


Circulating microRNA levels predict residual beta cell function and glycaemic control in children with type 1 diabetes mellitus

Nasim Samandari¹ · Aashiq H. Mirza^{1,2} · Lotte B. Nielsen¹ · Simranjeet Kaur¹ · Philip Hougaard³ · Siri Fredheim¹ · Henrik B. Mortensen^{1,4} · Flemming Pociot^{1,2,4} 

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Abstract

Aims/hypothesis We aimed to identify circulating microRNA (miRNA) that predicts clinical progression in a cohort of 123 children with new-onset type 1 diabetes mellitus.

Methods Plasma samples were prospectively obtained at 1, 3, 6, 12 and 60 months after diagnosis from a subset of 40 children from the Danish Remission Phase Cohort, and profiled for miRNAs. At the same time points, meal-stimulated C-peptide and HbA_{1c} levels were measured and insulin-dose adjusted HbA_{1c} (IDAA_{1c}) calculated. miRNAs that at 3 months after diagnosis predicted residual beta cell function and glycaemic control in this subgroup were further validated in the remaining cohort ($n=83$). Statistical analysis of miRNA prediction for disease progression was performed by multiple linear regression analysis adjusted for age and sex.

Results In the discovery analysis, six miRNAs (hsa-miR-24-3p, hsa-miR-146a-5p, hsa-miR-194-5p, hsa-miR-197-3p, hsa-miR-301a-3p and hsa-miR-375) at 3 months correlated with residual

beta cell function 6–12 months after diagnosis. Stimulated C-peptide at 12 months was predicted by hsa-miR-197-3p at 3 months ($p=0.034$). A doubling of this miRNA level corresponded to a sixfold higher stimulated C-peptide level. In addition, a doubling of hsa-miR-24-3p and hsa-miR-146a-5p levels at 3 months corresponded to a 4.2% ($p<0.014$) and 3.5% ($p<0.022$) lower IDAA_{1c} value at 12 months. Analysis of the remaining cohort confirmed the initial finding for hsa-miR-197-3p ($p=0.018$). The target genes for the six miRNAs revealed significant enrichment for pathways related to gonadotropin-releasing hormone receptor and angiogenesis pathways.

Conclusions/interpretation The miRNA hsa-miR-197-3p at 3 months was the strongest predictor of residual beta cell function 1 year after diagnosis in children with type 1 diabetes mellitus.

Keywords Biomarkers · Children · miRNA · Residual beta cell function

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✉ Flemming Pociot
flemming.pociot.01@regionh.dk

¹ Copenhagen Diabetes Research Center (CPH-DIRECT), Department of Paediatrics, Herlev and Gentofte Hospitals, University of Copenhagen, Herlev Ringvej 75, 2730 Herlev, Denmark

² Center for Non-coding RNA in Technology and Health, University of Copenhagen, Copenhagen, Denmark

³ Department of Statistics, University of Southern Denmark, Odense, Denmark

⁴ Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

Abbreviations

C _p	Crossing point
CLIP-seq	Cross-linking, immunoprecipitation and high-throughput sequencing
GO	Gene ontology
IDAA _{1c}	Insulin-dose adjusted HbA _{1c}
KEGG	Kyoto Encyclopedia of Genes and Genomes
miRNA	MicroRNA
PANTHER	Protein ANALYSIS THrough Evolutionary Relationship
RT-qPCR	Reverse transcription-quantitative PCR

Introduction

Over the past twenty years, the interest in the expression of circulating variations of microRNA (miRNA) has increased, specifically in regard to their potential role as biomarkers for several diseases. The leading hypothesis is that differential miRNA expression profiles may provide insight into pathological processes related to disease origin and thereby potentially serve as non-invasive predictive biomarkers for diagnosis and disease progression [1].

miRNAs belong to the class of evolutionarily conserved non coding RNAs (ncRNAs) and are small, single-stranded molecules (18–25 nucleotides). They predominantly regulate gene expression at a post-transcriptional level through partial base-pairing with their target mRNAs to the 3' untranslated region (UTR) [2, 3]. The binding of miRNA to target mRNA leads to either decreased translational efficiency or repression of mRNA levels, thus indicating that destabilisation of target mRNAs is the main reason for reduced protein output [4]. A fast-growing wealth of evidence shows the importance of miRNAs as fundamental master regulators in cell cycle processes, and their dysregulation is indicated to be involved in various pathophysiologicals, including autoimmune and metabolic disorders [5, 6]. Circulating, cell-free miRNAs are stabilised and protected from RNase degradation mainly by inclusion in microvesicles or exosomes [7]. Studies have also demonstrated that endogenous plasma miRNAs are protected from degradation in the RNase-rich environment of the blood by the argonaute 2 complex [8].

