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Abstract

BACKGROUND Cardiovascular disease (CVD), the leading causes of death worldwide, correlates with inflammation and shortens life span in type 1 diabetes (T1DM). In animals, miR-342 were shown to be anti-inflammatory in CVD. We hypothesise: proangiogenic miR-342-3p/-5p are downregulated in T1DM whereas inflammatory cytokines are upregulated.

METHODS We studied plasma/peripheral blood mononuclear cells (PBMCs) in 29 T1DM and 20 controls (HC). Vascular health was measured by Fibronectin Adhesion Assay (FAA), CD45dimCD34+133+ cells (flow cytometry) and Tissue inhibitor of metalloproteases (TIMP-1). Inflammatory markers IL-7, IL-8, TNFα and VEGF-C were assayed by Mesoscale assay, mRNA for chemokine receptor CXCR1/2, PANX2 and miR 342-3p/5p by qRT-PCR. Analysis: Unpaired Student t test, Pearson correlation and Ingenuity Pathway Analysis (IPA) to predict miR-342-3p/-5p targets.

RESULTS In T1DM, pro-inflammatory cytokines IL-8 and TNF-α, IL-7 and growth factor VEGF-C were increased versus HC; p=0.008, p=0.003, p=0.041 and p=0.013 respectively. MiR-342-3p/-5p were downregulated in PBMCs in T1DM versus HC; p=0.01, but not in plasma. PANX2, CXCR1 and CXCR2 mRNA were increased in PBMCs in T1DM versus HC; p=0.004, p=0.017 and p<0.001 respectively.

MiR-342-3p correlated negatively with TIMP-1, IL-6, IL-8, TNF-α, HbA1c; p=0.006, p=0.031, p=0.029, p=0.038, p=0.001, p=0.031 whilst miR-342-5p with TIMP-1, IL-6, IL-8, HbA1c, p=0.005, p=0.034, p=0.029, p=0.001, p=0.042 respectively. There was positive correlation between miR-342-3p and FAA, CXCR1, CXCR2, p=0.006, p=0.019, p=0.001 and between miR-342-5p and FAA, CXCR2; p=0.038, p=0.036 respectively. IPA predicted miR-342-3p/-5p to be anti-inflammatory through the indirect regulation of mitogen-activated protein 3 kinase 1, inhibitor of nuclear factor kappa B kinase regulatory subunit Gamma, Surfactant protein A1 and PANX2 as well as predicting IL-8, TNF-α, IL-7 and glucose to activate the inflammatory response. ROC curve analyses showed: miR-342-3p/-5p to be a biomarker for T1DM: p=0.01,
p=0.006 respectively (2) significant downregulation of miR-342-3p/-5p has occurred at HbA1c > 46.45 mmol/mol, p=0.006, p=0.005 respectively.

CONCLUSION
Our findings validated animal studies. Downregulation of miR-342-3p/-5p by hyperglycaemia and increase in cytokines confirmed inflammation in T1DM. MiR-342-3p/5p correlated with indices of vascular health and defined early CVD at HbA1c of 46.5 mmol/mol cut off point. MiR-342-3p/-5p based either intervention or monitoring may prove to be beneficial in CVD in T1DM.

Keywords
CXCR1/CXCR2, PANEX2, TNF-α, inflammation, miR-342-3p/-5p, therapeutic targets, TIMP -1, T1DM, CVD

Background
Inflammatory processes have been shown to be key to the development and complications of cardiovascular disease (CVD) [1]. CVD is currently the predominant cause of early mortality in type 1 diabetes patients. Data has suggested life expectancy decreases by approximately 13 years in type 1 diabetes patients [2]. Evidence suggests CVD is strongly correlated with pro-inflammatory cytokines, such as TNF-α. Inflammatory cytokines have been proven to act as biomarkers for diseases including cardiovascular disease however, other biomarkers are becoming apparent including microRNAs (miRNA) [3].

MiRNAs are highly conserved, non-coding RNA molecules of approximately 22 nucleotides long that exert post-transcriptional effects on gene expression[4]. They are expressed in a tissue- and cell-type specific manner and play essential roles in many biological processes including inflammation. Since the discovery of miRNAs in 1993 they have been established as novel biomarkers for many different conditions including cardiovascular diseases [5]. This group of gene expression regulators have also been described in Type 1 diabetes mellitus patients [6] and there are many miRNAs that have been seen to have potential to act as biomarkers for type 1 diabetes mellitus [7]. However, the understanding of specific miRNA roles in this disease is lacking.

