were female and 91% were HIV positive. We had self-reported ethnicity data on 31 participants, of whom 27 (87%) self-identified as Black African.

3.2 | Time intervals between AT-DILI presentation, biomarker sampling and study infusion

The median time from presentation with AT-DILI to collection of the pre-infusion sample was 2.7 days (interquartile range [IQR] 1.5-4.1 days). This first sample was taken at a median of 0.6 h (IQR 0.2-11 h) before study infusion commenced, and the second sample was taken at a median of 50 h (IQR 26-73 h) after commencement of study infusion. The median time interval between paired specimens was 68 h (IQR 47-77 h) and was similar between the NAC and placebo groups (Wilcoxon rank sum test $P = .08$).

3.3 | ALT and miR-122 concentrations before study infusion

The ALT and miR-122 concentrations and CVs are summarized in Table 1. The median ALT concentration before intravenous NAC/placebo infusion was 420 U/L (IQR 238-580 U/L) and the median miR-122 concentration was 0.58 pM (IQR 0.18-1.47 pM) (Figure 1). miR-122 and ALT concentrations before study infusion were correlated (Spearman's $\rho = .54$, $P = .0001$); Figure 2. The median cycle threshold (Ct) pre-infusion was 23.1 (IQR 20.8-24.7).

3.4 | ALT and miR-122 concentrations after study infusion

Post-infusion ALT and miR-122 concentrations were similar in the NAC and placebo groups (see Table 1 and Figure 1).Post-infusion miR-122 concentrations were correlated with ALT concentrations in both the NAC and placebo groups (Spearman's $\rho = .42$, $P = .031$ and Spearman's $\rho = .53$, $P = .020$, respectively) and when participants from both groups were analysed together (Spearman's $\rho = .45$, $P = .002$) (Figure 2).The median Ct post-infusion was 23.7 (IQR 22.0-24.7).

TABLE 1 Baseline characteristics and biomarker concentrations before and after study infusion in participants with antituberculosis drug-induced liver injury randomized to intravenous N-acetylcysteine or placebo

	NAC (n = 26)	Placebo (n = 19)	Total (n = 45)	*P* value[a]
Age, years, mean (±SD)	38 (±12)	38 (±7)	38 (±10)	.897
Female, *n* (%)	17 (65)	9 (47)	26 (58)	.360
Weight, kg, median (IQR)	55 (49-64)	57 (45-63)	56 (48-64)	.980
HIV positive, *n* (%)	22 (85)	19 (100)	41 (91%)	.126
CD4 count (HIV positive) cells/mm^3, median (IQR)	93 (54-277)	54 (7-132)	74 (19-161)	.222
Enrolment total bilirubin, μmol/L, median (IQR)	50 (17-91)	74 (35-191)	52 (28-117)	.112
Pre-infusion ALT, U/L, median (IQR)	361 (238-580)	427 (238-612)	420 (238-580)	.899
CV (%)	91	64	81	
Post-infusion ALT, U/L, median (IQR)	194 (119-293)	250 (111-419)	221 (119-342)	.550
CV (%)	93	98	95	
Pre-infusion miR-122, pM, median (IQR)	0.44 (0.17-1.47)	0.84 (0.31-2.79)	0.58 (0.18-1.47)	.251
CV (%)	167	152	169	
Post-infusion miR-122, pM, median (IQR)	0.18 (0.77-0.94)	0.33 (0.16-0.86)	0.26 (0.12-0.86)	.113
CV (%)	214	145	207	

Abbreviations: ALT, alanine aminotransferase; CV, coefficient of variation; HIV, human immunodeficiency virus; IQR, interquartile range; SD, standard deviation.

[a]Fisher's exact test for categorical variables, Student's *t*-test for parametric data, Wilcoxon rank sum test for nonparametric data.

