miR-409-3p is reduced in plasma and islet immune infiltrates of NOD diabetic mice and is differentially expressed in people with type 1 diabetes

Giuliana Ventriglia^{1,2,3} · Francesca Mancarella^{1,2} · Guido Sebastiani^{1,2} · Dana P. Cook³ · Roberto Mallone⁴ · Chantal Mathieu³ · Conny Gysemans³ · Francesco Dotta^{1,2}

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Abstract

Aims/hypothesis MicroRNAs (miRNAs) are a novel class of potential biomarkers emerging in many diseases, including type 1 diabetes. Here, we aim to analyse a panel of circulating miRNAs in non-obese diabetic (NOD) mice and individuals with type 1 diabetes.

Methods We adopted standardised methodologies for extracting miRNAs from small sample volumes to evaluate a profiling panel of mature miRNAs in paired plasma and laser-captured microdissected immune-infiltrated islets of recently diabetic and normoglycaemic NOD mice. Moreover, we validated the findings during disease progression and remission after anti-CD3 therapy in NOD mice, as well as in individuals with type 1 diabetes.

Results Plasma levels of five miRNAs were downregulated in diabetic vs normoglycaemic mice. Of those, miR-409-3p was also downregulated in situ in the immune islet infiltrates of diabetic mice, suggesting an association with disease pathogenesis. Target-prediction tools linked miR-409-3p to immune- and metabolism-related signalling molecules. In situ miR-409-3p expression correlated with insulinitis severity, and CD8⁺ central memory T cells were found to be enriched in miR-409-3p. Plasma miR-409-3p levels gradually decreased during diabetes development and improved with disease remission after anti-CD3 antibody therapy. Finally, plasma miR-409-3p levels were lower in people recently diagnosed with type 1 diabetes compared with a non-diabetic control group, and levels were inversely correlated with HbA_{1c} levels.

Conclusions/interpretation We propose that miR-409-3p may represent a new circulating biomarker of islet inflammation and type 1 diabetes severity.

Keywords Anti-CD3 therapy · Biomarker · Inflammation · MicroRNA · Progression · Type 1 diabetes

Giuliana Ventriglia and Francesca Mancarella are joint first authors.
Conny Gysemans and Francesco Dotta are joint senior authors.

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✉ Francesco Dotta
francesco.dotta@unisi.it

¹ Diabetes Unit, Department of Medicine, Surgery and Neurosciences, University of Siena, V.le Bracci, 16 – 53100 Siena, Italy

² Fondazione Umberto Di Mario ONLUS c/o Toscana Life Sciences, Siena, Italy

³ Clinical and Experimental Endocrinology (CEE), Katholieke Universiteit Leuven (KU LEUVEN), Leuven, Belgium

⁴ Inserm, U1016, CNRS, UMR8104, Paris Descartes University, Sorbonne Paris Cité, Cochin Institute, Paris, France

Abbreviations

aCD3	Anti-CD3
LCM	Laser-captured microdissected
mAb	Monoclonal antibody
miRNA	MicroRNA
NOR	Non-obese resistant
SOP	Standard operating procedure
TCM	Central memory T cell
TEM	Effector memory T cell
3'-UTR	3'-Untranslated region

Introduction

Circulating biomarkers for screening, diagnosis and monitoring type 1 diabetes include blood glucose level, HbA_{1c} and

Research in context

What is already known about this subject?

- Circulating microRNAs (miRNAs) have been shown to be altered in the plasma of individuals with type 1 diabetes
- Circulating miRNA profiles are associated with hyperglycaemia in people with type 1 diabetes
- Circulating miRNAs are deregulated in plasma-derived exosomes from people with type 1 diabetes

What is the key question?

- Can we identify circulating plasma-borne miRNAs that mirror in situ pancreatic inflammation in type 1 diabetes?

What are the new findings?

- miR-409-3p was reduced in plasma of recently diabetic NOD mice
- miR-409-3p was reduced in pancreatic immune infiltrates of recently diabetic NOD mice and correlated with insulinitis severity
- miR-409-3p was differentially expressed in the plasma of individuals with recently diagnosed type 1 diabetes

How might this impact on clinical practice in the foreseeable future?

- Circulating plasma-borne microRNAs may be clinically applicable biomarkers that could help to stage disease progression and to stratify individuals with type 1 diabetes

autoantibody measurements. They are often detected only during the late stages of disease development, when most beta cells are destroyed. Although genotyping and islet autoantibodies perform relatively well at predicting future type 1 diabetes, it remains challenging to accurately monitor islet inflammation during disease progression.

MicroRNAs (miRNAs), a class of small non-coding RNAs about 19–24 nucleotides long and processed from hairpin precursors by the drosha ribonuclease III (DROSHA)–DGCR8 microprocessor complex subunit (DGCR8) complex and dicer 1, ribonuclease III (DICER) [1], have been proposed as circulating biomarkers. miRNAs modulate gene expression by binding to complementary sites within 3′-untranslated regions (3′-UTRs) of target mRNAs, leading to translational repression or to mRNA degradation [2, 3]. Mature miRNAs can be either functional, to guide translational repression of target mRNAs, or released in extracellular fluids [4, 5][6]. The high stability of miRNAs in tissues and extracellular fluids and the possibility that alterations in cell-free circulating miRNAs may be paralleled by a dysregulation in a cellular subset support their potential use as biomarkers. The biological role of circulating miRNAs as mediators of cell-to-cell crosstalk is yet to be defined. Nonetheless, alterations in miRNA expression in body fluids can be detected before conventional biomarkers and have been associated with different pathophysiological conditions, such as cancer [7], autoimmune diseases [8, 9], and cardiovascular [10] and metabolic disorders, even though detailed information on their specific cells of origin is lacking in most reports.