Currently there are strong links between miRNA and their role in many cellular pathways including cell proliferation, apoptosis and immune response pathways to name a few [8]. This introduces the idea that miRNAs, may be responsible for regulation of inflammation associated with type 1 diabetes mellitus and the association between their regulation and cardiovascular health. Previous evidence has demonstrated a strong correlation between type 1 diabetes mellitus and increased risk of cardiovascular disease [9]. If miRNAs could modulate inflammatory cytokine concentration and cell adhesion for example, this in turn could have a positive impact on cardiovascular health and CVD risk in type 1 diabetes patients.

Out of 2000 miRNAs that have been discovered in humans, miR-342-3p/-5p appear to have great potential to repress inflammation in atherosclerosis [10][11]. As well as this, current studies have shown, therapeutic intervention of miR-342-3p led to the discovery this miRNA acts by increasing cell survival, motility and proliferation of osteoclast precursors [12]. Through these studies, it is clear this miRNA may play a role in type 1 diabetes and understanding its role in inflammation could lead to the new therapies to reduce the CVD risk in these patients.

We thus hypothesise that miR-342-3p/-5p are downregulated in type 1 diabetes mellitus patients and may be associated with deterioration of vascular health.
Methods

We recruited 29 patients with type 1 diabetes with inclusion criteria of HbA1c <8.5 % (69 mmol/mmol), absence of macrovascular disease or stage 3b renal impairment (eGFR <45 ml/min/1.73 m²) or active proliferative retinopathy. Those patients were matched with 20 age- and gender-matched non-diabetic healthy controls (HC). Patients with type 1 diabetes were recruited either from Queen Elizabeth Hospital, Gateshead or Royal Victoria Infirmary, Newcastle, UK. Power calculation was undertaken as part of previously published study to detect improvement in EPCs [13]. The minimum number of patients /healthy controls required was 20 in each group. The data from baseline from that study has been used for the comparison between type 1 diabetes patients and healthy controls in this sub-study.

Routine laboratory investigations (full blood count, U&Es, liver function test, thyroid function test, and HbA1c), 12-lead ECG, blood pressure, weight, height and BMI were performed.

Meso scale discovery (MSD) assay Plasma samples for patients and control were assayed using K15050D V-PLEX Cytokine Panel 1 human kit, K15049D V-PLEX Proinflammatory Panel 1 human kit, K15190D V-PLEX Angiogenesis Panel 1 human kit and K151JFC human tissue inhibitor of metalloproteinases-1 (TIMP-1) kit (Meso Scale Discovery, Rockville, MD) in accordance with the manufacturer protocol. Plates were read with MSD Sector Imager 2400 and data were analyzed by MSD Discovery Workbench version 2.0 software.

Flow cytometric evaluation of circulating endothelial progenitor cells Flow cytometry on BD FACS Canto™ II system was used previously by us to determine circulating epithelial progenitor cells (cEPCs) CD45dimCD34+CD133+[13].

Fibronectin adhesion assay (FAA) This assay was carried out as previously described.[13]

Extraction of microRNAs from plasma In order to extract microRNAs from plasma, blood samples collected were centrifuged for 15 minutes at 500x g to separate platelet-poor plasma. The platelet-poor plasma was extracted and centrifuged for a further 5 minutes at 13000x g. This plasma was stored at -80°C for further analysis. During this analysis the samples were tested for haemolysis. MicroRNA was extracted from the centrifuged plasma with a proprietary RNA isolation protocol optimized for serum/plasma by QIAGEN (Exiqon Services, Denmark). After extraction, the integrity of RNA samples was assessed using Agilent 2100 (Santa Clara, CA, USA) yielding RNA Integrity Numbers (RIN) between 9.1-10 (high).

Extraction of microRNA and mRNA from PBMCs To extract microRNAs and mRNAs from peripheral blood mononuclear cells (PBMC), peripheral blood was collected and PBMCs were isolated through Ficol separation after an overnight fast. After isolation, cells were lysed with Trizol lysis buffer and lysates were collected and stored at -80°C for analysis. Total RNA was extracted from PBMCs using miRNEasy Kit (QIAGEN, Hilden, Germany). After extraction, the integrity of RNA samples was assessed using Agilent 2100 (Santa Clara, CA, USA) yielding RNA Integrity Numbers (RIN) between 9.1-10 (high).

Assay of miRNA and mRNA using real-time quantitative PCR miRNAs were assayed in plasma and PBMCs with the miRCURY LNA RT Kit (QIAGEN, Hilden, Germany). 10 ng RNA was reverse transcribed resulting in cDNA which was diluted 100x and assayed in 10 µl PCR reactions in compliance with the procedure for miRCURY LNA miRNA PCR. Using qPCR on the microRNA Ready-To-Use PCR, each microRNA was assayed once with Pick and Mix using miRCURY LNA SYBR Green master mix. Both positive and negative controls from the reverse transcription reaction were performed and profiled similarly to the samples. An amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland) in 384 well plates. Using the Roche LC software, amplification curves were analysed to determine ΔCt values. Equation 1 was then used to calculate ΔΔCt values and Equation 2 to calculate fold change (see below for Equation 1 and 2).
IPA software version 9 (Ingenuity, Redwood City, CA, USA) aided in the identification of target genes, cellular functions and pathological states regulated by miR-342-3p or -5p.

