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AIMS

Liver-enriched microRNA-122 (miR-122) is a novel circulating biomarker for drug-induced liver injury (DILI). To date, miR-122 has been measured in serum or plasma venous samples. If miR-122 could be measured in capillary blood obtained from a finger prick it would facilitate point-of-care testing, such as in resource-limited settings that have a high burden of DILI.

METHODS

In this study, in healthy subjects, miR-122 was measured by polymerase chain reaction in three capillary blood drops taken from different fingers and in venous blood and plasma ($n = 20$). miR-122 was also measured in capillary blood obtained from patients with DILI ($n = 8$).

RESULTS

Circulating miR-122 could be readily measured in a capillary blood drop in healthy volunteers with a median (interquartile range) cycle threshold (Ct) of 32.6 (31.1–34.2). The coefficient of variation for intraindividual variability across replicate blood drops was 49.9%. Capillary miR-122 faithfully reflected the concentration in venous blood and plasma (Pearson $R = 0.89$, $P < 0.0001$; 0.88, $P < 0.0001$, respectively). miR-122 was 86-fold higher in DILI patients [median value 1.0×10^8 (interquartile range 1.89×10^7–3.04×10^9) copies/blood drop] compared to healthy subjects [1.85×10^6 (4.92×10^5–5.88×10^6) copies/blood drop]. Receiver operator characteristic analysis demonstrated that capillary miR-122 sensitively and specifically reported DILI (area under the curve: 0.96, $P = 0.0002$).

CONCLUSION

This work supports the potential use of miR-122 as biomarker of human DILI when measured in a capillary blood drop. With development across DILI aetiologies, this could be used by novel point-of-care technologies to produce a minimally invasive, near-patient, diagnostic test.

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Drug-induced liver injury (DILI) is a major healthcare challenge in western countries and in resource-limited settings.
- microRNA-122 (miR-122) has substantial promise as a sensitive and specific biomarker of hepatocyte injury when measured in venous samples.

WHAT THIS STUDY ADDS

- miR-122 can be quantified reliably in a capillary blood drop from a finger prick.
- Capillary miR-122 faithfully reflects the plasma and venous whole blood concentration.
- Capillary miR-122 can identify patients with DILI with high sensitivity and specificity.
- If combined with a novel point-of-care detection platform, capillary miR-122 could allow near patient testing for DILI.

Introduction

Drug-induced liver injury (DILI) presents a major burden to clinical medicine and is a common cause of drug failure during clinical development [1]. In western clinical medicine, about half of the cases of acute liver failure are caused by DILI [2]. In the developing world, cotreatment of human immunodeficiency virus (HIV) and tuberculosis (TB) is a major cause of DILI. Globally, an estimated 37 million people are HIV-positive, with eastern and southern Africa carrying the highest burden with an estimated 19 million people infected [3]. The South African, TB incidence is particularly high; new diagnoses being 834 per 100 000 *per annum* [4]. TB prevalence is high in people coinfected with HIV, with 42% of HIV-positive TB cases receiving both TB and antiretroviral treatment [5]. DILI complicates TB treatment in up to 33% of cases [6], and in South Africa the in-hospital mortality from DILI has been reported to be around 30% [5].

MicroRNAs (miRNAs) are small (~22 nucleotides long) nonprotein-coding RNAs involved in post-transcriptional gene regulation [7]. In the circulation, miRNAs are protected from degradation by binding to RNA protein complexes (such as argonaute 2) and high-density lipoproteins, and being encapsulated in extracellular vesicles such as exosomes [8, 9]. As miRNAs are amplifiable and some are tissue enriched [10], they have emerged as a reservoir for the discovery of biomarkers that report organ injury [11].

