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Article

Influence of disease duration on circulating levels of miRNAs in children and adolescents with new onset type 1 diabetes

Nasim Samandari[1], Aashiq H. Mirza[1], Simranjeet Kaur[1,2], Philip Hougaard[3], Lotte B. Nielsen[1], Siri Fredheim[1], Henrik B. Mortensen[1,4], Flemming Pociot[1,2,4,*]

[1] Copenhagen Diabetes Research Centre (CPH-DIRECT), Department of Paediatrics, Herlev and Gentofte Hospitals, Herlev Ringvej 75, 2730 Herlev, Denmark
[2] Steno Diabetes Center Copenhagen Gentofte, Denmark
[3] Research unit of epidemiology, biostatistics and biodemography, University of Southern Denmark, Odense, Denmark
[4] Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
* Correspondence: flemming.pociot@regionh.dk; Tel.: +45 39680800

Abstract: The objective of this study was to identify circulating miRNAs affected by disease duration in newly diagnosed children with type 1 diabetes. Forty children and adolescents from The Danish Remission Phase Cohort were followed with blood samples drawn at 1, 3, 6, 12 and 60 months after diagnosis. Pancreatic autoantibodies were measured at each visit. Cytokines were measured only the first year. miRNA expression profiling was performed by RT-qPCR and quantified for 179 human plasma miRNAs. The effect of disease duration was analyzed by mixed models for repeated measurements, adjusted for sex and age. Eight miRNAs (hsa-miR-10b-5p, hsa-miR-17-5p, hsa-miR-30e-5p, hsa-miR-93-5p, hsa-miR-99a-5p, hsa-miR-125b-5p, hsa-miR-423-3p and hsa-miR-497-5p) were found to significantly change expression (adjusted p-value < 0.05) with disease progression. Three pancreatic autoantibodies ICA, IA-2A, GADA65 and 4 cytokines IL-4, IL-10, IL-21, IL-22 were associated with the miRNAs at different time points. Pathway analysis revealed association with various immune-mediated signaling pathways. Eight miRNAs, involved in immunological pathways changed expression levels during the first five years after diagnosis in children with type 1 diabetes, and were associated with variations in cytokine and pancreatic antibodies, suggesting a possible effect on the immunological processes in the early phase of the disease.

Keywords: children, immunology, miRNA, partial remission phase, type 1 diabetes

1. Introduction

There is an increased interest in epigenetic mechanisms to understand disease pathogenesis and progression. Epigenetics involves the heritable modifications of gene expression without changes in the genome sequence, leading to an altered, sometimes unfavorable, phenotype e.g. resulting in metabolic diseases such as diabetes. While it is recognized that a genetic component is essential to the risk of developing type 1 diabetes (T1D), data from twin studies suggests that genetics alone cannot explain the disease development [1,2]. Furthermore, the increasing incidence of T1D in younger children is at a rate, which is too fast to be accounted for by a genetic change only. Thus, the role of other pathogenic influences, such as diet, environment, and epigenetic modifications has been suggested to contribute to the development of autoimmune diabetes [3-5].

MicroRNAs (miRNAs) are small non-coding RNAs with potent post-transcriptional regulatory properties which have shown potential as biomarkers for different pathophysiologies [6]. During recent years the promising features of miRNAs as biomarkers associated to insulin production, residual beta cell function and disease complications of diabetes have been investigated [7-9].

Knowledge emerging on the role of epigenetic control, such as miRNAs, DNA methylation patterns and histone modifications, indicates that these regulatory mechanisms have an important function in T1D [10-12].

An important stage in the natural history of T1D is the spontaneous remission phase, which can be either complete, with temporary insulin independence, or partial. During this phase, which can last from weeks to several months, a decreased requirement of exogenous insulin is experienced and an improved metabolic control is observed [13]. The partial remission phase (PR) is a potential temporal opening, to investigate the important immunoregulatory events taking place when the remaining beta-cells are still able to produce enough insulin. Currently, the strategies employed so far, have not yet been able to unravel the underlying mechanisms during this phase, making the development of future immunotherapies with the capabilities to extend the PR challenging.