Statistical analysis

Data were presented as mean ± SD, unless stated otherwise. The normality of the data was assessed by Shapiro-Wilk tests and were log transformed as appropriate. Comparison between two study groups were analysed using unpaired t-test or Mann Whitney U test. The associations were examined by Pearson correlation test. All statistical analyses were performed using IBM SPSS Statistics software version 25 (SPSSTM Inc., Armonk, NY, USA) at a significance level of $p = 0.05$.

**Results**

**Patient phenotypes** Type 1 diabetes patients were relatively well controlled ($HbA_1c$ 57.3±7.6 mmol/mol) with duration of diabetes of 22.4 ± 3.9 years. Patients were age- and sex-matched with healthy controls. (Table 1).

**MicroRNA-342 isoform expression**
Both miR-342-3p/-5p were significantly downregulated in type 1 diabetes patients versus HC; fold change = -1.4, $p=0.01$; fold change = -1.6, $p=0.01$ respectively in PBMCs but not in plasma (Fig 1f).

**Cytokine profiles** Pro-inflammatory cytokines IL-8 and TNF-α, homeostatic cytokine IL-7 and growth factor VEGF-C were increased in patients with type 1 diabetes (4.7±1.3 pg/ml, 1.6±0.2 pg/ml, 2.3±0.6 pg/ml and 63.2±20.3 pg/ml) respectively versus HCs (2.8±0.5 pg/ml, 1.4±0.2 pg/ml, 1.4±0.6 pg/ml, 50.8±48.2 pg/ml respectively; $p=0.003$, $p=0.041$, $p=0.008$ and $p=0.013$ respectively. In PBMCs CXCR1 and CXCR2 mRNA was significantly upregulated in type 1 diabetes compared to HCs; fold change = +3.16, $p = 0.017$ and fold change = +2.21, $p < 0.001$ respectively (Fig 1e). The CXCR1:CXCR2 mRNA ratio was unchanged between type 1 diabetes and HCs.

**Pannexin-2 (PANX2) (target of miR-342-3p) mRNA expression**
In PBMCs PANX2 mRNA was significantly upregulated in type 1 diabetes versus HCs; fold change = 2.26 $p=0.004$. (Fig 1e)

**Association between miR-342 isoforms and inflammatory markers**
In all studied subjects (type 1 diabetes patients and healthy controls), miR-342-3p was inversely correlated with IL-6 ($r=-0.496$, $p=0.031$), IL-8 ($r=-0.501$, $p=0.029$) and TNF-α ($r=-0.480$, $p=0.038$). There was also negative correlation between miR-342-5p and IL-6 ($r=-0.489$, $p=0.034$) and IL-8 (-0.500, $p=0.029$) (Fig 2a-d, h).

**Association between miR-342 isoforms and inflammatory receptors**
Both miR-342-3p/-5p were positively correlated with CXCR2; $r= 0.677$, $p=0.001$; $r=0.483$, $p=0.036$ respectively (Fig 2e-f). MiR-342-3p was also positively correlated with CXCR1 ($r=0.533$, $p=0.019$) (Fig 2g).

**Association between miR-342 isoforms and vascular health (FAA, CD45<sup>dim</sup>CD34<sup>+</sup>133<sup>+</sup> cells and TIMP-1)**
MiR-342-3p/-5p isoforms were both positively correlated with fibronectin adhesion assay (FAA); $r= 0.601$, $p=0.006$; $r= 0.479$, $p=0.038$ respectively. (Fig 3a-b). Moreover, miR-342-3p/-5p were positively correlated with endothelial progenitor cells, CD45<sup>dim</sup>CD34<sup>+</sup>CD133<sup>+</sup>; $r=0.635$, $p=0.004$, $r=0.523$, $p=0.022$ respectively. (Fig 3c and d). Whereas both isoforms were inversely correlated with TIMP-1; $r = -0.605$, $p= 0.006$; $r = -0.612$, $p=0.005$ respectively. (Fig 3e and f).
Correlation between miR-342 isoforms and HbA1c

Both miR-342-3p and -5p are negatively correlated with logHbA1c; r=-0.695, p=0.001; r=-0.564, p=0.002 respectively. (Fig 4a and b).

ROC analysis for miR342-3p and 342-5p

ROC curve analyses showed (1) miR-342-3p-to be a biomarker for T1DM (p=0.0105; AUC 0.8523) with sensitivity 75% and 91% specificity) (2) significant downregulation of miR-342-3p (p=0.0006, AUC 1) with sensitivity 100% and 85% specificity defined subclinical CVD at HbA1c > 46.45 mmol/mol, (6.4%), p=0.0006.