The liver enriched miRNA-122 (miR-122) is a circulating biomarker of DILI. miR-122 is released into the circulation when hepatocytes are injured and is a translational safety biomarker across zebrafish [12], rodents [13] and humans [14–16]. In humans, miR-122 is around 100-fold higher in paracetamol overdose patients with DILI compared to those patients without liver injury [17] and is able to report DILI soon after overdose when serum alanine transaminase (ALT) activity is still in the normal range [14, 15, 18]. Circulating miR-122 is not DILI specific but is specific for hepatocyte injury. It is also increased in patients with cholestyramine-induced liver injury [19], ischemic hepatitis [20], viral hepatitis [21] and cholestatic liver injury [22]. In these published studies serum or plasma venous samples have been analysed in specialist laboratories with time-consuming and expensive kits. There is an unmet need for assays that can rapidly and accurately measure miRNA at the point-of-care (POC) [23]. Ideally, a POC assay would measure miR-122 in a single blood drop from a finger prick, be affordable for use in resource-limited settings and suitable for use near, or actually in, a patient's home [24]. Such an assay could provide an early signal of DILI in patients at elevated risk, for instance, following prescription of antimicrobials with a significant DILI liability [25–27]. With development, serial monitoring of miR-122 could improve patient safety by allowing medication change before life-threatening liver failure develops and by supporting safe reintroduction of treatment after interruption. In commercial drug development, measurement of miR-122 using a finger prick could reduce the need for venepuncture, which is especially advantageous in certain groups such as children and when multiple serial measurements in the same person are required.

The aims of this study were to determine if miR-122 can be measured in a capillary blood drop from a finger prick and to compare the concentration with venous blood and plasma; to assess the intraindividual variability of capillary miR-122 concentration and to establish proof of concept as to whether capillary miR-122 can report DILI in patients.

Material and methods

The study was approved by the research ethics committee (East Midlands – Nottingham 1 Research Ethics Committee) and performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants.

Healthy volunteers

Healthy volunteers were eligible if they had no history of liver disease, were taking no medications and were willing to give blood samples by venepuncture and finger prick.

Drug-induced liver injury patients

A total of eight adult patients (age 24–82 years) admitted to the Royal Infirmary of Edinburgh, UK with DILI were entered into the study. In each patient, causality of liver injury was scored as *definitive* by the Roussel Uclaf Causality Assessment Method [28].

Blood collection

Blood was collected in EDTA tubes by venepuncture. Immediately, a 50 μL aliquot was collected in 1 mL of Qiazol (Qiagen, Venlo, Netherlands) for whole blood analysis. The remaining blood was centrifuged at 11 000 × *g* for 15 min at 4°C after which the supernatant was separated into aliquots and frozen at –80°C until miRNA extraction.

Table 1

Copy numbers of capillary miR-122 per blood drop (BD1: index finger; BD2: middle finger; BD3: ring finger) in healthy volunteers. The coefficient of variation (CV) across the three blood drops is presented

Healthy volunteer number	BD1 (copy/drop)	BD2 (copy/drop)	BD3 (copy/drop)	CV (%)
1	0.45×10^6	0.40×10^6	1.53×10^6	81.13
2	3.39×10^6	11.5×10^6	4.11×10^6	71.14
3	0.29×10^6	0.41×10^6	0.11×10^6	54.52
4	0.24×10^6	0.11×10^5	0.20×10^6	37.15
5	0.59×10^6	0.17×10^6	0.42×10^6	54.17
6	1.25×10^6	0.91×10^6	0.69×10^6	29.85
7	0.11×10^6	0.20×10^6	0.28×10^6	43.24
8	2.85×10^6	5.20×10^6	1.18×10^6	65.70
9	1.53×10^6	0.89×10^6	0.69×10^6	42.28
10	0.29×10^6	0.23×10^6	4.45×10^6	146.1
11	0.13×10^6	0.14×10^6	0.19×10^6	20.47
12	1.37×10^6	2.81×10^6	1.93×10^6	35.53
13	2.09×10^6	0.64×10^6	1.20×10^6	56.18
14	1.53×10^6	6.40×10^6	5.52×10^6	57.85
15	5.06×10^6	9.52×10^6	8.79×10^6	30.74
16	10.6×10^6	9.07×10^6	10.3×10^6	8.00
17	7.61×10^6	9.37×10^6	5.70×10^6	24.26
18	1.81×10^6	3.00×10^6	5.18×10^6	51.21
19	4.70×10^6	2.69×10^6	2.79×10^6	33.44
20	36.6×10^6	36.3×10^6	10.3×10^6	54.38
Mean	4.12×10^6	5.00×10^6	3.28×10^6	49.87