Recent studies analysed miRNA expression profiles in serum or plasma from individuals with type 1 diabetes in order to explore new approaches to monitor the development, progression or resolution of autoimmune diabetes [11–16]. Altered miRNA signatures were detected in these studies, and levels of specific circulating miRNAs correlated with beta cell function or other clinical variables in some of these studies. However, owing to the obvious difficulties related to tissue procurement and collection, none of the published studies identified altered miRNAs in both the circulation and the diseased organ, not even in the non-obese diabetic (NOD) mouse model of autoimmune diabetes.

We adopted standardised methodologies for extracting miRNAs from small sample volumes with the aim of evaluating a profiling panel of mature miRNAs in paired plasma and in laser-captured microdissected (LCM) immune-infiltrated islets of recently diabetic and normoglycaemic NOD mice.

Methods

Experimental workflow We analysed an miRNA profiling panel in plasma of age-matched (same week of age) normoglycaemic and recently diabetic NOD mice ($n = 5$ each) and validated the findings in matched LCM islet endocrine cells and immune infiltrates (key resources are shown in electronic supplementary materials [ESM] Table 1). Differentially expressed miRNAs were further investigated: (1) for in situ

expression according to insulinitis severity; (2) in the cellular subsets particularly enriched for the miRNA(s) of interest; (3) in plasma samples from an additional group of recently diabetic ($n = 12$), age-matched normoglycaemic ($n = 25$), 4-week-old ($n = 14$) and 8-week-old ($n = 14$) prediabetic NOD mice; and (4) for changes in expression after an in situ targeted therapeutic intervention. Finally, we analysed plasma miR-409-3p expression in two independent human cohorts: a first cohort ('Cohort 1') of non-diabetic individuals (control group) ($n = 25$), autoantibody-negative relatives of individuals with type 1 diabetes ($n = 9$), and individuals recently diagnosed with type 1 diabetes ($n = 18$), whose blood samples were collected and processed following a strict standard operating procedure (SOP); and a second, historical, cohort group ('Cohort 2') of individuals without diabetes (control group) ($n = 17$), individuals recently diagnosed with type 1 diabetes ($n = 23$) and individuals with longstanding type 1 diabetes ($n = 13$), as well as individuals with rheumatoid arthritis ($n = 18$).

Animals NOD mice were housed and inbred in the KU Leuven animal facility (Leuven, Belgium) since 1989 and kept under semi-barrier conditions, as described [17, 18]. Female mice were screened for diabetes by glycosuria (Diastix Reagent Strips; Bayer, Leverkusen, Germany) and venous blood glucose levels (Accu-Chek; Roche Diagnostics, Vilvoorde, Belgium). Mice were diagnosed as diabetic when positive for glycosuria and for two consecutive blood glucose measurements > 11.1 mmol/l. Recently diabetic NOD mice were 12 to 22 weeks old; age-matched normoglycaemic animals were used as controls. Non-obese resistant (NOR) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and killed at around 21 weeks of age. NOD mice of 4 and 8 weeks of age were used to evaluate miR-409-3p expression during the prediabetic phase.

For anti-CD3 (aCD3) monoclonal antibody (mAb) therapy, recently diabetic NOD mice were treated for 7 consecutive days with increasing i.v. doses (day 1, 0.42 μ g; day 2, 1.67 μ g; days 3–7, 4.46 μ g per mouse) of anti-mouse CD3 mAb (clone 145-2C11; BioXCell, West Lebanon, NH, USA) (key resources are shown in ESM Table 2). Blood was collected by heart puncture or submandibular bleeding and centrifuged at 2000 g for 10 min at 4°C to obtain plasma. Collected plasma was immediately stored at -80°C . Mice were bred and housed according to protocols approved by the Katholieke Universiteit Leuven Animal Care and Use Committee (Leuven, Belgium; project number 116/2015) and experiments complied with EU Directive 2010/63/EU for animal experiments.

Human donors In Cohort 1, individuals with recently diagnosed type 1 diabetes (< 1 year of diagnosis; $n = 18$) and autoantibody-negative non-diabetic first-degree relatives of individuals with type 1 diabetes ($n = 9$) and without diabetes

($n = 25$) were recruited in the outpatient diabetes centre, University Hospital UZ Leuven (Leuven, Belgium), and at the diabetes outpatient unit, Siena University Hospital (Siena, Italy) (Table 1). Informed written consent was obtained from all individuals enrolled in the study, which was approved by the local ethics committee (Comitato Etico Regionale per la Sperimentazione Clinica della Toscana – Area Vasta Sud Est – cod. INNODIA01).