Previously, we identified six miRNAs predictive for residual beta-cell function and metabolic control in children newly diagnosed with T1D [9]. In the present study we hypothesized that circulating miRNAs reflect the ongoing autoimmune process during the first year after disease onset. Thus, the objective of this study was to identify circulating miRNAs, which hold the potential to serve as biomarkers for the autoimmune/immunological status in newly diagnosed children and adolescents with T1D.

2. Results

2.1 Technical controls prior to miRNA profiling

Demographic and anthropometric data characterizing the study cohort is presented in Table 1, and has also recently been described [9]. Plasma samples from a subset of children and adolescents (n=40) from this cohort collected at different time-points (1, 3, 6, 12 and 60 months) after diagnosis were subjected to miRNA profiling. The steady expression levels of the RNA spike-ins, added in the RNA isolation and complementary DNA (cDNA) synthesis steps, indicate that the processes of RNA extraction, reverse transcription and qPCR were efficient and no inhibitors were present in the samples. Haemolysis of the samples varied between 5 and -2, thus indicating that the samples were not affected by haemolysis

Table 1 Characteristics of 40 children and adolescents (study population) from The Danish Remission Phase Cohort and the entire cohort, respectively

Study population	**1 month**	**3 months**	**6 months**	**12 months**	**60 months**
n (girls/n)	40 (24)	37 (22)	33 (19)	34 (20)	38 (23)
Age at diagnosis (years)	8.7 (3.4)	-	-	-	-
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 (12.0)	62 (17.5)	63 (12.0)	68 (11.0)
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)	12.0 (1.9)
Insulin dose (units/kg/24h)	0.45 (0.27)	0.47 (0.23)	0.58 (0.26)	0.78 (0.27)	0.88 (0.32)
Autoantibody positivity (%)					
GAD65A	55	62	61	56	55
IA	80	100	100	100	78
IA-2A	73	68	70	68	74
ICA	93	92	94	94	92
ZnT8tripleAb	65	70	75	76	26

Data are mean (SD), unless otherwise stated. SCP: stimulated C-peptide; IDAA1c = HbA1c (per cent) + [4 x insulin dose (units per kilogram per 24 h)]. GAD65A: Autoantibodies against antigen GAD. IA: Insulin

autoantibodies. IA-2A: Islet antigen 2 antibody. ICA: Islet-cell antibody. ZnT8tribleAb: Zinc transporter 8 triple mix antibody variant containing the isotypes glutamin, arginine and tryptophan. Missing data are either due to no visit, missing or lost sample, or drop-out.

2.2 miRNA quantification in 182 plasma samples

A selected panel of 179 human serum/plasma miRNAs was analyzed over the five time points after diagnosis. An average of 163 miRNAs was quantified and 100 were quantified in all samples.

2.3 Effect of disease duration on miRNA expression levels during the first five years after diagnosis

Analysis by mixed model for repeated measurements indicated, that 28 miRNAs varied in expression levels during the first five years after diagnosis ($p<0.05$). After correcting for multiple testing, eight miRNAs (hsa-miR-10b-5p, hsa-miR-17-5p, hsa-miR-30e-5p, hsa-miR-93-5p, hsa-miR-99a-5p, hsa-miR-125b-5p, hsa-miR-423-3p and hsa-miR-497-5p) showed variation in expression levels during the first five years after disease onset ($p<0.05$), Table 2. The results of these eight candidate miRNAs were adjusted for age and sex. After adjustments, data revealed that age only had an effect on hsa-miR-423-3p ($p=0.0088$, data not shown). Neither HbA1c nor SCP correlated with any of the quantified miRNAs. The average expression levels of the eight candidate miRNAs with altered expression levels during the first five years are shown in Figure 1.