ROC curve analyses showed (1) miR-342-5p-5p to be a biomarker for T1DM (p=0.006) (AUC 0.8750) with sensitivity 75% and 91% specificity) (2) significant downregulation of miR-342-5p (AUC 0.89) with sensitivity 86% and 83% specificity defined subclinical CVD at HbA1c > 46.45 mmol/mol, (6.4%), p=0.005.

Discussion

We have validated in the current research animal findings on miR-342-3p/-5p. Furthermore we have confirmed a presence of inflammation in otherwise healthy patients within type 1 diabetes by showing the increased plasma concentrations of inflammatory IL-7, TNF-α, IL-6 as well as IL-8. In addition, we are reporting for the first-time downregulation of isoforms miR-342-3p/5p in PBMCs but not in plasma in patients with type 1 diabetes compared to HC. Moreover to our knowledge, we are the first to demonstrate negative correlation between miR-342-3p/5p and glycaemic control, TIMP-1 and TNF-α as well as positive correlations between miR-342-3p/-5p and vascular health (Fibronectin adhesion assay (FAA)), receptors of pro-inflammatory cytokine (CXCR1 and CXCR2 mRNA) and CD45dim CD34+ CD133+cells otherwise known as early endothelial progenitor cells.

Inflammatory markers in type 1 diabetes

Our results showed an increase in IL-7 in type 1 diabetes which implicated IL-7 has a role in inflammation in type 1 diabetes and this is concordant with other studies. In a recent mechanistic study it has been found that downregulation of IL-7 in intra-epithelial lymphocytes in colitis reduced inflammation, confirming its role in the inflammatory process. [14] Furthermore through blocking of receptor IL-7R one was able not only prevent but also reverse onset of diabetes in mice [15].

IL-8 was also seen to be increased in current study being concordant with others and our previous research [16] [17]. This cytokine plays a role in activating inflammatory cells including neutrophils and is modulated by differential activation of CXCR1/2 [18]. In line with our results, we observed increased CXCR1 mRNA and CXCR2 mRNA levels in type 1 diabetes. The upregulated CXCR1 and CXCR2 mRNA upon elevated IL-8 concentration acts as a positive feedback to provide continuous stimulation of inflammatory pathway.

As well as IL-7 and IL-8 being raised in our subjects, other pro-inflammatory cytokine, TNF-α was also increased. TNFα is a well-established cytokine involved in the inflammatory response. It presents during inflammation and is reported to affect other inflammatory markers [19]. Thus our results on IL-7, IL-8, CXCR1, CXCR2, and TNFα confirm type 1 diabetes to be an inflammatory disease Anti-TNF therapies are currently being used to treat patients with inflammatory diseases with good safety profiles [20]. Furthermore anti-inflammatory therapies are being pioneered in T1DM (still unpublished).

MiR-342-3p expression in PBMC in type 1 diabetes.

We are the first to report downregulation of both isoforms of miR-342-3p and 5p in type 1 diabetes. Although previous studies have described a decreased expression of miR-342 in regulatory T cells compared with T cells in type 1 diabetes, no information is available on isoforms miR-342-3p or 5p [21]. It is important to note that the miRNA isoforms discussed in our paper, miR-342-3p/-5p, are only significantly expressed in PBMCs and not in plasma. It is to our knowledge that we are the first group to identify this. Plasma in patients with type 1 diabetes appears to have insignificant results for many miRNAs, as found by us through Next Generation Sequencing (NGS) analysis (data not shown).
In type 1 diabetes mellitus patients, the downregulation of miR-342-3p/5p may account for the increased concentration of inflammatory cytokines. It has been investigated in another study on atherosclerosis that following the inhibition of nuclear enriched abundant transcript 1 (NEAT1) and subsequent overexpression of miR-342-3p, reduction of inflammation was observed [11]. The overexpression of miR-342-3p in response to the inhibition of NEAT1 inhibited the release of inflammatory cytokines IL-6, IL-1β, TNFα and cyclooxygenase-2 (COX-2). This strongly supports our finding in patients and can be translated into the causal relationship of miR-342-3p downregulating inflammatory cytokines in our study.

Furthermore, after inputting miR-342-3p, miR-342-5p, IL-7, IL-8, TNF-α, VEGF-C and glucose into IPA software simulating diabetic state, it was predicted through published knockout studies that miR-342-3p had anti-inflammatory effects. miR-342-3p was predicted to activate mitogen-activated protein 3 kinase 1 (MAP3K1), inhibitor of nuclear factor kappa B kinase regulatory subunit gamma (IKBKγ), and platelet derived growth factor subunit B (PDGFB) (Figure 5). These kinases and growth factors indirectly inhibit inflammation, heart failure and cardiomyopathy. IPA predicted these causal relationships from knockout studies such as IKBKγ gene knockout resulting in an increased inflammation of liver etc. Similarly, IPA also predicted IL-7, IL-8, and TNF-α to activate the inflammatory response, as well as confirming that elevated glucose concentrations caused increased TNF-α and IL-8 expression.