All non-diabetic individuals were negative for islet autoantibodies and for signs of ongoing endocrine dysfunction or of other autoimmune diseases. A SOP was followed to collect plasma samples (see 'Human donors – blood collection procedure' section in ESM Methods). Additional plasma samples were analysed; these were derived from Cohort 2 composed of non-diabetic individuals (control group) ($n = 17$), individuals recently diagnosed with type 1 diabetes (< 1 year of diagnosis, $n = 23$) and individuals with longstanding type 1 diabetes (> 2 years of diagnosis, $n = 13$), in addition to individuals with rheumatoid arthritis (ESM Table 3). All individuals with type 1 diabetes were positive for at least one autoantibody. Autoantibodies were analysed as described previously [19].

Plasma RNA extraction Total RNA, including miRNAs, was extracted from 50 μ l NOD mouse plasma or from 100 μ l human plasma using miRNeasy kit (Qiagen, Hilden, Germany) (see ESM Methods).

Extracellular circulating miRNA profiling and data analysis miRNA profiling was performed using TaqMan miRNA array rodent microfluidic cards (Panel A v2.1; Life Technologies, Carlsbad, CA, USA). Megaplex reverse transcriptase reaction and miRNA arrays were performed according to the manufacturer's protocols (Life Technologies). Samples were checked for housekeeping stability and haemolysis rate (see ESM Methods; ESM Fig. 1a,b).

LCM and insulinitis grading Islet endocrine cells and juxtaposed infiltrating immune cells were microdissected separately as described previously [17]. LCM islet endocrine cells and immune infiltrates were pooled separately according to insulinitis score: score 0 (no infiltration) and 1 (peri-insulinitis) were pooled together; tissues with score 2 (infiltration in $< 50\%$ of the islet area) and 3 (infiltration in $\geq 50\%$ of the islet area) were pooled separately (see ESM Methods; ESM Fig. 2).

Cell staining, flow cytometry and cell sorting Single cell suspensions were prepared from pancreases in digestion medium (RPMI medium + 5% FCS, 2 mmol/l L-glutamine, 0.05 mmol/l β -mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mg/ml collagenase-VIII and 0.02 mg/ml DNase-I; Life Technologies) for 15 min in a shaking incubator at 150 rpm at 37°C. The phenotype analysis was performed with flow cytometry by staining the cells with anti-CD4,

Table 1 Main characteristics of the non-diabetic control group, first-degree relatives and individuals with type 1 diabetes in Cohort 1

Characteristic	Control participants			Type 1 diabetes
	Unrelated ^a	UFM	Total	
<i>n</i>	25	9	34	18
Clinical site (Siena: Leuven), <i>n:n</i>	13:12	8:1	21:13	3:15
Age, years	28.0±7.0	31.0±8.8	29.0±7.3	31.0±10.2
Male sex (female sex), <i>n</i>	12 (13)	1 (8)	13 (21)	14 (4)
Fasting blood glucose, mmol/l	ND	ND	ND	11.9±6.8
Insulin dose, U/day	NA	NA	NA	45±12.0
BMI, kg/m ²	ND	ND	ND	21±2.0
HbA _{1c} , %	ND	ND	ND	13.3±2.1
HbA _{1c} , mmol/mol	ND	ND	ND	120.9±23.3
C-peptide, nmol/l	ND	ND	ND	0.07±0.05
ICA ⁺ , <i>n</i>	0	0	0	6
IAA ⁺ , <i>n</i>	0	0	0	2
GADA ⁺ , <i>n</i>	0	0	0	14
IA-2A ⁺ , <i>n</i>	0	0	0	7
ZnT8A ⁺ , <i>n</i>	0	0	0	4

Data are shown as mean ± SD unless stated otherwise

^a Control participant without familiarity for type 1 diabetes

GADA, GAD autoantibody; IAA, insulin autoantibody; IA-2A, insulinoma-associated 2A autoantibody; ICA, islet cell autoantibody; NA, not applicable; ND, not determined; UFM, unaffected family member; ZnT8A, zinc transporter 8 autoantibody

-CD8, -CD44 and -CD62L mAbs, all from eBioscience (San Diego, CA, USA) (ESM Fig. 3). Cells were sorted directly into Trizol-LS (Life Technologies). RNA was extracted, quantified and quality controlled as above.

miRNA and mRNA single-assay real-time PCR To validate miRNA expression in the reverse transcription (RT)-PCR single-assay reactions, 1 ng of RNA extracted from LCM islet immune infiltrates or flow-sorted immune cells, RNA extracted from 50 µl NOD mouse plasma or 100 µl human plasma was reverse transcribed using the Megaplex protocol with pre-amplification, and analysed using real-time PCR through specific TaqMan miRNA assays in 96 well plates (all from Life Technologies). To analyse mRNA expression from LCM islet endocrine cells and immune infiltrates, reverse transcription reactions were performed using ImProm-II RT (Promega, Madison, WI, USA) and TaqMan gene expression assays (Life Technologies), adopting a pre-amplification step following the manufacturer's protocol (see ESM Methods).

miR-409-3p absolute quantification Tenfold serial dilutions from 10⁻² to 10⁻⁸ nmol/l of the synthetic miR-409-3p (mirVana miRNA mimic, Life Technologies) were reverse transcribed and assayed in parallel with RNA extracted from human plasma samples (ESM Fig. 4). Single-assay real-time PCR reactions were carried out as reported above (see ESM Methods).