Three finger-prick blood drops (BD1: index finger, BD2: middle finger, BD3: ring finger) per healthy volunteer were obtained using disposable lancets that are used in routine clinical practice for glucose measurement (Accu-Chek, Roche, Basel, Switzerland – adjustable depth settings 1.8 mm). In DILI patients, one blood drop from the index finger was collected. After blood drop collection, Qiazol (1 ml) was added to each sample. All samples were stored at –80°C until analysis.

MicroRNA extraction

MicroRNA was extracted using miRNeasy Serum/Plasma kit (Qiagen), following the manufacturer's instructions. For venous blood and plasma, 50 µl of sample was used in combination with 150 µl nuclease free water. For capillary blood, 200 µl nuclease free water was added to the Qiazol containing each blood drop.

Real-time polymerase chain reaction

From each sample, 2.5 µl of RNA eluate was reverse transcribed into cDNA using the miScript II RT Kit (Qiagen) following manufacturer's instructions. The synthesized cDNA was 5-fold diluted and used for cDNA template in combination with the miScript SYBR Green polymerase chain reaction (PCR) Kit (Qiagen) using the specific miScript assays (Qiagen). Real-time PCR was performed in duplicate on a Light Cycler 480 (Roche) using the recommended miScript cycling parameters.

Absolute quantitation of miRNA was achieved by generating a standard curve using synthetic target. Standard curves were generated by reverse transcribing known concentrations of miScript miRNA mimics (Qiagen, Venlo, The Netherlands) in 0.1X TE buffer spiked with 10 ng μl^{-1} Poly-C (Sigma–Aldrich, Gillingham, UK). The resulting cDNA was measured using serial dilutions on three different plates on 3 different days to demonstrate minimal variability [interassay coefficient of variation (CV): 3.4%]. The calibration curve was linear in the cycle threshold (Ct) range of 20.0–36.1. A Ct value of 37.1 was obtained in water control.

Statistical analysis

Statistical differences, correlations and receiver operator characteristic (ROC) curve analyses were performed using Graphpad Prism (GraphPad Software, La Jolla California, USA). Nominal statistical significance was set at $P < 0.05$.