The pathogenesis of type 1 diabetes mellitus still remains unclear but it is well established that the immune system initiates a cascade of events that lead to the destruction of insulin-producing beta cells [9, 10]. The end-stage condition is a lack of endogenous insulin production, resulting in elevated blood glucose levels and lifelong insulin treatment. Clinical challenges in the early and precise diagnosis of children and adolescents with type 1 diabetes are obvious, and optimal treatment is crucial to obtain the best prognosis and avoid severe late complications such as microvascular and macrovascular injuries, often associated with the disease [11].

Over the past few years, evidence has accumulated for the role of specific miRNAs in type 1 diabetes mellitus [12–19]. However, the prognostic potential of miRNAs in type 1 diabetes has been largely unexplored. We hypothesised that biomarkers, in the form of miRNAs in plasma from children with recent-onset type 1 diabetes mellitus would be able to predict the decline or regeneration of endogenous residual beta cell function. Therefore, the aim of this study was to identify circulating miRNA that predicts clinical progression as assessed by stimulated C-peptide, HbA_{1c} and insulin-dose-adjusted HbA_{1c} (IDAA_{1c}) in children with new-onset type 1 diabetes.

Methods

Study cohort The Danish Remission Phase Cohort is a longitudinal multicentre study that was conducted prospectively in four paediatric outpatient clinics over 2004–2005. A total of 126 children (61 girls) younger than 17 years old and with newly diagnosed type 1 diabetes mellitus were included in the study. Demographic and anthropometric data, as well as blood samples for centralised measurement of HbA_{1c} and meal-stimulated C-peptide, were collected prospectively 1, 3, 6 and 12 months after diagnosis (± 1 week) for all individuals, and also after 60 months in a subset of 40 patients. For three individuals, there was insufficient material for miRNA analysis. Compared with the entire cohort ($n = 123$), the 40 patients in the subgroup who completed follow-up at a diabetes duration of 5 years were slightly younger, with a lower BMI, and a lower postprandial C-peptide levels 12 months after diagnosis. However, the subgroup was similar to the entire cohort in terms of sex distribution and HbA_{1c} levels. Causes for dropout or loss at the 5 year follow-up were transferral of adolescents to adult clinics, changes of residence and unwillingness to participate. A complete overview of the characteristics of the original study cohort can be found in Table 1 and earlier publications [20, 21]. Insulin dose ($\text{U kg}^{-1} 24 \text{ h}^{-1}$) and IDAA_{1c} were recorded. IDAA_{1c} is an indirect HbA_{1c}- and insulin-weighted measure of residual beta cell function and was calculated on basis of the actual insulin dose and HbA_{1c} as follows: $\text{IDAA}_{1c} = \text{HbA}_{1c} (\%) + (4 \times \text{insulin dose} [\text{U kg}^{-1} 24 \text{ h}^{-1}])$ [22]. Plasma samples were collected using a highly standardised protocol in order to avoid preanalytical variations, and were stored at -80°C until further use [23]. The diagnosis of diabetes was classified according to World Health Organization criteria [24]. Ninety-six per cent of samples were positive for at least one autoantibody 1 month after diagnosis (GAD65A 60.3%, insulin autoantibody 82.5%, insulinoma-associated antigen 2 antibody 69.0% and zinc transporter 8 autoantibody 62%) [20].

Exclusion criteria included suspected non-type 1 diabetes mellitus (type 2 diabetes, MODY or secondary diabetes), decline of enrolment by patients or parents, and initial treatment outside of the study centre for more than 5 days (if patients were initially treated outside of the study centre for ≤ 5 days, the patient was included and the data necessary for the study was requested from the referring hospital). The study was performed according to the criteria of the Helsinki II Declaration and was approved by the Danish National Committee on Biomedical Research Ethics (journal number H-KA-04010-m). Older children gave their assent and all parents or guardians gave written informed consent.

miRNA isolation and reverse transcription-quantitative PCR miRNA isolation was performed on a total of 182 samples from the 40 children who participated in the 60 month visit.

Table 1 Clinical and demographic data of participants in the Danish Remission Phase Cohort