MiR-342-5p expression in PBMCs in type 1 diabetes
Similarly, to miR-342-3p, miR-342-5p was also downregulated in type 1 diabetes patients. Both correlated with similar molecules however, miR-342-5p was not correlated with CXCR1 or TNF-α. This could suggest that miR-342-5p may regulate similar pathways to miR-342-3p but miR-342-3p is more pro-angiogenic than miR-342-5p.

Through generating predicted targets using IPA, there is some uncertainty as to whether miR-342-5p is pro-inflammatory or anti-inflammatory. This is because one study has shown miR-342-5p to be pro-inflammatory at late stages of atherosclerosis [23]. However, IPA also predicted miR-342-5p to be anti-inflammatory and inhibit inflammation through targeting surfactant protein A1 (SFTPA1). The data was born out of experiment in mutant mouse SFTP1 knockout resulting in increased lung inflammation. Several other causations described SFTP1 to be anti-inflammatory through gene knockouts in mice supporting miR-342-5p is anti-inflammatory. Hence, the prediction shown in Figure 6 demonstrates miR-342-5p is inhibiting the inflammatory response through the activation of SFTP1A and PDGFB.

Interestingly, PDGFB was activated by both miR-342-3p/5p further suggesting both may regulate similar pathways. PDGFB has been described to inhibit heart failure through a mutant mouse PDGFB gene knockout resulting in an increased congestive heart failure. As type 1 diabetes has been found to be linked to cardiovascular disease, this prediction of inhibition of heart failure through miR-342-3p/5p may prove to be beneficial to type 1 diabetes patients.

Inflammatory markers associations with miR-342 We have shown there was negative correlation between miR-342-3p and IL-6 IL-8 and TNF-α. Interestingly, miR-342-5p showed negative correlation with IL-6 and IL-8 however, unlike miR-342-3p, not with TNF-α. Therefore, as miR-342-3p/5p increases, these pro-inflammatory cytokines are shown to be downregulated confirming our findings that miR-342-3p/5p have potential anti-inflammatory effects.

Other studies support our conclusion that miR-342-3p is protective against atherosclerosis as when miR-342-3p was downregulated in endothelial cells in mice, this led to a rise in chitinase 3 like 1 (Chi3l1), mediator of endothelial inflammation [10]. This resulted in the subsequent vascular inflammation.
In contrast to this, in a state of established atherosclerosis (atherosclerotic plaques), miR-342-5p has been described to induce pro-inflammatory mediators including nitric oxide synthase 2 (Nos2) and IL-6 [24]. This is converse to our study as it was performed in PBMCs from type 1 diabetes, therefore it is understandable that the results differ and miR-342-5p may exert different effects at different stages of inflammatory diseases. Reviewing miR-342-5p expression in established atherosclerosis is likely to be complex as many other factors may be involved at late stages of the atherosclerotic process.

A positive correlation was also observed between miR-342-3p/5p and cytokine receptors CXCR1 and CXCR2 mRNA. The latter being the receptors of IL-8, CXCR1 and CXCR2 are responsible for trafficking of inflammatory mediators [18]. This positive correlation between miR-342-3p/5p and CXCR2 as well as miR-342-3p and CXCR1 provides further evidence miR-342-3p/5p may play a part in regulating the inflammatory response. Increasing the concentration of receptors involved in removing cytokines from site of inflammation may lead to a decrease in inflammation. As inflammation in type 1 diabetes mellitus had been found to be associated with an increased risk of cardiovascular disease, regulating inflammation may decrease this risk.

**Vascular health and miR-342** We found positive correlations between miR-342-3p/5p with fibronectin adhesion assay (FAA). Cell adhesions are crucial for cell regeneration, motility and angiogenesis [26][27]. This suggests that the greater miRNA concentration, the greater cell adhesion. This association can be converted to the causal relationship since the upregulation of miR-342-3p in recent study has led to the promotion of cell survival and motility [28].

In addition, we have reported that miR-342-3p/5p were positively correlated with CD45^{dim}CD34^{+}CD133^{+} cells otherwise known as circulating early endothelial progenitor cells (EPCs). It is well established, including by us, that in both type 1 and type 2 diabetic patients, there are fewer of circulating EPCs than in healthy controls confirming increased CVD risk [29][13]. EPCs have been proven to promote vascular repair via transmigrating to promote new vessel growth and predict future CVD events [30][31]. It is well known that almost all risk factors for CVD are associated with dysfunction or decrease of EPCs validating our findings [32].