Prediction and pathway enrichment analysis of miRNA targets Putative targets of miR-409-3p, as well as pathway enrichment analysis, were investigated employing miRWalk v.3.0 (<http://mirwalk.umm.uni-heidelberg.de>). The results were visualised in Cytoscape v.3.6.1. using the ClueGo plugin [20, 21].

Statistical Analysis Statistical analyses were performed using GraphPad Prism Version 7.00 (GraphPad Software, La Jolla, CA, USA). Two-tailed Mann–Whitney *U* tests were used for comparisons between groups; two-tailed Wilcoxon signed-rank tests were used for comparisons between paired samples. Data are reported as mean ± SEM. For all analyses, a *p* value of ≤0.05 was considered significant. Randomisation and blinding were not carried out.

Results

A distinct signature of five unique downregulated miRNAs is identified in plasma of recently diabetic mice Expression profiling of 384 miRNAs was performed on five plasma samples obtained from age-matched normoglycaemic and recently diabetic NOD mice (between 12 and 22 weeks of age). The expression of the 182 mature miRNAs that were detected in at least one sample for each group is depicted in the heatmap hierarchical clustering tree (Fig. 1a). We identified seven

Fig. 1 Circulating miRNA profiling of plasma samples deriving from recently diabetic and normoglycaemic NOD mice. **(a)** Hierarchical clustering heatmap showing colour-coded expression levels of miRNAs (blue, high expression; red, low expression) in plasma from recently diabetic and age-matched normoglycaemic NOD mice. **(b)** Volcano plot showing changes in miRNA levels between recently diabetic and normoglycaemic NOD mice. Levels in normoglycaemic mice were set at 0. Red lines indicate fold change and p value cut-offs. The larger data

points indicate differentially expressed miRNAs. **(c–i)** Single-assay real-time RT-PCR validation of differentially expressed miRNAs was performed in the same plasma samples as those used for the miRNA screening. Values are expressed as $2^{-\Delta C_t}$. Data are presented as mean $\pm$ SEM; * $p \leq 0.05$, ** $p \leq 0.01$, Mann–Whitney U test. mmu, *Mus musculus*; NG, normoglycaemic; rno, *Rattus norvegicus*

differentially expressed miRNAs (miR-126a-3p, miR-126a-5p, miR-155, miR-188-3p, miR-204, miR-218 and miR-409-3p). All of them were downregulated in the plasma of diabetic vs normoglycaemic mice (Fig. 1b and ESM Fig. 5). Using single-assay real-time PCR, five out of seven miRNAs were validated as differentially expressed (miR-126a-3p, miR-126a-5p, miR-155, miR-204 and miR-409-3p) (Fig. 1c–i).

miR-409-3p is reduced in pancreatic immune infiltrates of NOD mice In order to investigate whether these miRNA alterations were also present in matched pancreas samples, LCM was performed to separately capture islet endocrine cells and juxtaposed immune infiltrates from the same mice. miR-126a-3p, miR-126a-5p, miR-155 and miR-204 were not differentially expressed in islet endocrine cells captured from diabetic

vs normoglycaemic mice nor in LCM juxtaposed immune infiltrates (ESM Fig. 6a–d). Interestingly, miR-409-3p was significantly downregulated in LCM immune infiltrates captured from recently diabetic vs normoglycaemic NOD mice, while no significant differential expression was detected in islet endocrine cells (Fig. 2a,b).

To investigate the potential target genes of miR-409-3p, we employed a conservative strategy by adopting the online target-prediction tool miRWalk. This resulted in three modules that were enriched with the following functions: immune-related pathways; metabolic pathways; and vesicle transport and cell adhesion. The metabolic module was the most significant, with 19 pathways involved (ESM Table 4). Major immune genes were identified in the immune module, such as *Pten*, *Mapk1*, *Nfatc1*, *Nfatc2*, *Ccr5* and *Il6ra*. Additionally,

Fig. 2 In situ miR-409-3p expression analysis. Expression analysis of miR-409-3p in (a) LCM-captured islet endocrine cells from normoglycaemic ($n = 5$) and recently diabetic NOD mice ($n = 5$) (all islets had an insulinitis score of 2–3) and (b) LCM-captured islet-juxtaposed immune infiltrates from normoglycaemic ($n = 5$) and recently diabetic NOD mice ($n = 5$). Values are expressed as $2^{-\Delta C_t}$ plotted on a log10 scale. Data are presented as mean $\pm$ SEM. $**p \leq 0.01$ Mann-Whitney U test NG, normoglycaemic

target genes (e.g. *Gad2*) and pathways (e.g. antigen processing and presentation genes, T cell receptor signalling pathway and chemokine signalling pathway) known to play a role in type 1 diabetes were detected (Fig. 3).