Table 2 Effect of disease duration on miRNA expression level

miRNA	Unadjusted p-value	Adjusted p-value
hsa-miR-99a-5p	<0.00001	0.00022
hsa-miR-30e-5p	<0.00001	0.00027
hsa-miR-497-5p	<0.00001	0.00041
hsa-miR-10b-5p	0.00001	0.00072
hsa-miR-423-3p	0.00001	0.00131
hsa-miR-125b-5p	0.00003	0.00252
hsa-miR-17-5p	0.00011	0.01057
hsa-miR-93-5p	0.00012	0.01094
hsa-miR-146a-5p	0.00094	0.08678
hsa-miR-484	0.00195	0.17716
hsa-miR-185-5p	0.00208	0.18726
hsa-miR-24-3	0.00291	0.25943
hsa-miR-660-5p	0.00944	0.83059
hsa-miR-25-3p	0.01088	0.94673
hsa-let-7b-5p	0.01133	0.97420
hsa-miR-320a	0.01236	0.98546
hsa-miR-223-3p	0.01248	0.98546
hsa-let-7g-5p	0.01553	0.98546
hsa-miR-20a-5p	0.01815	0.98546
hsa-let-7i-5p	0.02298	0.98546
hsa-miR-142-3p	0.02412	0.98546
hsa-miR-32-5p	0.02544	0.98546
hsa-miR-486-5p	0.02989	0.98546
hsa-miR-142-5p	0.04400	0.98546
hsa-miR-320b	0.04421	0.98546

hsa-miR-145-5p	0.04545	0.98546
hsa-miR-221-3p	0.04598	0.98546
hsa-miR-106a-5p	0.04701	0.98546

The table show the 28 statistically significant miRNAs values with altered expressed levels among 100 miRNAs investigated during observation time before correction for multiple testing and after correction (n=8), sorted according to the lowest adjusted p-value.

The normalized expression levels (ΔCp) of hsa-miR-17-5p, hsa-miR-30e-5p, hsa-miR-93-5p and hsa-miR-423-3p declined within the first 12 months after diagnosis and subsequently increased from one to five years after diagnosis. By contrast, the levels of hsa-miR-10b-5p, hsa-miR-99a-5p, hsa-miR-125b-5p and hsa-miR-497-5p increased during the first 12 months and subsequently decreased between one and five years after diagnosis. Five miRNAs (hsa-miR-10b-5p, hsa-miR-17-5p, hsa-miR-99a-5p, hsa-miR-125b-5p and hsa-miR-497-5p) were expressed with a lower ΔCp value compared to the average of all samples at each time point and the remaining three miRNAs (hsa-miR-30e-5p, hsa-miR-93-5p and hsa-miR-423-3p) were more abundantly expressed compared to the average of all samples at all time-points, Figure 1.