Cohort	1 month	3 months	6 months	12 months	60 months
Discovery cohort					
<i>n</i> (girls, <i>n</i>)	40 (24)	37 (22)	33 (19)	34 (20)	38 (23)
Age at diagnosis (years)	8.7 (3.4)	8.6 (3.5)	8.8 (3.5)	8.6 (3.5)	8.5 (3.5)
HbA _{1c} (DCCT, %)	9.1 (1.3)	7.3 (1.1)	7.8 (1.6)	7.9 (1.1)	8.4 (1.0)
HbA _{1c} (IFCC, mmol/mol)	76 (14.2)	56 (12)	62 (17.5)	63 (12)	68 (11)
C-peptide (pmol/l)	629 (371)	528 (323)	436 (333)	257 (251)	62 (76)
IDAA _{1c}	10.8 (2.0)	9.2 (1.5)	10.1 (2.2)	11.0 (1.8)	12 (1.9)
Insulin dose (U kg ⁻¹ 24 h ⁻¹)	0.45 (0.27)	0.47 (0.23)	0.58 (0.26)	0.78 (0.27)	0.88 (0.32)
<i>HLA-DQ</i> risk group (<i>n</i>)					
Very high risk ^a	12	12	11	9	12
High risk ^b	20	17	14	18	18
Moderate risk ^c	6	6	6	5	6
Low risk ^d	2	2	0	2	2
Validation cohort					
<i>n</i> (girls, <i>n</i>)	80 (35)	83 (37)	80 (36)	81 (37)	
Age at diagnosis (years)	10.7 (4.1)	10.7 (4.0)	10.9 (4.0)	10.7 (4.1)	
HbA _{1c} (DCCT, %)	9.3 (1.2)	6.9 (0.7)	7.1 (1.0)	7.6 (1.2)	
HbA _{1c} (IFCC, mmol/mol)	79 (13)	51 (8)	54 (11)	59 (13)	
C-peptide (pmol/l)	742 (414)	762 (477)	648 (439)	448 (398)	
IDAA _{1c}	11.1 (1.6)	8.6 (1.3)	9.3 (1.8)	10.4 (1.9)	
Insulin dose (U kg ⁻¹ 24 h ⁻¹)	0.46 (0.21)	0.43 (0.23)	0.55 (0.27)	0.71 (0.32)	
<i>HLA-DQ</i> risk group (<i>n</i>)					
Very high risk ^a	33	34	33	32	
High risk ^b	30	33	33	33	
Moderate risk ^c	8	8	7	8	
Low risk ^d	4	4	3	4	

Data are mean (SD), unless otherwise stated

For *HLA-DQ* risk grouping, missing data (discovery cohort: *n* = 2 at 6 months; validation cohort: *n* = 5 at 1 month, *n* = 4 at 3 months, *n* = 4 at 6 months and *n* = 4 at 12 months) is a result of: (1) samples could not be drawn at visit; (2) samples missing; (3) too little material for the analysis

^a *HLA-DQB1*0201/DQB1*0302*

^b *HLA-DQB1*0302/X, DQB1*0201/X, DQB1*0302/DQB1*0604, DQB1*0304/DQB1*0201*

^c *HLA-DQB1*0603/DQB1*0302, DQB1*0301/DQB1*0302, DQB1*0604/DQB1*0304, DQB1*0201/DQB1*0603, DQB1*0201/DQB1*0301, DQB1*0604/X, DQB1*0304/X, X/X*

^d *HLA-DQB1*0602/DQB1*0304, DQB1*0602/DQB1*0302, DQB1*0602/DQB1*0201, DQB1*0603/DQB1*0301, DQB1*0604/DQB1*0301, DQB1*0301/X, DQB1*0602/X DQB1*0603/X*

Total RNA was extracted from 200 µl EDTA-plasma using the miRCURY RNA isolation kit biofluids (Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions. Carrier RNA 1 µg per 60 µl Lysis Solution and RNA spike-in mixture were added to each sample (Exiqon). RNA (7 µl) was reverse-transcribed in 35 µl reactions using the miRCURY LNA Universal RT miRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon). cDNA was diluted 50× and assayed in 10 µl reaction volumes using the miRCURY LNA Universal RT miRNA PCR human serum/plasma panel (v3; Exiqon), which included 179 specific human miRNAs (several of which targeted immunological pathways), with ExiLent SYBR Green master mix (Exiqon). For the subsequent validation

study, RNA was extracted and reverse-transcribed as described above. Reverse transcription-quantitative PCR (RT-qPCR) was performed using a Custom Pick-&-Mix panel and ExiLent SYBR Master Mix (Exiqon). All RT-qPCR assays were performed using a LightCycler480 Real-Time PCR System (Roche, Hvidovre, Denmark) in 384-well plates.

Quality control Quality control was carried out on a subset of samples using synthetic spike-in RNAs to analyse the robustness of the RNA isolation process and quality of isolated miRNA. The RNA isolation controls (UniSp2, UniSp4 and UniSp5; Exiqon) were added to the purified RNA and were used to detect differences in extraction efficiency. The cDNA

synthesis control (UniSp6; Exiqon) was added in the reverse transcription reaction, to evaluate the efficacy of the reverse transcription process. In addition, a DNA spike-in (Sp3; Exiqon) was present on all panels. Haemolysis was assessed by the ratio between hsa-miR-451a (which is expressed in erythrocytes) and hsa-miR-23a-3p (which is relatively stable in plasma and serum and not affected by haemolysis) [25]. The difference in crossing point (ΔC_p) between miR-23a-3p and miR-451a provides a good measure of the degree of haemolysis, with values >5 indicating possible erythrocyte miRNA contamination [25].

Normalisation All individual samples ($n = 182$) were run on a predefined assay panel of 179 specific human miRNAs. From the initial profiling, the 100 miRNAs with complete data were employed for the global mean method for normalisation of the data as this was found to be the most stable normaliser [26].