miR-409-3p expression in islet immune infiltrates correlates with insulinitis severity Based on the resulting miRNA-target interaction network linking miR-409-3p mainly to immune signalling molecules, we hypothesised a potential association of miR-409-3p expression with insulinitis severity. Therefore, we micro-dissected islet immune infiltrates from five new pairs of recently diabetic and age-matched normoglycaemic NOD mice and separated them based on their insulinitis score (either 1 for mild insulinitis or 2/3 for severe insulinitis) (Fig. 4a,b). The decreased level of miR-409-3p in plasma (Fig. 4c) and immune infiltrates (Fig. 4d) from this additional group of recently diabetic mice compared with normoglycaemic animals confirmed the results of the previous analysis. By separating the islet immune infiltrates in the normoglycaemic mice by insulinitis score, we observed a significant downregulation of miR-409-3p expression in severe insulinitis vs mild insulinitis (Fig. 4e). Moreover, miR-409-3p expression in immune infiltrates with score 2/3 in the normoglycaemic mice was comparable to those with score 2/3 in recently diabetic mice, further confirming that in situ miR-409-3p expression was linked to insulinitis severity (Fig. 4e).

miR-409-3p expression in plasma gradually decreases during autoimmune diabetes progression and is mainly enriched in CD8⁺ central memory T cells We further validated the expression of miR-409-3p in plasma samples derived from a new group of recently diabetic NOD mice, age-matched normoglycaemic NOD mice, age-matched diabetes-resistant NOR mice and 4 and 8 week old NOD mice, with various degrees of islet infiltration (Fig. 5a). The analyses confirmed the lower expression of miR-409-3p in plasma from diabetic

vs normoglycaemic mice (Fig. 5b). Furthermore, miR-409-3p expression was already downregulated in plasma of 8-week-old vs 4-week-old mice and progressively decreased until disease onset. Of note, age-matched normoglycaemic NOR mice retained: (1) similar miR-409-3p levels to those of 8-week-old NOD mice; and (2) higher miR-409-3p levels vs age-matched normoglycaemic NOD mice (Fig. 5b). Thus, higher miR-409-3p levels were inversely associated with insulinitis severity in NOD mice (Fig. 5a). Additionally, miR-409-3p expression levels inversely correlated with blood glucose levels, indicating a link with disease progression (ESM Fig. 7).

To identify the cellular source of in situ miR-409-3p expression, we purified the most abundant immune-cell subsets from dissected samples of pancreas from longstanding normoglycaemic NOD mice by flow cytometry according to established surface markers [22]. The major cell type in human autoimmune infiltrates is the lymphocyte, with a dominance of CD8⁺ over CD4⁺ T cells [23–25]. Here, we also studied the CD4⁺ and CD8⁺ T cell frequencies both in peripheral blood and in pancreas during disease progression in the NOD mouse model. First, we confirmed the predominance of CD8⁺ over CD4⁺ T cells in the pancreas (data not shown). We did not observe relevant changes in CD4⁺ T cell frequencies during disease progression (Fig. 5c,d). Moreover, we noticed that the proportion of CD8⁺ T cells, particularly the CD8⁺ effector memory T (TEM; CD44⁺CD62L⁻) cells, gradually increased in the periphery and especially in the pancreas, with beta cell mass waning (Fig. 5e,f). More interestingly, the proportion of CD8⁺ central memory T (TCM; CD44⁺CD62⁺) cells decreased in both periphery and pancreas during disease progression (Fig. 5e,f), paralleling circulating miR-409-3p decrease.

Based on a previously reported association of miR-409-3p with human memory T cells [26], we focused on both islet-associated CD4⁺ and CD8⁺ TEM and TCM cells and assessed miR-409-3p enrichment in these immune subsets. The expression of miR-409-3p was significantly enriched in islet-associated CD8⁺ over CD4⁺ T cells, more specifically in TCM vs TEM subsets (Fig. 5g).

Plasma miR-409-3p expression improves after disease intervention therapy with anti-CD3 antibody We treated recently diabetic NOD mice with increasing i.v. doses of aCD3 mAbs, which have been shown to change the anti-islet immune response and stabilise disease progression [27–29]. We assessed plasma miR-409-3p at disease onset (day 0) and 7 days after the last aCD3 injection (day 14) (Fig. 6a). At day 14, plasma miR-409-3p levels were increased as compared with day 0 in aCD3-treated mice, while no significant changes were observed in untreated mice (Fig. 6b,c). Additionally, miR-409-3p levels did not correlate with blood glucose levels upon aCD3 treatment, thus suggesting an intrinsic association with

Fig. 3 miR-409-3p target genes pathway analysis. Enrichment of biological functions of predicted and validated miR-409-3p target genes retrieved with miRWalk 3.0 set using Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis in ClueGo. ClueGo revealed correlations among terms as nodes based on their κ score level (≥ 0.3). The most significant term per group is reported and each node represents a biological function. Different colours represent different paths. When the same gene is enriched in different terms, the term is plotted in a variety of

colours. The lines represent connections between the nodes and indicate close relatedness of the node's target genes. AMPK, 5'-AMP-activated protein kinase; ERBB, epidermal growth factor receptor; FOXO, forkhead box O; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; STAT, signal transducer and activator of transcription; Th1, T helper 1; Th2, T helper 2; Th17, T helper 17

immune modulation rather than with metabolic status (ESM Fig. 8a,b). Indeed, by assessing CD4⁺ and CD8⁺ TCM and TEM frequencies in the pancreases of untreated and aCD3-treated mice vs the recently diabetic control group, we observed that aCD3 decreased CD8⁺ TEM cells, while increasing CD8⁺ TCM cells in the pancreas, as compared with recently diabetic mice but not the untreated control group (Fig. 6e). Frequencies of CD4⁺ TEM and TCM in the pancreas of aCD3-treated mice either non-significantly increased or decreased, respectively, compared with untreated recently diabetic NOD mice (Fig. 6d). Collectively, these results suggest that plasma miR-409-3p levels parallel the immune modulation by aCD3 therapy, which has been shown to affect not only T cells but also the proinflammatory milieu in the islets [29].