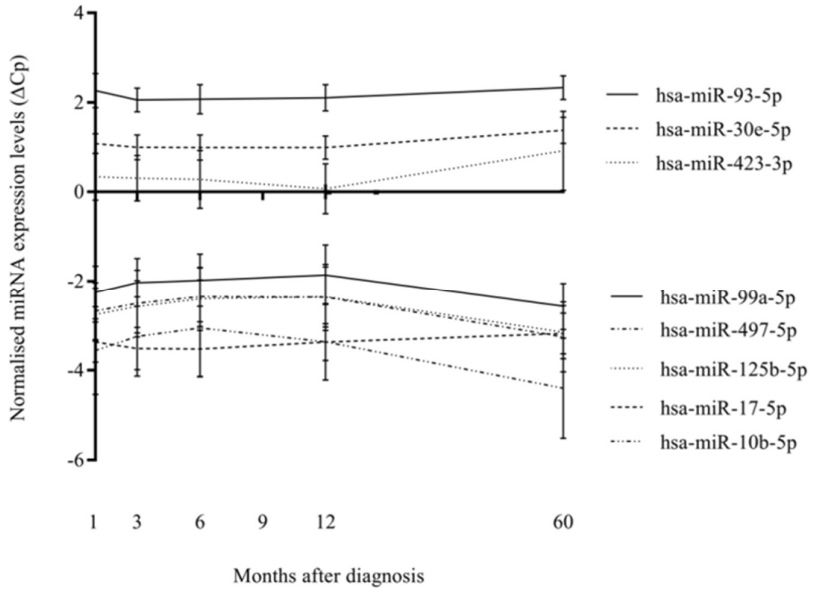

Figure 1 The eight candidate miRNAs with changing expression levels during the first five years after diagnosis. Graph illustrating the mean normalized expression levels (ΔCp) of the eight candidate miRNAs at 1, 3, 6, 12 and 60 months after diagnosis. Normalization is based on the global mean method. Age and sex are not accounted for in this figure.

2.4 Identification of miRNA target genes

Validated target genes and the associated pathways for the eight candidate miRNAs were retrieved from miRWalk [14] and PantherDB [15]. The pathway analysis revealed association of these miRNA targets with various signaling pathways including epidermal growth factor (EGF), fibroblast growth factor (FGF), Integrin, platelet-derived growth factor (PDGF), transforming growth factor (TGF) beta, vascular endothelial growth factor (VEGF), Toll-receptor, apoptosis and interleukin signaling pathways as shown in Table 3.

2.5 PR and immunological status

Using the new definition for PR with IDAA1c ≤ 9 in the study population of 40 children and adolescents, 19 (51%) children were in PR 3 months after diagnosis, and 4 (12%) and 1 (3%) were in PR after 12 and 60 months, respectively.

Table 3 Common pathways associated with the validated target genes of the eight miRNAs with altered expression levels during the first five years after diagnosis

PANTHER Pathway	miRNAs
EGF receptor signalling pathway	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-497-5p, hsa-miR-93-5p, hsa-miR-99a-5p
Endothelin signalling pathway	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-497-5p, hsa-miR-93-5p, hsa-miR-99a-5p
FGF signalling pathway	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-497-5p, hsa-miR-93-5p, hsa-miR-99a-5p
Heterotrimeric G protein signalling pathway Gi alpha and Gs alpha mediated pathway	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-30e-5p, hsa-miR-423-3p, hsa-miR-93-5p, hsa-miR-99a-5p
Insulin/IGF pathway protein kinase B signalling cascade	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-497-5p, hsa-miR-93-5p, hsa-miR-99a-5p
Integrin signalling pathway	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-497-5p, hsa-miR-93-5p, hsa-miR-99a-5p
PDGF signalling pathway	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-497-5p, hsa-miR-93-5p, hsa-miR-99a-5p
Parkinson disease	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-30e-5p, hsa-miR-423-3p, hsa-miR-93-5p, hsa-miR-99a-5p
Toll receptor signalling pathway	hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-30e-5p, hsa-miR-423-3p, hsa-miR-497-5p, hsa-miR-93-5p, hsa-miR-99a-5p
Angiogenesis	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-93-5p, hsa-miR-99a-5p
Apoptosis signalling pathway	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-30e-5p, hsa-miR-423-3p, hsa-miR-93-5p
B-cell activation	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-497-5p, hsa-miR-93-5p
Huntington disease	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-93-5p, hsa-miR-99a-5p
Hypoxia response via HIF activation	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-93-5p, hsa-miR-99a-5p
Interleukin signalling pathway	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-93-5p, hsa-miR-99a-5p
TGF beta signalling pathway	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-93-5p, hsa-miR-99a-5p
T-cell activation	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-497-5p, hsa-miR-93-5p

VEGF signalling pathway	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-93-5p, hsa-miR-99a-5p
p53 pathway	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-93-5p, hsa-miR-99a-5p
Ubiquitin proteasome pathway	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-93-5p, hsa-miR-99a-5p
Ras Pathway	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-497-5p, hsa-miR-93-5p
p53 pathway by glucose deprivation	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-30e-5p, hsa-miR-423-3p, hsa-miR-93-5p
p53 pathway feedback loops 2	hsa-miR-10b-5p, hsa-miR-125b-5p, hsa-miR-17-5p, hsa-miR-423-3p, hsa-miR-93-5p, hsa-miR-99a-5p