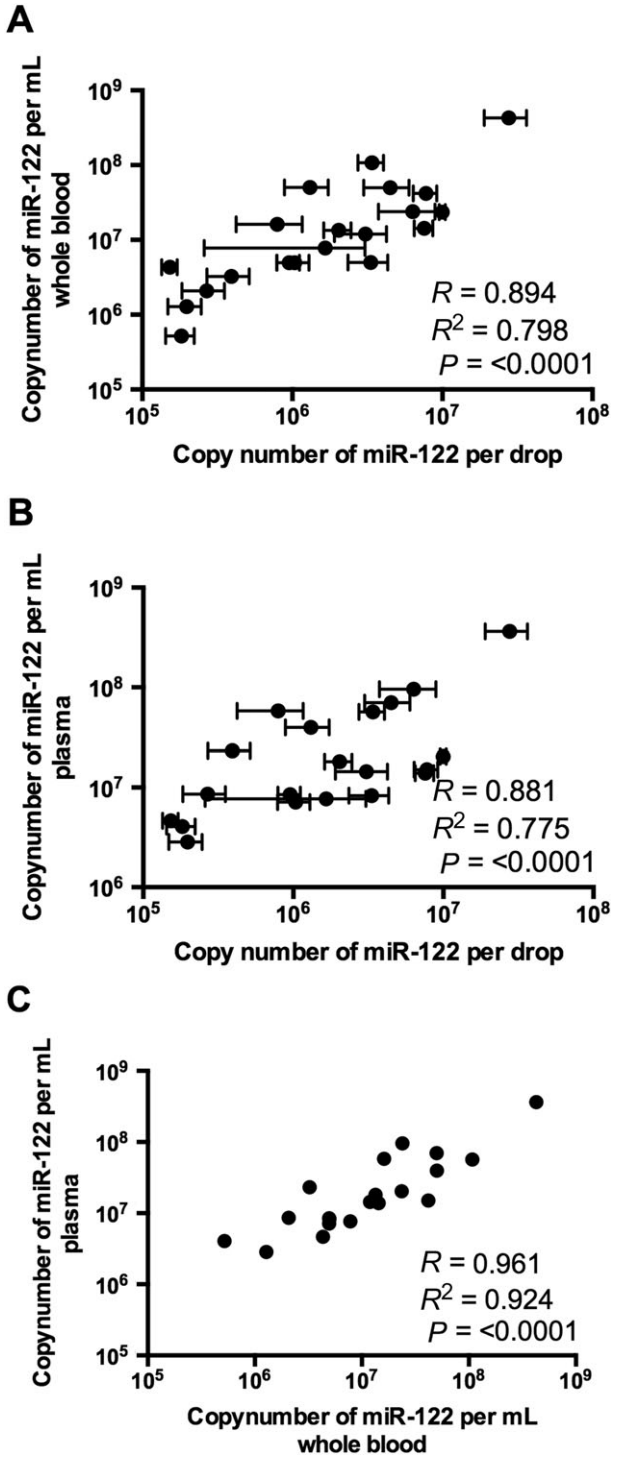

Figure 1

Scatter graphs. Correlation between copy numbers of miR-122 per mL (A) venous blood or (B) plasma and copy numbers of miR-122 per capillary blood drop. (C) correlation between copy numbers of miR-122 per mL venous blood and plasma in each healthy volunteer (n = 20). Pearson R values are 0.89 ($P < 0.0001$), 0.88 ($P < 0.0001$) and 0.92 ($P < 0.0001$), respectively (Pearson's correlation test). Blood drop values represent the mean copy number measured in three drops, error bars represent standard errors of the mean

Results

Capillary miR-122 can be measured in a finger prick blood drop

A total of 20 adults (14 females, median age 24 years; range 21–31 years) were recruited to this study. First it was determined whether a capillary blood drop yields sufficient miR-122 for robust quantification. Capillary blood Ct values (obtained by quantitative PCR) were all within the linear range of the calibration curve [mean (range) 32.6 (29.1–35.4)]. Copy numbers of miR-122 per blood drop in the healthy controls are presented in Table 1. Across the replicate drops from different fingers the mean CV (± standard deviation) was 49.9 ± 28.9%. The CV ± standard deviation of duplicate PCR measurements of the same blood drop was 0.94 ± 1.29%.

Capillary miR-122 correlates with venous blood and plasma

Across the healthy volunteers, the relationship between copy numbers of miR-122 per blood drop and copy numbers of miR-122 per mL of venous blood and plasma was determined. Copy number of miR-122 per blood drop significantly correlated with miR-122 measured in venous blood and plasma ($P < 0.0001$, Figure 1A, B). The correlation coefficients (R^2) were 0.80 and 0.78 and the Pearson R values [95% confidence interval (CI)] were 0.89 (0.75–0.96) and 0.88 (0.72–0.95), both $P < 0.0001$, in venous blood and plasma, respectively. As would be expected there was a significant correlation between venous blood and plasma miR122 (Figure 1C).

Liver-enriched miRNA-122 is higher in ALI patients

Capillary miR-122 was measured in blood drop samples obtained from patients with DILI (n = 8) and compared with healthy volunteers (n = 20). Clinical parameters of the DILI patient cohort are summarized in Table 2, along with their capillary miR-122 concentrations. In the single case of nonparacetamol DILI (induced by nitrofurantion) other causes of liver disease such as viral hepatitis (A–E) were excluded. miR-122 was increased 86 fold in DILI patients [median 1.58×10^8 (interquartile range 4.67×10^6–4.51×10^9) copies/blood drop] compared to healthy volunteers [1.85×10^6 (1.53×10^5–2.77×10^7) copies/blood drop] P = 0.004 (Figure 2). ROC analysis was performed to determine the sensitivity and specificity of miR-122 for detecting DILI (Figure 3). Capillary miR-122 had high sensitivity and specificity (area under the curve; 0.96 (95%CI 0.89–1.04), P = 0.0002, sensitivity: 86% at 90% specificity).