Plasma miR-409-3p expression is downregulated in individuals with recently diagnosed type 1 diabetes To investigate whether plasma miR-409-3p is differentially expressed in human type 1 diabetes, we analysed both the relative and absolute miR-409-3p levels in the plasma of individuals recently diagnosed with type 1 diabetes (from the Cohort 1, see Methods section). Plasma samples were collected from

individuals recently diagnosed with type 1 diabetes (< 1 year of diagnosis) and a healthy non-diabetic (autoantibody-negative) control group. Plasma miR-409-3p levels were reduced in individuals recently diagnosed with type 1 diabetes vs the non-diabetic control group using both the relative and absolute quantification methods (Fig. 7a,b).

A similar miR-409-3p expression pattern was observed in plasma samples obtained from Cohort 2 of individuals with type 1 diabetes with less than 1 year since diagnosis vs a non-diabetic control group (Fig. 7c). Individuals with longstanding type 1 diabetes (> 2 years of diagnosis) and individuals with rheumatoid arthritis did not show a reduction in plasma miR-409-3p levels vs the non-diabetic control group (Fig. 7c). Instead, an increased level of miR-409-3p was observed in plasma obtained from individuals with rheumatoid arthritis compared with those with type 1 diabetes and the non-diabetic control group (Fig. 7c). No significant correlation was found between plasma miR-409-3p expression and age, BMI, insulin dose, blood glucose level or C-peptide (ESM Fig. 9a-f). However, a negative correlation ($n = -0.52$, $p = 0.04$) with HbA_{1c} was identified in individuals recently diagnosed with type 1 diabetes (ESM Fig. 9d). Overall, these results suggest that miR-409-3p expression is downregulated

Fig. 4 Characterisation of miR-409-3p expression in NOD lymphocytic infiltrates. **(a)** Representative images of H&E-stained LCM-captured inflamed islets with an insulitis score of 1, 2 or 3; scale bars, 175 μ m. **(b)** Insulitis scoring analysis. **(c–e)** Expression analysis of miR-409-3p in plasma samples **(c)**, LCM-captured immune infiltrates from islets with insulitis score 1–3 (pooled) **(d)** or LCM-captured islet immune infiltrates separated by insulitis score (score 1 or 2/3) **(e)**, from recently diabetic ($n = 5$) and normoglycaemic ($n = 5$) NOD mice. Values are expressed as $2^{-\Delta C_t}$ plotted on a linear scale **(c)** or a log10 scale **(d, e)**. Data are presented mean $\pm$ SEM. * $p \leq 0.05$, ** $p \leq 0.01$, Mann–Whitney U test. NG, normoglycaemic;

in plasma samples from individuals recently diagnosed with type 1 diabetes vs the non-diabetic control group, and correlates with glycaemic control, similar to that observed in the NOD mouse.

Discussion

A systematic discovery and characterisation of circulating miRNAs using paired peripheral blood plasma/serum and the diseased tissue are still missing in the field of autoimmune diabetes [30, 31]. However, circulating miRNAs hold great potential as a novel class of non-invasive biomarkers reflecting in situ molecular alterations; the identification of their cellular source could provide new insights into the molecular mechanisms of disease pathogenesis. Thus, circulating miRNAs may be regarded as fingerprints of the affected tissue. We profiled the expression of 384 miRNAs in plasma of recently diabetic and age-matched normoglycaemic NOD mice. We identified five miRNAs (i.e. miR-126a-3p, miR-126a-5p, miR-155, miR-204 and miR-409-3p) differentially expressed in plasma of diabetic vs normoglycaemic mice. All were significantly downregulated in plasma of diabetic vs normoglycaemic mice, in line with previous publications in which miRNA expression levels were mainly downregulated [32–34]. Some of these miRNAs identified in plasma of

diabetic mice were previously discovered in other autoimmune diseases, such as systemic lupus erythematosus, inflammatory bowel disease and rheumatoid arthritis [35–37]. In the case of human type 1 diabetes, conflicting data have been published regarding the role of these circulating miRNAs. Seyhan et al reported high plasma expression of miR-126 in individuals with type 1 diabetes compared with control participants, while Osipova et al did not find differences in plasma miR-126 levels between paediatric type 1 diabetic participants and age- and sex-matched control participants [15, 38]. Assmann et al documented upregulated expression of miR-155 in the plasma of individuals with type 1 diabetes for <5 years compared with non-diabetic control participants, while no alterations were detected between the control participants and those with >5 years of disease onset [39].

In the present study, miR-409-3p showed consistent reduced expression in paired plasma samples and LCM immune-infiltrated islets of recently diabetic NOD mice, especially in the immune infiltrates belonging to severely infiltrated islets. Moreover, we observed reduced miR-409-3p expression in the plasma of individuals recently diagnosed with type 1 diabetes.