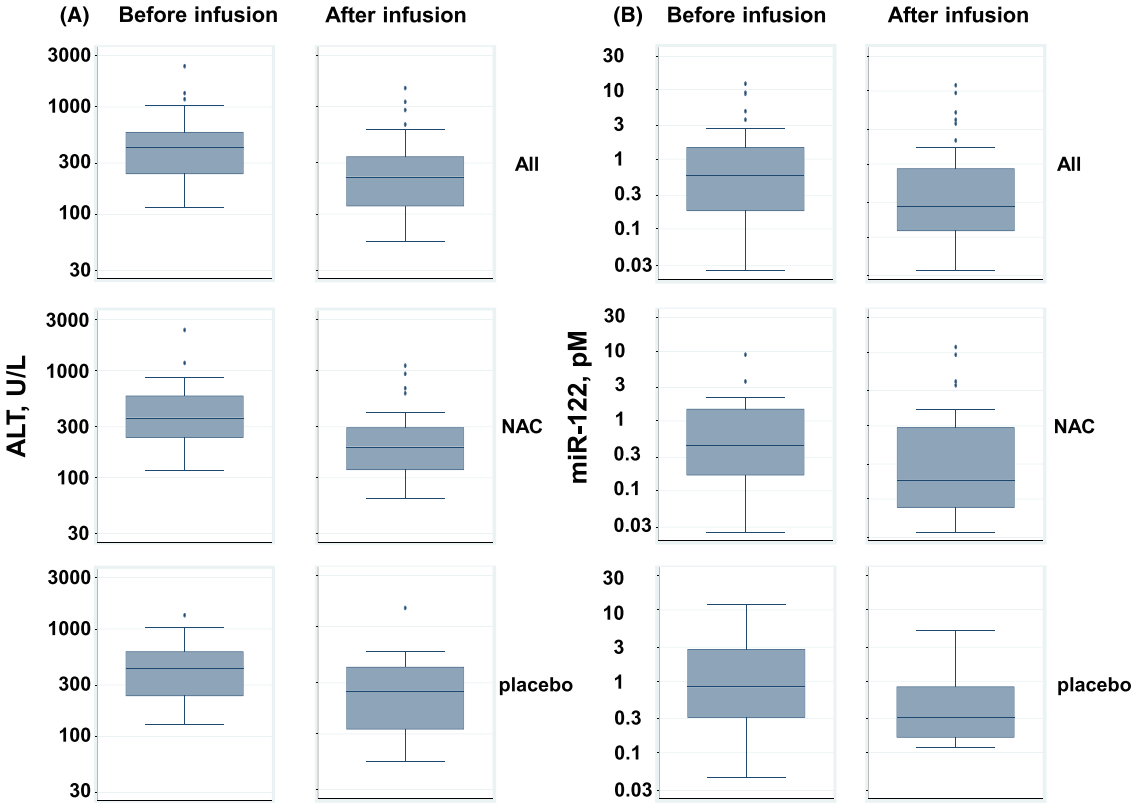

FIGURE 1 Box and whisker plots of (A) alanine aminotransferase (ALT) and (B) microRNA-122 (miR-122) serum concentrations before and after study infusion in 45 participants in a randomized placebo-controlled trial of intravenous N-acetylcysteine (NAC) in the management of antituberculosis drug-induced liver injury

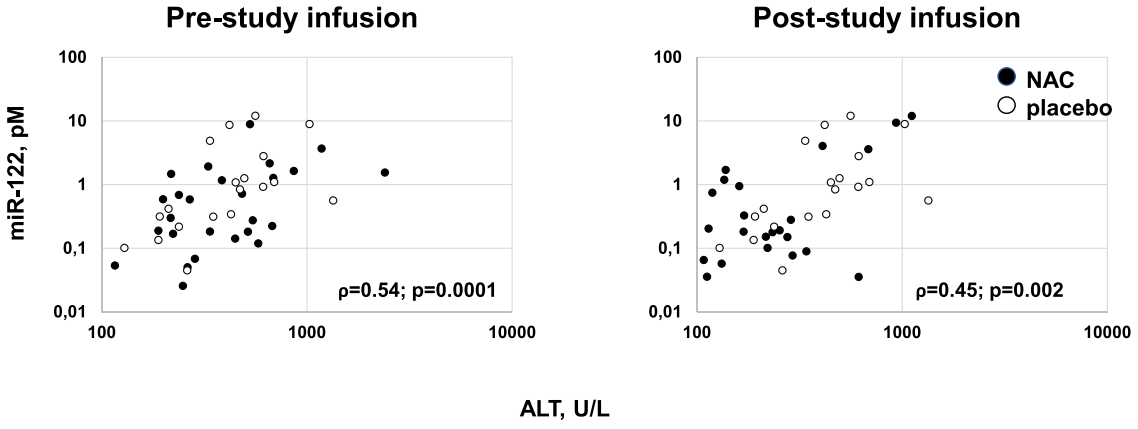

FIGURE 2 Correlation of pre- and post-study infusion serum alanine aminotransferase (ALT) and microRNA-122 (miR-122) concentrations in 45 participants with antituberculosis drug-induced liver injury randomized to intravenous N-acetylcysteine (NAC) or placebo

3.5 | Change in ALT and miR-122 concentrations

ALT concentrations decreased between samples in 43 (96%) participants. In contrast, the magnitude and direction of change in miR-122 concentrations varied substantially between participants, and miR-122 concentrations increased in 16 (36%) participants (10/26 in NAC group and 6/19 in placebo group) (Figure 3).