Discussion

This study has demonstrated, for the first time, that capillary miR-122 can be measured in a single blood drop to report DILI. This facilitates point-of-care measurement out with hospital, such as in the developing world where the burden of DILI is substantial.

Table 2

Clinical parameters of the patient cohort with drug-induced liver injury (DILI).

Patient	Age (years)	Sex	ALT activity ($U\ l^{-1}$)	INR	Serum creatinine ($\mu mol\ l^{-1}$)	ALP activity (U/L)	Bilirubin ($\mu mol\ dl^{-1}$)	Aetiology	Copies miR-122/ drop
1	82	F	3475	2	50	74	29	Paracetamol	1.58×10^8
2	44	F	10 543	6.9	47	101	106	Paracetamol	4.51×10^9
3	54	F	1210	8.9	66	236	162	Nitrofurantoin	4.09×10^7
4	24	F	1608	1.4	79	239	224	Paracetamol	1.58×10^8
5	29	M	1143	1.7	52	43	17	Paracetamol	5.64×10^7
6	25	M	1653	1.9	72	147	58	Paracetamol	1.66×10^9
7	45	M	1131	1.5	65	79	34	Paracetamol	3.50×10^9
8	35	M	1101	1.8	57	141	32	Paracetamol	1.16×10^7

ALT, alanine aminotransferase; INR, international normalized ratio; serum creatinine; ALP, alkaline phosphatise; bilirubin and aetiology of DILI are presented

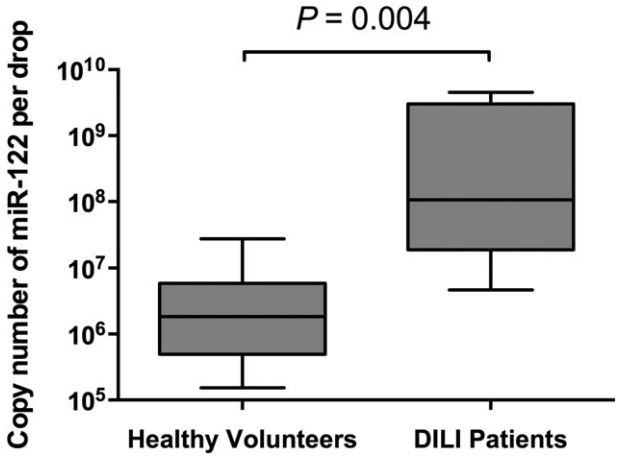

Figure 2

Copy number of miR-122 per blood drop from healthy volunteers (n = 20) and drug-induced liver injury patients (n = 8). Data are presented as a Tukey plot. In healthy volunteers, the mean copy number measured in three drops was used

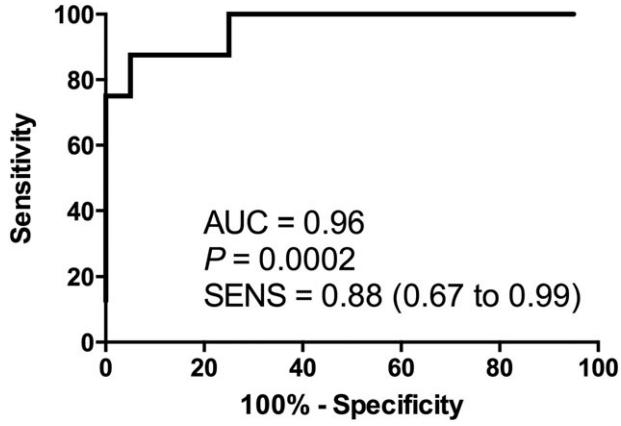

Figure 3

Receiver operator characteristic curve analysis with respect to blood drop miR-122 as a discriminator of drug-induced liver injury patients from healthy volunteers. Area under the curve (AUC), statistical significance and sensitivity (SENS) at 90% specificity (95%CI) are presented