Statistical analysis and identification of candidate miRNAs Statistical analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC, USA). Stimulated C-peptide and miRNA levels were studied on a logarithmic scale (\log_e).

Prediction of stimulated C-peptide, IDAA_{1c} and HbA_{1c} at 6 and 12 months was done by multiple linear regression analysis for each miRNA separately, including the covariates for sex and age. For each outcome (stimulated C-peptide, IDAA_{1c} and HbA_{1c}) at each time point (6 and 12 months) separately, multiple testing correction was applied across 688 analyses (172 miRNAs at time points 1, 3, 6 and 12 months) using the Hochberg method [27]. Values of $p < 0.05$ were considered significant. Samples below the detection limit (the number of C_p cycles reaching 37) were included by imputing the covariate value as the detection limit.

Pearson's correlation coefficient and Pearson's test p values were used for scatter plots and regression lines for the correlation between age and hsa-miR-197-3p. Partial correlation coefficients between \log_e C-peptide and hsa-miR-197-3p accounting for sex and age were calculated.

Validation of candidate miRNAs Six candidate miRNAs with significantly predictive value for stimulated C-peptide, HbA_{1c} and IDAA_{1c} in the subgroup of 40 children were analysed in the remaining cohort of 83 children in samples taken 3 months after diagnosis. As only six miRNAs were analysed, two additional miRNAs (hsa-miR-21-5p and hsa-miR-425-5p) were selected as endogenous controls for normalisation based on results from the initial profiling analysis. These two miRNAs showed high stability (NormFinder, <http://cancerres.aacrjournals.org/content/64/15/5245.long>, accessed December 2013) and strong correlation with the global mean ($r^2 = 0.99$). p values in the validation study

were not corrected for multiple testing, although adjustments were made for sex and age.

miRNA target prediction and pathway analysis The miRNA targets were retrieved from MiRWALK (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/>, accessed 15 April 2016) [28] and miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/>, accessed 06 June 2016) [29]. miRNA target interaction maps were retrieved based on cross-linking, immunoprecipitation and high-throughput sequencing (CLIP-Seq) from starBase database v2.0 (<http://starbase.sysu.edu.cn/>, accessed 27 October 2016) [30]. Target genes were selected based on a minimum of three prediction tools and CLIP-Seq overlap with biological complexity ≥ 1 (a measure of reproducibility between biological replicates or experiments in order to further reduce the number of false-positive results). The target genes were further filtered based on a CLIP-Seq read number ≥ 1 . Gene ontology (GO) and pathway-based over-enrichment tests for miRNA target genes were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.jp/kegg/>, accessed 18 August 2016) [31] and the Protein ANalysis THrough Evolutionary Relationship (PANTHER; <http://pantherdb.org/>, 18 August 2016) [32]. The enrichment for GO terms in the biological process, molecular function and cellular component category was identified based on the binomial test in PANTHER. The KEGG and PANTHER pathways were used for pathway-based enrichment of the target genes. The total protein coding genes in the human genome were used as the reference list.

A complete overview outlining the study design is shown in Fig. 1.

Results

Quality control of samples using ‘spike-ins’ (technical controls) The RNA spike-ins showed low variation in C_p between samples in RNA isolation and cDNA synthesis, indicating that extraction, reverse transcription and qPCR were successful and none of the samples contained inhibitors. The ratio between the miRNAs hsa-miR-451a and hsa-miR-23a-3p varied between 5 and -2 , indicating that the samples were not affected by haemolysis. Thus, overall, these findings show a good and similar level of sample quality and reproducibility of processes for miRNA profiling.

Candidate miRNAs associated with residual beta cell function and glycaemic control Out of the 179 miRNAs tested, 100 were detectable across all samples (1, 3, 6, 12 and 60 months), with an average of 163 miRNAs detectable per sample (see electronic supplementary material [ESM] Table 1). Multiple linear regression analysis showed that six