The median fold-change in ALT concentrations between samples was 0.56 overall (IQR 0.43-0.69) and was similar in the NAC and placebo groups: median 0.55 (0.39-0.78) and 0.60 (0.46-0.69), respectively (Wilcoxon rank sum test $P = .40$) (Supporting Information Figure S2). The median fold change in miR-122 concentrations was 0.75 (IQR 0.23-1.53) overall and was similar in the NAC and placebo groups; median 0.78 (IQR 0.23-1.53) and 0.54 (IQR 0.20-1.62),

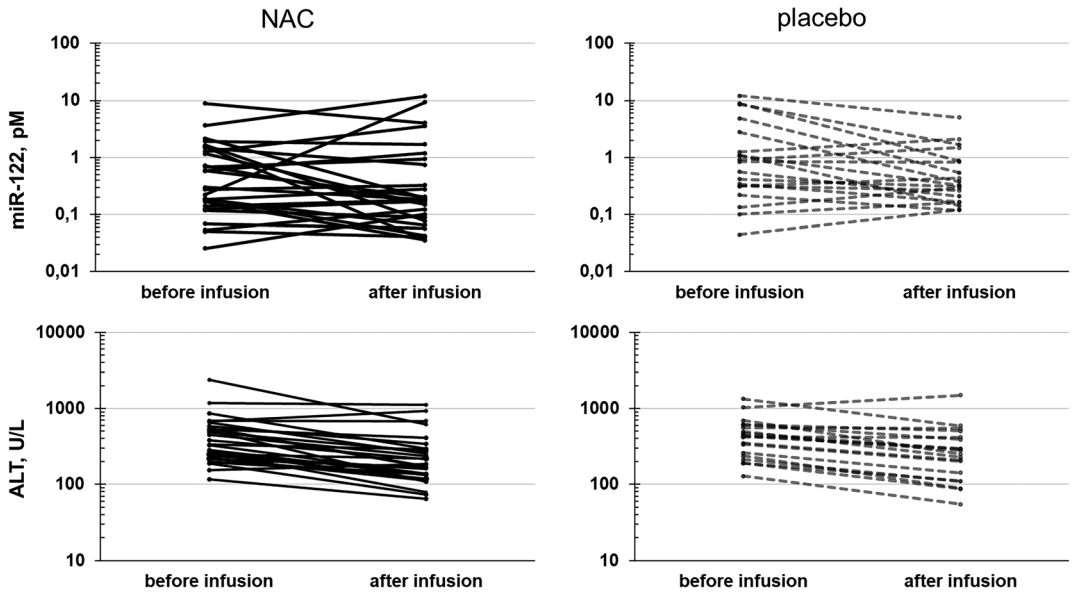

FIGURE 3 Change in serum alanine aminotransferase (ALT) and microRNA-122 (miR-122) concentrations after study infusion in 45 participants with antituberculosis drug-induced liver injury randomized to intravenous N-acetylcysteine (NAC) or placebo

respectively (Wilcoxon rank sum test $P = .68$) (Supporting Information Figure S2; note that fold change value greater than 1 corresponds to an increase).

3.6 | Change in ALT and miR-122 concentrations per day

ALT concentrations changed a median of 0.79-fold per day (IQR 0.75-0.83-fold) between the two biomarker samples. miR-122 concentrations changed a median of 0.89-fold per day (IQR 0.49-1.17-fold) between the two biomarker samples.

4 | DISCUSSION

The miR-122 concentrations observed in our participants with AT-DILI were much higher than those previously observed in healthy volunteers[20] or participants on antituberculosis therapy without DILI.[23] We found that serum ALT and miR-122 concentrations were correlated at both sampling occasions in participants with AT-DILI. ALT concentrations declined in almost all participants at the post-infusion sampling occasion. In contrast, changes in miR-122 concentrations were more variable, showing an increase in over a third of participants. The fold change in miR-122 concentrations between samples did not differ between NAC and placebo arms.

The median miR-122 concentration before study infusion in our cohort was 0.58 pM, which is 10 times higher than the upper limit of normal (ULN) of the reference range for healthy volunteers derived from the European Safer and Faster Evidence based Translation (SAFE-T) cohort, and 20 times the ULN from the Critical Path Institute's Predictive Safety Testing Consortium (PSTC) in the United States.[20] Only two of our participants had concentrations below the ULN derived from the SAFE-T cohort, and none were below the PSTC ULN.