When measured in capillary blood, the miR-122 Ct values were all within the quantifiable range of the PCR assay that had a linear calibration curve up to a Ct of 36. Intraindividual variability was tested by comparing three different blood drops taken from the same volunteer and resulted in an average CV of around 50%. Respectively, the intra-assay and interassay CVs of the PCR assay were only 0.94% and 3.4%, therefore the CV across the blood drops probably represents the variable volumes of the blood drops obtained during the collection procedure. The intraindividual CV of blood drop volume obtained from a finger has been reported to be 83% [29]. This is comparable to the intraindividual CV of miR-122 measured in our study. In a future POC assay, the variability of sample volume could be reduced by automated microchip sample processing technologies [30, 31] as already applied in test strips for international normalized ratio (INR) POC testing in the context of warfarin dosing [32]. Furthermore, as the circulating concentration of miR-122 increases up to a 100 fold in DILI patients [14], a CV of 50% would be expected to have little effect on the detection of DILI.

The concentration of capillary miR-122 measured in blood drops strongly correlated with blood and plasma obtained from venepuncture. This provides reassurance that our data reflect circulating concentrations. Furthermore, capillary miR-122 was significantly higher in blood drops from patients with DILI compared to healthy volunteers with a median fold increase of 86 and a ROC curve area under the curve of 0.96. These data confirm that the dynamic changes and the sensitivity to report DILI is similar between blood drops and earlier reported results from serum/plasma venous samples [14, 15]. A challenge in using circulating miRNAs as

biomarkers for human pathology is that the contribution of different tissues to the circulating pool is often unknown. Most miRNAs are expressed in multiple cell types, by contrast miR-122 is highly specific for the liver [33, 34]. miR-122 is not expressed in platelets, T-cells, B-cells, granulocytes or erythrocytes, which contain a wide variety of other miRNA species [35]. This makes miR-122 suitable for accurate measurement in whole blood without need for plasma or serum isolation.

There is an urgent need for improved DILI monitoring in the developing world where cotreatment of HIV and TB is a common cause [5]. However, despite the need, DILI monitoring in resource-limited settings is often restricted by practical concerns. The requirement for expensive tools and highly trained technicians can mean testing is only done in centralized or regional laboratories [24]. Moreover, many patients undergoing TB treatment in resource-limited settings have a negative association with venepuncture itself, do not have a primary care physician and do not value regular visits to a health care professional for health maintenance, which reduces potential participation in DILI monitoring [36]. A rapid POC test for measuring miR-122 from a single blood drop would mean that the patient undergoing TB and/or HIV treatment could use the assay at home (or near home). This study has demonstrated that a blood drop can be used as the matrix to measure miR-122. Recently, substantial effort has been spent in developing highly sensitive, rapid, reliable and low-cost methods for measuring miRNAs in minimal sample volumes. Electrochemical DNA hybridization sensors have potential as detection techniques in a POC test because this technology can detect specific miRNAs in the attomolar range without PCR amplification [37]. Other promising miRNA detection methods include nanoparticle-based optical technologies [38], surface plasmon resonance [39, 40] and amplification-free fluorescence-based assays [41, 42]. As miR-122 is a relatively high-concentration, organ-specific, circulating miRNA with a large dynamic range in disease it represents an ideal target for assay development with line of sight on a commercial product tackling a global health need.

This is an early phase proof of concept study that predominately used paracetamol toxicity as the model of DILI (seven of eight patients). Work is now required to determine whether capillary miR-122 has clinical utility in DILI caused by other drugs, especially antimicrobials. In conclusion, this work supports the potential use of miR-122 as biomarker of human DILI when measured in a blood drop from a finger prick. This could be used by novel POC technologies to produce a minimally invasive, near patient, diagnostic for DILI that has enhanced sensitivity and specificity compared to current tests.

Competing Interests

All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization for the submitted work; 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.

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Contributors

The experiments were performed by A.D.B.V., C.B. and C.P. Analysis was by M.K.K. and the study was co-ordinated by J.W.D.

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