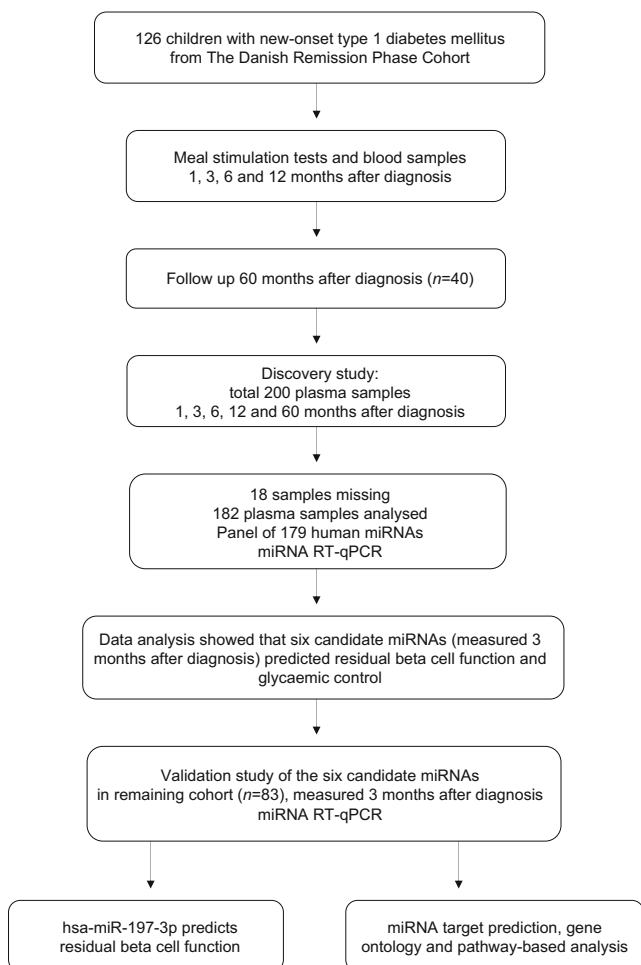


Fig. 1 The flowchart depicts the study design and the experimental workflow, including data analysis

miRNAs (hsa-miR-24-3p, hsa-miR-146a-5p, hsa-miR-194-5p, hsa-miR-197-3p, hsa-miR-301a-3p and hsa-miR-375) 3 months after diagnosis predicted the levels of stimulated C-peptide, HbA_{1c} or IDAA_{1c} 6 or 12 months after diagnosis (Table 2). Only significant observations are shown in Table 2. At 1 month after diagnosis, no miRNAs had any predictive value.

Stimulated C-peptide at 12 months was predicted by hsa-miR-197-3p measured 3 months after diagnosis ($p=0.034$) (Table 2). C-peptide is considered on a natural log scale, and therefore the value of 1.80 for C-peptide (12 months) corresponds to a factor of C-peptide of $e^{1.80}=6.05$. However, when the baseline C-peptide value (1 month after diagnosis) was included in the statistical model as a response, the significance did not remain after correction for multiple testing (unadjusted p value 0.00019, adjusted p value 0.13; data not shown). Additionally, a doubling of the miRNAs hsa-miR-24-3p and hsa-miR-146a-5p measured at 3 months corresponded to a 4.23% and 3.49% decrease in IDAA_{1c} 12 months after diagnosis ($p<0.014$ and $p<0.022$, respectively).

None of the measured miRNAs could predict residual beta cell function or glycaemic control 60 months after diagnosis. Only a limited number of miRNAs ($n=8$) showed a differential expression over time ($p<0.05$; ESM Table 1, marked with an asterisk). Whether this is an effect related to age or duration of disease has not yet been investigated. However, there was no overlap between these eight differentially expressed miRNAs and the six candidate miRNAs identified with predictive value.

Age had no significant effect on the level of hsa-miR-197-3p, both variables being measured 3 months after diagnosis ($r=0.22$, $p=0.22$) (Fig. 2).

Validation of the candidate miRNAs The six miRNAs (hsa-miR-24-3p, hsa-miR-146a-5p, hsa-miR-194-5p, hsa-miR-197-3p, hsa-miR-301a-3p and hsa-miR-375) were further validated in the remaining cohort ($n=83$). This analysis confirmed the initial finding that the level of hsa-miR-197-3p at 3 months predicted stimulated C-peptide concentrations 6 and 12 months after diagnosis ($p<0.02$; (Table 2)), with an estimated log_e C-peptide of 0.65 at 6 months, and 0.75 at 12 months, suggesting a 1.9-fold and 2.1-fold higher stimulated C-peptide level at 6 months and 12 months, respectively.

Scatter plots comparing the levels of hsa-miR-197-3p and stimulated C-peptide in both the discovery and validation cohorts at 6 and 12 months are shown in Fig. 3. These indicate a significant association between the two variables in both cohorts, with partial correlation coefficients accounting for sex and age (r values between 0.27 and 0.66).

Identification of miRNA target genes In total, 825 unique target genes were identified, with CLIP-Seq overlap for the six candidate miRNAs predicting residual beta cell function and IDAA_{1c}. The functional annotation of the target genes based on GO analyses revealed significantly enriched GO terms in biological processes, molecular function and the cellular component category (Table 3). The pathway-based annotation revealed enrichment for the gonadotropin-releasing hormone receptor pathway ($p=3.91 \times 10^{-3}$) and angiogenesis ($p=2.37 \times 10^{-2}$) (Table 3). We also observed significant enrichment for various transcriptional, signalling and metabolic processes in the biological process category (Table 3).

Out of ~400 predicted target genes for hsa-miR-197-3p, only six genes are supported by strong experimental evidence, such as Reporter Gene Assay data [28, 29]; *TUSC2*, *NSUN5*, *CD82*, *BMF*, *PMAIP1*, and *MTHFD1*. *TUSC2* and *CD82* are cancer-related genes, *NSUN5* functions as a methyltransferase, and *BMF* and *PMAIP1* are involved in various apoptosis pathways. *MTHFD1* is involved in the metabolism of vitamins and cofactors and histidine degradation.