Multiple lines of evidence suggest a significant link between miR-409-3p and immune dysregulation in autoimmune diabetes. First, the *miR-409-3p* gene is located on human chromosome 14q32 (or on its mouse homologue 12qF1), a type 1

Fig. 5 Circulating miR-409-3p gradually decreases during diabetes development in NOD mice. **(a)** Pancreatic sections were stained with H&E and insulinitis was scored. **(b)** Circulating miR-409-3p expression analysis in 4-week-old NOD mice ($n = 14$), 8-week-old NOD mice ($n = 14$), 17- to 25-week-old normoglycaemic NOD mice ($n = 25$), 12- to 22-week-old recently diabetic NOD mice ($n = 12$) and 21-week-old NOR mice ($n = 6$). Values are reported as $2^{-\Delta C_t}$. **(c–f)** Percentage of CD44⁺CD62L[−] (TEM) and CD44⁺CD62L⁺ (TCM) within CD4⁺ (**c, d**) and CD8⁺ (**e, f**) T cell gates in peripheral blood (**c, e**) and pancreas (**d, f**) of 4-week-old NOD

mice ($n = 3$), 8-week-old NOD mice ($n = 4$), 17- to 25-week-old normoglycaemic NOD mice ($n = 4$), 12- to 22-week-old recently diabetic NOD mice ($n = 5$) and 21-week-old NOR mice ($n = 8$). **(g)** Expression levels of miR-409-3p in CD4⁺ and CD8⁺ TEM and TCM cells sorted from the pancreases of 26-week-old normoglycaemic NOD mice. Each dot represents a pool of three mice. Data are reported as $2^{-\Delta C_t}$, with values normalised using the small RNA *Rnu6* and small nucleolar RNA *sno135* and *sno202*. Data are presented as mean $\pm$ SEM. *** $p \leq 0.01$, *** $p \leq 0.001$, Mann–Whitney U test. NG, normoglycaemic

diabetes susceptibility locus [40], which hosts a gene cluster that contains multiple miRNAs that target major type 1 diabetes autoantigens or that are involved in immune-mediated inflammation [41]. Second, our bioinformatics analysis revealed that miR-409-3p targets a large number of genes involved in immune-related pathways (e.g. *Pten*, *Mapk1*, *Nfatc1*, *Nfatc2*, *Ccr5*) or in immune metabolism (e.g. *Gapdh*, *Gpi1*, *Pfkfb3*, *Pfkfb3*). In addition, the phosphoinositide 3-kinase (PI3K)/Akt/forkhead box O (FOXO) pathway is enriched for miR-409-3p target genes and overlapped with the metabolic pathway genes (e.g. *Akt3*, *Ccnd2*, *Cdk6*, *Creb5*, *Mapk1*, *Pten*, *Cpt1a*).

Recent studies have highlighted the importance of miRNA-mediated regulation of transcription factors, immune

checkpoints and metabolism-triggered molecules during the differentiation of various immune-cell subsets [42–45]. In detail, *Socs3* (suppressor of cytokine signalling 3) is a direct target of miR-409-3p and is involved in the differentiation of CD8⁺ T cells [46]. *Cpt1a*, a previously demonstrated regulator of memory CD8⁺ T cell lipid metabolism and immune response in autoimmune diseases [47], is a conserved predicted target of miR-409-3p. Although additional functional studies are needed, these findings already suggest that dysregulated levels of miR-409-3p in plasma and in islet immune infiltrates may be involved in the pathogenesis of type 1 diabetes by inducing aberrant expression of genes involved in metabolic and immune pathways that may modulate the islet microenvironment and regulate the islet-

Fig. 6 aCD3 treatment increases plasma circulating levels of miR-409-3p. **(a)** Treatment and sample collection scheme for the administration of cumulative doses (from day 0 to day 7) of aCD3 in recently diabetic NOD mice followed up to day 14. **(b, c)** Expression levels of miR-409-3p in plasma collected longitudinally at day 0 (diabetes onset) and at day 14 in untreated ($n = 6$) **(b)** and aCD3-treated ($n = 6$) **(c)** groups. Values are expressed as $2^{-\Delta C_t}$. The connecting lines indicate paired data from the same NOD mice. **(d, e)** Percentage of CD44⁺CD62L⁺ (TEM) and CD44⁺CD62L⁺ (TCM) within CD4⁺ **(d)** and CD8⁺ **(e)** T cell gates in the pancreas of untreated ($n = 5$) and aCD3-treated ($n = 6$) groups, compared with recently diabetic mice ($n = 5$). Data are presented as mean $\pm$ SEM. * $p \leq 0.05$, Wilcoxon-signed rank test **(b, c)** or Mann–Whitney U test **(d, e)**

derived immune phenotype. A third line of evidence comes from data obtained during the natural course of diabetes progression and after diabetes remission by aCD3 therapy. While islet-specific inflammation gradually progresses during type 1 diabetes development [48], we observed a gradual reduction in miR-409-3p expression in the plasma of prediabetic NOD mice until disease onset. Importantly, plasma levels of miR-409-3p were significantly higher in normoglycaemic diabetes-resistant NOR mice compared with those in age-matched recently diabetic and normoglycaemic NOD mice. It is unlikely that circulating miR-409-3p expression levels rely simply on blood glucose levels. Indeed, normoglycaemic NOR mice, having a lower degree of insulinitis, had higher plasma miR-409-3p levels compared with age-matched normoglycaemic NOD mice, suggesting that other factors modulate miRNA expression and secretion.