In line with our hypothesis, we have established a negative correlation between miR-342-3p/5p and TIMP-1. As TIMP-1 is a matrix metalloproteinases (MMP) inhibitor, decreasing TIMP-1 would lead to an increase in cell motility and hence increased vascular repair [33]. Trans-endothelial migration is dependent upon the degradation of basement membranes. This degradation process relies on the production of enzymes capable of degrading the collagenous membranes, such as matrix metalloproteinases (MMPs). TIMP-1 is an inhibitor of many MMPs such as MMP-9 and therefore negative correlation with microRNA family miR-342 may suggest that TIMP-1 regulation is dependent on miR-342-3p/5p [34]. Furthermore, another study had reported a decrease of nitric oxide (NO) and MMPs in type 1 diabetes patients consistent with reduced vascular repair [35] which further supported our study.

**miR-342 affecting hyperglycaemia** We found inverse correlation between miR-342-3p/5p and logHbA1c. These data further support our findings that these miRNAs are downregulated by hyperglycaemia. This is validated by findings also in expression study where hyperglycaemia reduced expression of miR-342-3p and blocked vasculogenesis [36]. To our knowledge, no group examined miR-342-3p/5p in relation to HbA1c. This may be because other studies tested miRNAs in plasma whereas we have demonstrated that miR-342-3p is not significantly expressed in plasma and is better to be tested in PBMCs. Although our patients were relatively well controlled, the negative relation between glycaemic control across all subjects and miRNA-342 3p/5p confirms increased CVD risk in type 1 diabetes. We can postulate that this miRNA can act as CVD risk indicator for monitoring purposes.

**ROC analysis**
Although ROC curve analyses showed miR-342-5p-5p to be a significantly discriminating test for T1DM HbA1c is much better indicator of hyperglycaemia with ROC AUC here of 1.0. We are not claiming here to replace HbA1c with a new test for T1DM, however to establish at what level of HbA1c downregulation of miR-342-5p occurs. Our data showed that defining point of our miR downregulation (associated with T1DM) defined subclinical CVD/T1DM and this occurred at HbA1c > 46.45 mmol/mol, (6.4%). We can thus postulate that miR-342-3p/5p is a biomarker for early subclinical CVD as the difference in miR-342-3p/5p expression could be attributed to the onset of hyperglycaemia. It is of interest that, the value of HbA1c 46.5 mmol/mol (6.4%) achieved from ROC curve analysis also signifies the turning point for development of diabetes and commonly phrased as prediabetes state associated with the development of microvascular complication(37,38).

In this study of well characterised controls and diabetic patients, we found independently by using miR-342-3p/5p, the biochemical set point for diabetic complications. This cut-off defined an increased CVD risk and appeared to coincide within the pre-diabetes range (42-47 mmol/mol or 6.0-6.49%). Although this is the small study, this independent analysis can be used to confirm that miR342-3p/ 5p is a biomarker for the beginning of subclinical CVD.

Our finding has a clinical application, as reliable diagnosis of subclinical CVD is notoriously difficult without using invasive methods. Therefore, we hypothesize that miR-342-3p/5p can act as either biomarker of CVD onset or CVD progression or both. The downregulation of miR-342-3p/5p emphasises the increasing cardiovascular risk with the presence of T1DM even in the absence of clinical CVD.

**Pannexin-2 mRNA target of miR-342-3p** Through inputting our significant mRNAs into miR gene target filter in IPA (Fig. 6), it was predicted that Pannexin 2 (PANX2) was the mRNA target for miR-342-3p. We found PANX2 to be increased in PBMCs and predicted to be activated by miR-342-3p. PANX2 is one of the newly discovered 3-member family of proteins expressed in brain and tissue. The upregulation of PANX2 was predicted to indirectly result in the increased release of ATP. ATP has many functions in different pathways and may be pro- or anti-inflammatory. However, IPA generated inhibitory causation between ATP and inflammation in response to miR-342-3p involvement [39]. IPA also predicted a link between an influx of calcium and the influx of ATP which also led to an indirect anti-inflammatory response. Other studies have presented PANX2 might be involved in providing a flux pathway for ions such as ATP and Calcium [40]. Berchtold et al found that expression of PANX2 is downregulated by pro-inflammatory cytokines in rat islets and INS-1E cells [41]. Bond and Naus further supported involvement of PANX2 in inflammation as well as ATP signalling and their absence has been explicitly verified in macrophages [42]. Unfortunately, the exact role of PANX2-silenced cells and their role in STAT3 signalling is still uncertain and further research to understand the specific role of PANX2 in type 1 diabetes is required.