Table 2 miRNAs at 3 months associated with residual beta cell function and glycaemic control

Outcome 6 and 12 months after diagnosis	miRNA	<i>p</i> value	Adjusted <i>p</i> value	Estimate ^a	SE
Initial miRNA profiling, discovery (<i>n</i> = 40)					
Log _e C-peptide (6 months)	hsa-miR-375	3.6×10^{-5}	0.018	-0.42 ^b	0.09 ^b
HbA _{1c} (6 months)	hsa-miR-301a-3p	3.3×10^{-5}	0.017	-1.08 ^c	0.22 ^c
Log _e C-peptide (12 months)	hsa-miR-197-3p	4.9×10^{-5}	0.034	1.80 ^b	0.38 ^b
IDAA _{1c} (12 months)	hsa-miR-24-3p	2.0×10^{-5}	0.014	-4.23	0.83
	hsa-miR-146a-5p	3.3×10^{-5}	0.022	-3.49	0.71
	hsa-miR-194-5p	5.0×10^{-5}	0.038	1.22	0.26
Validation of miRNA profiling (<i>n</i> = 83)					
Log _e C-peptide (6 months)	hsa-miR-197-3p	1.3×10^{-2}	N/A	0.65 ^b	0.26 ^b
Log _e C-peptide (12 months)	hsa-miR-197-3p	1.8×10^{-2}	N/A	0.75 ^b	0.31 ^b

Six miRNAs measured 3 months after diagnosis showed an association with residual beta cell function in the subset of 40 patients who completed follow-up at 60 months. Validation of the candidate miRNAs was performed in the remaining cohort

^a Estimate is sex- and age-adjusted

^b pmol/l

^c Per cent of total haemoglobin

MicroRNAs are measured on a scale where a change of one unit corresponds to a doubling of the content. Thus, the estimate of -1.08 for HbA_{1c} (6 months), for example, means that a doubling of microRNA content in a linear model implies a predicted HbA_{1c} value that is 1.08% (11.7 mmol/mol) lower. Whereas, for C-peptide, a doubling in microRNA content gives a value of 1.80 (12 months), which corresponds to a factor of $e^{1.80} = 6.05$, since C-peptide is considered on a natural log scale.

N/A, not applicable

Discussion

The rate of decline of endogenous insulin production in children and adolescents with newly diagnosed type 1 diabetes mellitus varies considerably between individuals. Biomarkers that can predict the rate of loss of residual beta cell function will be of immense clinical value for the optimisation of current therapies and design of new treatment modalities to prolong endogenous insulin production. To our knowledge, this is the first prospective study to comprehensively investigate which miRNAs may influence beta cell function in terms of stimulated C-peptide production and glycaemic control after diagnosis.

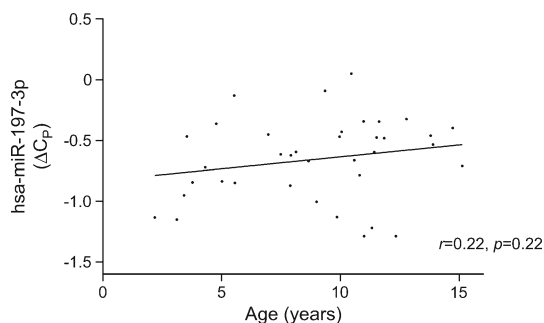


Fig. 2 The effect of age on hsa-miR-197-3p 3 months after diagnosis. The graph shows the relationship between age at disease onset of the study participants and the normalised expression levels of hsa-miR-197-3p, both measured 3 months after diagnosis. No significant effect of age on hsa-miR-197-3p level ($r=0.22$, $p=0.22$) was seen at this time point. ΔC_p , miRNA expression level normalised to the global mean

Six miRNAs were associated with residual beta cell function and glycaemic control during the first year after disease onset in the 40 patients who completed follow-up at disease duration of 5 years. In the entire cohort, only hsa-miR-197-3p at 3 months was able to predict stimulated C-peptide levels at 6 and 12 months after diagnosis. This was also illustrated by the significant partial correlation between hsa-miR-197-3p and stimulated C-peptide levels in the discovery and validation cohorts. However, when baseline C-peptide at 1 month was included in the statistical model, it weakened the impact of miR-197-3p for predicting stimulated C-peptide levels. At 1 month after diagnosis none of the miRNAs had any predictive value for residual beta cell function. This finding may suggest that the metabolic dysregulation was not completely recovered at this time point. At 3 months, most of the patients were in partial remission with improved residual beta cell function, as assessed by increased levels of stimulated C-peptide. Age of onset had no impact on the hsa-miR-197-3p level 3 months after diagnosis, which indicates that hsa-miR-197-3p level is independent of age at this time point. This implies that the hsa-miR-197-3p level can be referenced to other children without age adjustment. By contrast, age has a strong influence on stimulated C-peptide, which has to be taken into consideration when evaluating disease severity [23].