In the ALISTER study cohort in Scotland, participants starting antituberculosis therapy without liver injury had similar miR-122 concentrations to healthy volunteers. miR-122 concentrations increased slightly after starting antituberculosis therapy but remained considerably lower (median 0.004 pM) than the miR-122 concentrations observed in our participants with AT-DILI.[23]

There are limited data on miR-122 concentrations in patients with AT-DILI. Two participants in the ALISTER cohort developed DILI with ALTs of 431 and 194 U/L, and miR-122 concentrations of 0.06 and 0.34 pM, respectively. In both these participants, miR-122 concentrations increased considerably from baseline, by 15- and 20-fold, respectively. A recent Indian study by Bakshi et al found miR-122 expression to be 75% lower in participants with AT-DILI compared to healthy controls,[24] but miR-122 was only sampled at enrolment and participants had less severe DILI than in our study, with a median ALT of 120 U/L. Despite these differences, the reason for the conflicting results is unclear and requires further exploration.

miR-122 concentrations varied widely between participants in our study, as did the intra-individual change in miR-122 between sampling occasions. In the absence of other clinical evidence, it is unclear whether these changes in miR-122 concentrations indicate worsening or improvement of the liver injury or merely reflect high intra- and inter-individual variability. In the PSTC healthy volunteer cohort who were sampled repeatedly over 3 weeks, both inter-subject and intra-subject coefficients of variation for miR-122 were high, at 91% and 94%, respectively.[20] Intra-individual variability was particularly high in Black participants.[20] A North American healthy volunteer

cohort also found high intra- and inter-donor variability in miR-122 expression, particularly in participants who identified as non-Caucasian.[25] Inter-individual variability poses challenges to defining cut-offs for abnormal elevation of miR-122. Intra-individual variability complicates interpretation of changes observed in miR-122 concentration over time. Inter-individual and intra-individual variability may therefore limit utility of miR-122 as a biomarker in diagnosing AT-DILI and monitoring AT-DILI progression or recovery.

We observed an increase in miR-122 concentrations between the two sampling occasions in a third of our study participants. The hepatocellular injury in AT-DILI[26] may be more sustained than injury due to oxidative stress injury in paracetamol overdose, and miR-122 may therefore decline more slowly in AT-DILI. Even among studies of paracetamol-induced hepatotoxicity, there are some that describe increased miR-122 concentrations from 3 to 14 days after DILI onset.[15,17]

Our study has limitations. We only included RCT participants with two biomarker samples (approximately half of the cohort), which may have introduced selection bias. However, baseline characteristics and liver biochemistry did not differ substantially between those RCT participants included in this study and those not included. HIV prevalence was high in our cohort and therefore our findings may not be generalizable to other populations with lower HIV prevalence. We only quantified miR-122 at two time points. We did not have a control group without AT-DILI drawn from the same population to allow for comparison. Different quantification and normalization methods used in different studies may influence comparisons between our results and results from other studies.

5 | CONCLUSION

miR-122 concentrations were markedly higher in our cohort of AT-DILI than previously observed in healthy controls and in participants on antituberculosis therapy without liver injury. miR-122 may therefore be a useful biomarker to diagnose AT-DILI in this population. However, high intra-individual and inter-individual variability in miR-122 concentrations may limit its utility. To characterize miR-122 concentrations prior to liver injury onset and in early AT-DILI, a large prospective cohort study collecting repeated samples for biomarker quantification from patients on antituberculosis therapy, with frequent clinical review, would be required because AT-DILI occurs in a small subset of patients on antituberculosis therapy. Further larger studies monitoring miR-122 over the full course of recovery from AT-DILI are required to characterize changes in this biomarker over time, and associations between concentrations observed and outcomes.

COMPETING INTERESTS

All authors declare that they have no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work. MP has received partnership funding for the following: MRC Clinical Pharmacology Training Scheme (co-funded by MRC and Roche, UCB, Eli Lilly and Novartis) and a PhD studentship jointly funded by EPSRC and AstraZeneca. He also has unrestricted educational grant support for the UK Pharmacogenetics and Stratified Medicine Network from Bristol-Myers Squibb. He has developed an HLA genotyping panel with MC Diagnostics but does not benefit financially from this. He is part of the IMI Consortium ARDAT (www.ardat.org). None of this funding has been used for the current paper.

CONTRIBUTORS

Muhammed Shiraz Moosa, Karen Cohen, Gary Maartens, Dan Carr and Munir Pirmohamed conceived and designed the study. Giusy Russomanno, Chandni Patel, Eithne Costello and Christopher Goldring were responsible for performance and oversight of miR-122 assays and interpretation of laboratory results. Christopher Goldring, Karen Cohen and Jeffrey R. Dorfman formulated the data analysis strategy. Muhammed Shiraz Moosa and Jeffrey R. Dorfman analysed data. Muhammed Shiraz Moosa wrote the first draft of the manuscript. All authors reviewed the manuscript and contributed to revising and finalizing the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are stored in a controlled access repository and are not openly available due to reasons of sensitivity and patient confidentiality. Data are available from the corresponding author (Karen Cohen) upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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