The results from both our study and others including Wang et al suggest the downregulation of miR-342-3p could contribute to one of the world’s most prevalent diseases; cardiovascular disease[11]. As cardiovascular disease has been proven to be accelerated by type 1 diabetes, these results help support the idea that miR-342-3p could form the basis of therapeutic interventions to impact CVD risk in type 1 diabetes [43]. Our cross sectional findings need be further validated in a prospective longitudinal studies on progression of CVD

**Conclusions** We have validated animal research showing that miR-342-3p/5p are significantly downregulated in Type 1 diabetes confirming an increased risk of CVD. In addition we have confirmed that type 1 DM, even well controlled, can be characterised by an inflammatory state. Furthermore as miR 342-3p/5p were found to be markers of early CVD defining it at HbA1c > 46.45 mmol/mol, (6.4%), they can be used for either diagnosis of early CVD or monitoring of CVD Whilst miR 324-3p/5p are associated with markers of vascular health and inflammation in all subjects their beneficial effects can promote further research into pro-miR-342-3p/5p as therapeutic interventions for type 1 diabetes patients.

**List of abbreviations**
T1DM  Type 1 diabetes mellitus
CVD   Cardiovascular disease
PBMCs Peripheral blood mononuclear cells
FAA   Fibronectin Adhesion Assay
TIMP-1 Tissue inhibitor of metalloproteases
CXCR1/2 Chemokine receptor
IPA   Ingenuity Pathway Analysis
MAP3K1 Mitogen-activated protein 3 kinase 1
IKBKG Inhibitor of nuclear factor kappa B kinase regulatory subunit Gamma
SFTPA1 Surfactant protein A1

**Declarations**

**Ethics approval and consent to participate**

All subjects gave informed consent and the study was performed in accordance with the Helsinki Declaration. The study was approved by the NHS Health Research Authority, NRES Committee North East-Sunderland, UK (Research Ethics Committee Reference Number 12/NE/0044).

**Consent for publication**

All authors consent to this publication.

**Availability of data and materials**

The data has been submitted as a supplement.

**Competing interests**

The authors declare no conflicts of interest.

**Funding**

Funding was provided by the Diabetes Research and Wellness Foundation UK [open funding 2011], and the Diabetes Research Fund at Queen Elizabeth Hospital, Gateshead, UK.

**Authors’ contributions**

S.L.R., D.J.C., M.LY.Y. analysis and interpretation of the data and writing of the manuscript. S.B., laboratory experiments interpretation of the data and drafting of the manuscript. J.U.W., conception and design of the study, securing the funding, recruitment of patients, laboratory experiments, acquisition of data, analysis and interpretation of the data and writing of the manuscript. J.U.W. is the guarantor of this work and, as
such, had full access to all the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the final manuscript.

Acknowledgements

The author thanks Dr Fahad Ahmed, for the collection of the clinical and metabolic characteristics and the cytokine profiles of the subjects in the previous study. Our gratitude is expressed to the patients and healthy controls for their participation in this research. We thank the Clinical Research Facility and its staff at Royal Victoria Infirmary, Newcastle, for use of the facility.

References


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<th>Healthy controls (n=20)</th>
<th>Type 1 diabetes (n=29)</th>
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<td>Age (years)</td>
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<td>47.2 +/- 12.7</td>
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<td>BMI (kg/m^2)</td>
<td>26.0 +/- 4.5</td>
<td>28.4 +/- 6.7</td>
</tr>
<tr>
<td>Gender (M/F) n</td>
<td>9/11</td>
<td>14/15</td>
</tr>
<tr>
<td>Length of Diabetes (years)</td>
<td></td>
<td>22.4 +/- 13.9</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>35.1 +/- 2.8</td>
<td>57.3 +/- 7.6</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.4 +/- 0.3</td>
<td>7.4 +/- 0.7</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.6 +/- 1.0</td>
<td>9.7 +/- 3.7</td>
</tr>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>14.3 +/- 1.2</td>
<td>14.5 +/- 1.2</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.4 +/- 0.7</td>
<td>0.9 +/- 0.4</td>
</tr>
<tr>
<td>Alanine Aminotransferases (IU/L)</td>
<td>21.3 +/- 6.1</td>
<td>22.8 +/- 11.7</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>117.5 +/- 14.0</td>
<td>127.5 +/- 9.4</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mmHg)</td>
<td>74.8 +/- 8.3</td>
<td>76.8 +/- 8.8</td>
</tr>
<tr>
<td>CD45^dimCD34^+ CD133^+ per 100 lymphocytes</td>
<td>0.009 +/- 0.03</td>
<td>0.02 +/- 0.01</td>
</tr>
</tbody>
</table>