In type 2 diabetes mellitus, which is characterised by impaired beta cell function and insulin resistance, one study identified a unique plasma miRNA profile comprising ten miRNAs [33]. Interestingly, hsa-miR-197-3p was also found

Fig. 3 Correlation between hsa-miR-197-3p expression and \log_e C-peptide levels at 6 and 12 months. Scatter plots comparing expression levels (normalised to the global mean [ΔC_p]) of hsa-miR-197-3p and \log_e C-peptide (pmol/l) in (a) the Discovery cohort (6 months), $r=0.36$, $p=0.048$, (b) the Validation cohort (6 months), $r=0.28$, $p=0.013$, (c) the Discovery cohort (12 months), $r=0.66$, $p<0.0001$, and (d) Validation cohort (12 months), $r=0.27$, $p=0.018$. r values represent partial correlations accounting for sex and age

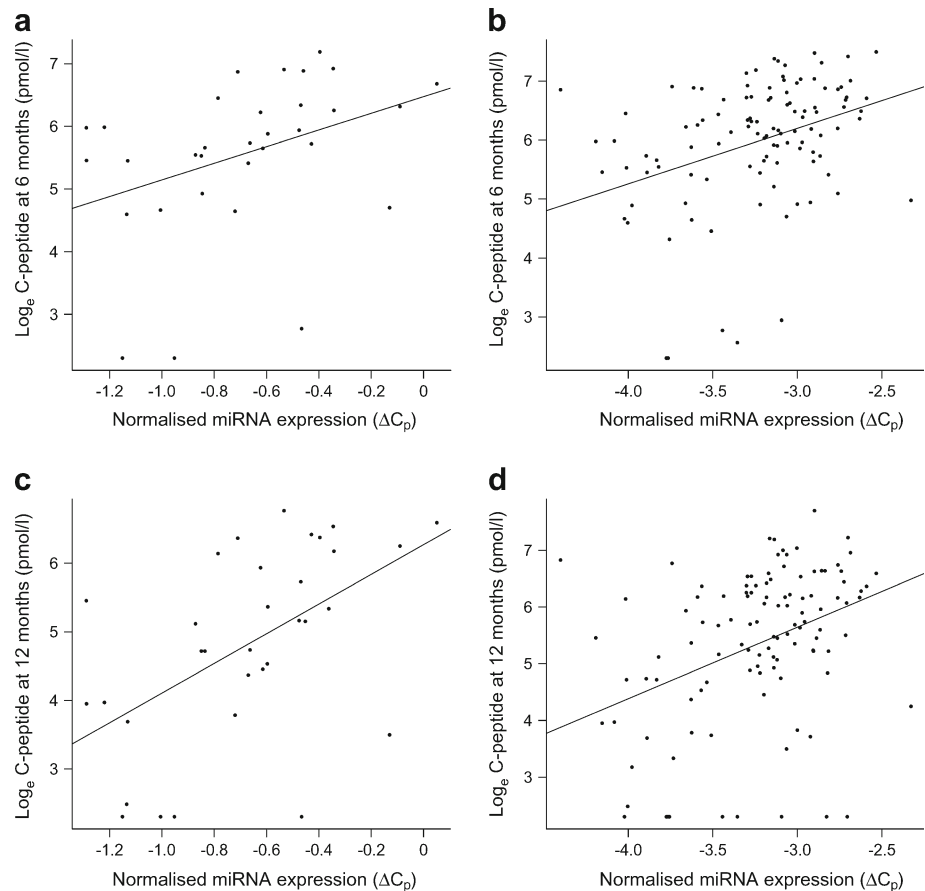


Table 3 GO and pathway-based analysis of target genes for six miRNAs

Enriched pathways/GO terms	Reference	Count	Expected	Fold enrichment	p value
Pathways					
Gonadotropin-releasing hormone receptor pathway	235	30	12.79	2.35	3.91×10^{-3}
Angiogenesis	176	23	9.58	2.4	2.37×10^{-2}
GO biological process					
Cell cycle	901	78	49.02	1.59	1.33×10^{-2}
Embryo development	155	22	8.43	2.61	1.58×10^{-2}
I- κ B kinase/NF κ B cascade	72	16	3.92	4.08	8.58×10^{-4}
Intracellular signal transduction	991	91	53.92	1.69	3.29×10^{-4}
Immune response	717	17	39.01	0.44	1.19×10^{-2}
Intracellular protein transport	1025	87	55.77	1.56	9.93×10^{-3}
Protein transport	1048	88	57.02	1.54	1.30×10^{-2}
MAPK cascade	316	34	17.19	1.98	4.74×10^{-2}
Regulation of transcription from RNA polymerase II promoter	976	83	53.1	1.56	1.40×10^{-2}
Metabolic process	6817	460	370.88	1.24	4.44×10^{-6}
Transcription from RNA polymerase II promoter	1219	115	66.32	1.73	3.00×10^{-6}
Transcription, DNA-dependent	1521	133	82.75	1.61	1.64×10^{-5}
Sensory perception	666	11	36.23	0.30	1.66×10^{-4}