In humans, miR-409-3p has been reported to be expressed in both peripheral CD4⁺ and in CD8⁺ T cells [26]. Here, we found that miR-409-3p was also expressed in both islet-associated CD4⁺ and CD8⁺ T cells, with specific enrichment in CD8⁺ T cells

Fig. 7 Relative and absolute miR-409-3p quantification in plasma of non-diabetic control participants and individuals recently diagnosed with type 1 diabetes from different cohorts. **(a, b)** Relative (reported as $2^{-\Delta C_t}$) **(a)** and absolute (reported as nmol/l values interpolated using a synthetic miR-409-3p standard curve) **(b)** quantification of plasma circulating miR-409-3p in non-diabetic individuals (control group; $n = 34$) and recently diagnosed individuals with type 1 diabetes (<1 year since diagnosis; $n = 18$) (Cohort 1). Red data points indicate autoantibody-negative unaffected family members of individuals with type 1 diabetes. **(c)** Relative quantification of plasma circulating miR-409-3p in non-diabetic individuals (control; $n = 17$), recently diagnosed individuals with type 1 diabetes (<1 year of diagnosis; $n = 23$) and individuals with longstanding type 1 diabetes (>2 years of diagnosis, $n = 13$) (second, historical cohort [Cohort 2]). Individuals with rheumatoid arthritis ($n = 18$) were also included in the analysis. Data are expressed as $2^{-\Delta C_t}$ or nmol/l and presented as mean values plotted on a log₁₀ scale. * $p \leq 0.05$, Mann–Whitney U test. CTR, control; RA, rheumatoid arthritis; T1D, type 1 diabetes

having a central memory phenotype (CD8⁺ TCM cells). Interestingly, we observed a gradual decrease in the frequency of islet-associated CD8⁺ TCM cells during disease progression, coinciding with the reduction in circulating plasma-borne miR-409-3p levels. We also found that plasma levels of miR-409-3p were retained following aCD3 treatment in recently diabetic NOD mice, indicating remodelling of the autoimmune process. Interestingly, we observed that aCD3 therapy increased the frequency of islet-associated CD8⁺ TCM cells, corresponding with the increase in cell-free plasma-borne miR-409-3p levels. Again, miR-409-3p levels were not dependent on blood glucose levels, thus reflecting possible dependence on immune modulation. Concordantly, we and others found that aCD3 therapy does not block insulinitis but rather changes the composition and regulatory balance of the autoimmune infiltrates [17, 18, 28, 49]. Although our data suggest that islet-associated CD8⁺ TCM cells may be the potential secretors of miR-409-3p, we do not claim that the miR-409-3p plasma pool derives exclusively from these islet-infiltrating immune cells. Other cell types may also contribute

to the circulating miR-409-3p pool and specific mechanisms leading to its downregulation in plasma should be further elucidated.

Finally, similarly to what was observed in the NOD mouse model, miR-409-3p levels were lower in plasma of adults recently diagnosed with type 1 diabetes compared with non-diabetic control groups in two independent cohorts. Despite the high overlap of the plasma miR-409-3p levels between individuals recently diagnosed with type 1 diabetes and the healthy non-diabetic participants, the reduction was statistically significant. Future miRNAs analyses could focus on specific plasma components (e.g. exosomes) to reduce this variability. Interestingly, the miR-409-3p reduction was not observed in plasma samples from individuals with longstanding type 1 diabetes, thus suggesting a potential association with disease aggressiveness and insulinitis severity. Moreover, miR-409-3p levels correlated with HbA_{1c} at disease onset, further implying a potential association with insulinitis severity and progression.

Of relevance, miR-409-3p expression in plasma samples from individuals with rheumatoid arthritis was higher compared with those recently diagnosed with type 1 diabetes and the non-diabetic control group. It should be emphasised that the samples from people with rheumatoid arthritis were not obtained at disease onset and these individuals were taking disease-modifying anti-rheumatic drugs combined with low-dose corticosteroids. If miR-409-3p is a circulating biomarker of tissue inflammation, it may not be surprising that individuals with rheumatoid arthritis had higher circulating miR-409-3p levels compared with individuals recently diagnosed with type 1 diabetes. Again, these findings suggest the involvement of miR-409-3p in immune modulation. Still, additional studies will be needed to further elucidate the biological role and cellular origin of miR-409-3p. Although we suggest a potential role for miR-409-3p as a novel biomarker of islet inflammation and immune dysregulation in autoimmune diabetes, we recognise the following limitations: (1) the elevated overlap observed between individuals recently diagnosed with type 1 diabetes and the control group; (2) the absence of a paediatric cohort with type 1 diabetes; and (3) the lack of evidence that specifically demonstrates the cellular origin of miR-409-3p. However, we envision a combined type 1 diabetes biomarker signature including, but not limited to, miR-409-3p alongside other variables comprising additional miRNAs and/or small RNAs.

In conclusion, circulating plasma-borne miR-409-3p may represent a candidate biomarker of islet inflammation in both type 1 diabetic mice and humans and could be potentially useful for serial tracking of islet inflammation or response to immune therapies, such as teplizumab aCD3 therapy, in intervention and prevention trials [50, 51].

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