Table 1: Subject’s clinical and metabolic characteristics. *p < 0.05, **p < 0.01, ***p < 0.001.
Fig. 1 Comparison of data between patients with type 1 diabetes and healthy controls (a) IL-7 expression, (b) IL-8 expression, (c) TNF-α expression, (d) VEGF-C expression (e) CXCR1, CXCR2 and PANX2 mRNA in PBMCs (f) miR-342-3p and miR-342-5p expression in peripheral blood mononuclear cells (PBMCs). IL-7, IL-8, TNF-α, VEGF-C, CXCR1 mRNA and CXCR2 mRNA. Data are presented as mean±SD, analysed by unpaired t-tests or Mann-Whitney U test accordingly. *p < 0.05, **p<0.01 ***p<0.001.
**Fig. 2** Correlation between miR-342-3p/-5p and inflammatory markers in type 1 diabetes and healthy controls combined. (A) miR-342-3p and IL-6; r=-0.496, p=0.031. (B) miR-342-5p and IL-6; r=-0.489, p=0.034. (C) miR-342-3p and IL-8; r=-0.501, p=0.029. (D) miR-342-5p and IL-8; r=-0.500, p=0.029. (E) miR-342-3p and chemokine receptor type 2 (CXCR2); r=0.677, p=0.001. (F) miR-342-5p and CXCR2; r=0.483, p=0.036. (G) miR-342-3p and chemokine receptor type 1 (CXCR1); r=0.533, p=0.019. (H) miR-342-3p and TNF-α; r=-0.480, p=0.038.
Fig. 3 Correlation between miR-342-3p/5p and vascular health in type 1 diabetes and healthy controls combined. (a) miR-342-3p and LogFAA; \( r = 0.601, \ p = 0.006 \). (b) miR-342-5p and Log Fibronectin Adhesion Assay (FAA); \( r = 0.479, \ p = 0.038 \). (c) miR-342-3p and CD45dimCD34+CD133+; \( r = 0.635, \ p = 0.004 \). (d) miR-342-5p and CD45dimCD34+CD133+; \( r = 0.523, \ p = 0.022 \). (e) miR-342-3p and Log Tissue inhibitor of metalloproteinases (TIMP1); \( r = -0.605, \ p = 0.006 \). (f) miR-342-5p and LogTIMP1; \( r = -0.612, \ p = 0.005 \).

Fig. 4 Correlation between miR-342-3p/5p and glycaemic control in type 1 diabetes and healthy controls (HC) combined. (a) miR-342-3p and logHbA1c; \( r = -0.695, \ p = 0.001 \). (b) miR-342-5p and logHbA1c; \( r = -0.681, \ p = 0.001 \).
Fig. 5 Ingenuity Pathway Analysis (IPA) prediction network of miR-342-3p/5p and cardiovascular disease.

The downregulation is shown in green and the upregulation in red. Orange signifies predicted activation of a molecule or biological function. Paler orange signifies a prediction of less activation. Blue signifies inhibition of a molecule or biological function. Orange lines represent stimulation, blues lines represent inhibition and grey lines represent reciprocal stimulation and inhibition. Solid un-interrupted lines represent a direct action and dashed interrupted lines represent an indirect action. miR-342-5p is predicted, through animal knockout studies, to activate surfactant protein A1 (SFTPA1) and platelet derived growth factor subunit B (PDGFB) which in turn inhibit heart failure and inflammation. miR-342-3p activates inhibitor of nuclear factor kappa B kinase regulatory subunit gamma (IKBKG), mitogen activated 3 kinase 1 (MAP3K1) and also PDGFB which are also responsible for inhibiting heart failure and inflammation. D-glucose is predicted to activate cardiomyopathy, TNF-α and IL-8. IL-8 is predicted to activate inflammation whilst TNF-α activates VEGF-C as well as activating inflammation. As there are some studies that suggest otherwise to VEGF-C activating inflammation, this arrow appears in grey. IL-7 is predicted to activate TNF-α resulting in activation of inflammation whilst Phosphoinositide 3-kinase (PI3), when activated, is predicted to inhibit TNF-α and IL-8.
Ingenuity Pathway Analysis (IPA) prediction network representing activation of PANX2 by miR-342-3p and its effect on inflammatory response. Orange signifies predicted activation of a molecule or biological function. Paler orange signifies a prediction of less activation. Blue signifies inhibition of a molecule or biological function. Orange lines represent stimulation and blue lines represent inhibition. Solid un-interrupted lines represent a direct action and dashed interrupted lines represent an indirect action. miR-342-3p was predicted to inhibit PANX2. However, when PANX2 is activated, it activates ATP which is predicted to be responsible for indirectly activating calcium (Ca2+) release to inhibit inflammation. ATP itself is also predicted to indirectly inhibit inflammation. TNF-α is predicted to activate process of atherosclerosis indirectly and also activates IL-8 which activates inflammation.