The common pathways are listed for the validated targets of the eight candidate miRNAs which change expression levels over time. The selected pathways are based on association with target genes of a minimum of 6 out of 8 miRNAs identified in this study. EGF: Epidermal growth factor. FGF: Fibroblast growth factor. IGF: Insulin-like growth factor. PDGF: platelet-derived growth factor. HIF: Hypoxia-inducible factors. TGF: Transforming growth factor. VEGF: Vascular endothelial growth factor.

Of the five autoantibodies tested, seroconversion was most profound for ZnT8tripleAb during the study period. IA increased from 80 to 100% during the first year after diagnosis (Table 1) and remained positive in all the children between three and twelve months in the study population and the study cohort. Seroconversion for IA was observed in twenty-two percent of the children five years after diagnosis (Table 1). The Spearman correlation analysis performed on the association of the eight candidate miRNAs and the anti-islet autoantibodies measurements showed that three of the candidate miRNAs were statistically significantly ($p<0.036$) associated with the measured autoantibodies before correcting for multiple testing. ICA was negatively associated with hsa-miR-99a-5p at 3 months after diagnosis. At the same time point ICA and IA-2A were negatively associated with hsa-miR-125b-5p. Finally, GADA65 was positively associated with hsa-miR-17-5p 6 months after diagnosis, Table 4.

Table 4 Association between the eight candidate miRNAs and autoantibodies titres

Months after diagnosis	miRNA	Autoantibody	Spearman correlation (r_s)	Unadjusted p-value	Adjusted p-values
3	hsa-miR-99a-5p	ICA	-0.35	0.036	N/S
3	hsa-miR-125b-5p	ICA	-0.36	0.030	N/S
3	hsa-miR-125b-5p	IA-2A	-0.35	0.032	N/S
6	hsa-miR-17-5p	GAD	0.37	0.033	N/S

The association between the eight candidate miRNAs and autoantibody titres measured at the five time points after diagnosis by Spearman correlation (r_s). Only the statistically significant associations are shown ($p\leq0.05$). The significant values are before correcting for multiple testing. After correction no significant association was seen (N/S). GAD65A: Autoantibodies against antigen GAD. IA-2A: Islet antigen 2. ICA: Islet-cell antibody. ZnT8(tripleAb): Zink transporter 8 (triple mix variant containing the isotypes glutamin, arginine and tryptophan).

2.6 Association of the eight candidate miRNAs to cytokines

The Spearman correlation analysis performed between the eight candidate miRNAs and 35 measured cytokines at 1, 3, 6, and 12 months after diagnosis, showed before correction for multiple testing that hsa-miR-10b-5p correlated negatively with IL-21(r= -0.63, p=0.0051) at 3 months, whereas hsa-miR-17-5p at 1 month correlated positively with IL-21 (r=0.62, p=0.0048). Likewise hsa-miR-17-5p was positively associated with IL-10 and IL-4 at 1 month (r=0.68, p=0.0015; r=0.70, p=0.0008), respectively. Finally hsa-miR-30e-5p correlated positively with IL-22 at 6 months (r=0.71, p=0.0015).