The enriched pathways and GO terms in the biological process category are listed for the 1117 target genes for six miRNAs

The GO terms are followed by number of genes having the enriched term in the reference list ('Reference'), the number of genes in the input list having the enriched term ('Count'), the expected value (Reference divided by total number of genes in human genome), fold enrichment and Bonferroni corrected p values, MAPK, mitogen-activated protein kinase

to correlate with impairments in glycaemic control in individuals with type 2 diabetes, as observed in the present study with type 1 diabetes mellitus. An elevated level of hsa-miR-197-3p at 3 months in patients who completed follow-up at a diabetes duration of 5 years was associated with a sixfold increase in residual beta cell function, as assessed by stimulated C-peptide 12 months after diagnosis. This finding was replicated in the remaining study population of 83 children. Together, these findings suggest a role for hsa-miR-197-3p in beta cell function in both type 1 and type 2 diabetes. Interestingly, some of the best validated target genes for hsa-miR-197-3p, i.e. *BMF* and *PMAIP1*, are involved in the beta cell apoptosis pathways [34, 35]. Furthermore, hsa-miR-197-3p is involved in different immune pathways in other autoimmune diseases, including multiple sclerosis [36], coeliac disease [37] and Graves' disease [38], and in patients with the metabolic syndrome [39].

In the subgroup of the 40 children who participated at the 60 month visit, we found that stimulated C-peptide, IDAA_{1c} and HbA_{1c} at 6 and 12 months were predicted by hsa-miR-375, hsa-miR-301a-3p, hsa-miR-24-3p, hsa-miR-194-5p and hsa-miR-146a-5p levels at 3 months. Several reports have implicated these miRNAs in beta cell growth, development, differentiation and function [40–45]. These observations are in agreement with our findings; hsa-miR-375 levels were negatively associated with residual beta cell function 6 months after diagnosis, hsa-miR-24-3p indicated a better preserved beta cell function and/or insulin sensitivity 12 months after diagnosis, hsa-miR-146a-5p was associated with improved IDAA_{1c} level after 12 months, and hsa-miR-194-5p was associated with a poorer IDAA_{1c} level after 12 months. Whether these miRNAs reflect cell turnover (and if so, that of beta cells, insulin-dependent cells or immune cells) or result from the diabetic metabolic state cannot be deduced from the present study. We did not identify any miRNAs that predicted residual beta cell function at 60 months. This is likely because of the low degree of beta cell function remaining after 60 months (<10% of that at diagnosis), requiring a much larger sample size to detect a (small) difference. We were also not able to replicate our previous observation of hsa-miR-25 being a marker for residual beta cell function in the international Hvidoere cohort [13], possibly because of differences in the analytical platforms used in the two studies. However, in the present study, hsa-miR-25-3p was differentially expressed after diagnosis, suggesting a possible effect of disease progression on miRNA levels (unadjusted $p=0.0015$, adjusted $p=0.16$; data not shown). Thus, we cannot rule out an influence of hsa-miR-25-3p on disease development in children with newly diagnosed type 1 diabetes mellitus.

The strengths of the present study are the phenotypically well-characterised cohort of children with new-onset type 1 diabetes mellitus followed closely for the first 5 years after diagnosis with repeated measurements. A limitation of the study is the reduced number of individuals (40 patients) who

completed the follow-up after a diabetes duration of 5 years, thus reducing the power to identify more predictive markers for beta cell function that may have had a smaller effect size. We only included those miRNAs that were identified in all samples during the normalisation analysis and, therefore, we cannot exclude the fact that miRNAs not fulfilling this stringent criterion may have predictive value. Another limitation is that, although the prospective study design enabled an analysis of associations between clinical characteristics and miRNAs, the observational feature makes the investigation of causality impossible.

In summary, this study suggests that hsa-miR-197-3p at 3 months can predict residual beta cell function 12 months after diagnosis. These findings may be of clinical relevance and could suggest new therapeutic avenues for intervention therapies, including miRNA-based patient stratification aimed at preserving and/or regenerating beta cell function in new-onset type 1 diabetes mellitus.

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Contribution statement LBN, HBM and FP contributed to the idea and the design of the study. NS, AHM, LBN, SK, HBM and FP researched the data and drafted, revised and edited the manuscript. PH performed the statistical analyses (except for Table 3, which was carried out by AHM and SK) and contributed to interpretation of the results and critical review of the manuscript. SF was responsible for acquisition of data, contributed to discussions, and reviewed and critically revised the manuscript. All authors reviewed and approved the manuscript to be published. FP is the guarantor of the manuscript and, as such, had full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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