3. Discussion

Aberrantly circulating miRNA levels in plasma, serum and urine samples from children with T1D were assessed recently by Osipova et al [16] and the number of studies exploring the prognostic potential of miRNAs in different settings is steadily increasing [17-19]. Our group recently showed, in the same cohort as the one used in present study, that six miRNAs predicted stimulated C-peptide, HbA1c and the insulin dose adjusted HbA1c (IDAA1c) the first year after disease onset [9]. In the present study we identified miRNAs, which expression levels were influenced by disease progression, but not associated to decline in beta-cell function. In this prospective study, disease progression had a statistically significant effect on the expression levels of eight candidate miRNAs, irrespective of age and sex. Further bioinformatics evaluation of the validated targets of these miRNAs, and the observed cytokine and pancreatic antibody associations support a possible immunological impact reflected by circulating miRNAs during the PR. However, cytokine profiles were only quantified in about half of the study participants in the Danish Remission Phase Cohort due to technical reasons. Thus, the study was under-powered to fully explore these potential associations. The immune-mediated pathways associated with the target genes for these eight candidate miRNAs, include interleukin signaling, TGF-beta signaling, insulin/IGF pathway, Toll-receptor signaling and apoptosis signaling and angiogenesis.

Toll-like receptors (TLRs) are membrane-bound receptors that participate in the activation of innate immunity by inducing production of proinflammatory cytokines and play significant roles in inflammation, immune cell regulation, survival, and proliferation [20]. TNFAIP3, target gene for hsa-miR-125b-5p; RELA (NF-kappa-B subunit), target gene for hsa-miR-30e-5p; MAPK3 and IRAK1, target genes for hsa-miR-423-5p; MAP2K1, target gene for hsa-miR-497-5p; JUN, MAPK9 and TOLLIP, target genes for hsa-miR-93-5p; IKBKB and PTGS2, target genes for hsa-miR-99a-5p are all key components of Toll receptor signaling pathway. Moreover, four target genes (MAP3K8, IRAK1, MAPK9 and PTGS2) for hsa-miR-17-5p are also associated with Toll-receptor signaling cascades. Another validated target for hsa-miR-17-5p is c-Myc (a transcription factor), which is associated with cell growth, apoptosis and metabolism [21]. STAT3, a potential target gene for hsa-miR-93-5p and hsa-miR-125b-5p plays an essential role in angiogenesis, Ras pathway, EGF receptor signaling, Interleukin signaling and PDGF signaling pathways. MTOR (a serine/threonine protein kinase), which is a target gene for hsa-miR-99a-5p is associated with Interleukin signaling pathway. MTOR is a central regulator of cellular metabolism, growth and survival [22]. The activation of MTOR regulates various effector functions of CD4+ and CD8+ T cells and MTOR inhibitors (i.e. rapamycin) are used as potential immunosuppressive therapeutics for organ transplants, metabolic diseases and various autoimmune disorders [23]. Furthermore, MTOR have been demonstrated to be involved in the onset and progression of autoimmune disorders including diabetes [24]. PIK3CD, a target gene for hsa-miR-10b-5p is a known mediator of immune responses and is associated with several pathways including angiogenesis, apoptosis signaling, EGF receptor signaling, PDGF signaling, FGF signaling, Integrin signaling, insulin/IGF signaling cascade, B-cell and T-cell activation. PIK3CD, a target gene for hsa-miR-10b-5p is a known mediator of immune responses and is associated with several pathways including angiogenesis, apoptosis signaling, EGF receptor signaling, PDGF signaling, FGF signaling, Integrin signaling, insulin/IGF signaling cascade, B-cell and T-cell activation.

One of the strengths of this study is the phenotypically thorough characterization of the study participants, who were followed with repeated measurements initially after diagnosis and during the first five years hereafter. The present study also provides data from pathway analysis together with cytokine and pancreatic antibody variations associated with miRNAs during PR to suggest that miRNAs regulating genes that control immune recognition could be involved in the autoimmune processes during the initial progression of the disease. In our previous study [9], six miRNAs analyzed in the same population could predict residual beta-cell function and glycemic control (SCP, HbA1c and IDAA1c) in children with new onset T1D the first five years after diagnosis. The current study further provides data, in the same study population, on different miRNAs, which change expression levels during the first five years after diagnosis and have a possible involvement in immunological pathways. A weakness of this study is the limited number of study participants followed five years after diabetes diagnosis. This decreases the statistical power to conclude on the potential role of the eight candidate miRNAs during the PR. The observed variability of the candidate miRNAs need further validation in larger cohorts and also their immunological function in autoimmune diabetes needs to be elucidated in further details.

In conclusion, this study identified eight circulating miRNAs changing expression levels irrespective of age and sex during the first five years after diabetes diagnosis, which have the potential to be used as biomarkers for the immunological progression and define the optimal state for immune intervention during the initial phase of T1D.

4. Materials and Methods

4.1 Study population and samples

The Danish remission phase cohort comprised 129 children (66 boys) with T1D. The study was a longitudinal multicenter investigation conducted in four pediatric out-patient clinics with enrolment during the years 2004 to 2005. Plasma samples were drawn on fasting state after a meal stimulation test at 1, 3, 6, 12 and 60 months after diagnosis and stored in a bio-bank at –80 Celsius degrees until further use. Diabetes diagnosis was classified according to the World Health Organization criteria [25]. Reasons for dropouts from the cohort or loss of follow up at the 5-year visit were either adolescent transferred to adult clinics, or changed residence, or unwillingness to participate. Exclusion criteria were suspicion of other types of diabetes (maturity-onset diabetes of the young (MODY), type 2 diabetes or secondary diabetes), and/or if patients were initially treated outside the center for more than five days. The 40 participants who completed follow-up at five years diabetes duration were slightly younger with a lower BMI, and had lower postprandial C-peptide levels 12 months after diagnosis compared to the full cohort, but they were similar in terms of sex distribution, HbA1c and hormone levels.

4.2 Partial Remission (PR) Phase

Different definitions for the PR phase have been suggested. Previously it was defined as HbA1c near or within the normal range, and a daily insulin dose requirement of < 0.5 units/kg/day [26]. A new definition combining both values has recently been proposed: insulin dose-adjusted HbA1c, IDAA1c, which suggests that a value ≤ 9 indicates partial remission [27-28]. This definition has been recommended by the International Society for Pediatric and Adolescent Diabetes (ISPAD) [29].

4.3 Pancreatic anti-islet autoantibodies

Islet autoantibody measurements were measured in serum samples from all included children in the Danish Remission Phase Cohort. Titers of the anti-islet autoantibodies (Insulin antibodies (IA), insulinoma associated antigen-2 autoantibody (IA-2A), autoantibodies against glutamic acid decarboxylase (GAD65A) and islet-cell autoantibodies (ICA)) were analyzed at 1, 3, 6, 12 and 60 months after diagnosis according to the methods described earlier [30]. The zinc transporter 8 variant

containing all three isotypes arginine, tryptophan and glutamine (ZnT8RWQ or ZnT8tripleAb) were analyzed by a so called triple mix Radioligand Binding Assay (RBA) as previously described [31-32] at the time points 1, 3, 6 and 12 months after diagnosis. Another method was used for analyzing the 'triple mix' ZnT8 autoantibodies (ZnT8tripleAb) 60 month after diagnosis [33].

The cut-off values for anti-islet autoantibody positivity were 5.36 relative units (RU) for GAD65A, 0.77 RU for IA-2A, 2.5 Juvenile Diabetes Foundation units (JDFU) for ICA and 2.80 RU for IA. The cut-off limit for the triple mix assay, used the first year after diagnosis, was 58 U/mL, and for the assay by Salonen et al. [33] used five years after diagnosis, the cut-off value was set to 0.61 RU.

4.4 Cytokine measurements

Cytokine status was measured for half of the recruited study participants in the Danish Remission Phase cohort at 1, 3, 6 and 12 months after diagnosis. Cytokine measurements were performed using an in-house developed and validated multiplex immunoassay (Laboratory of Translational Immunology, University Medical Center, Utrecht, The Netherlands) based on Luminex technology (xMAP, Luminex Austin TX, USA) [34].

4.5 miRNA expression profiling and normalization method

miRNAs were quantified from a total of 182 plasma samples drawn from the subgroup of 40 children and adolescents, who participated in the 5-year visit. RNA extraction was performed using the miRCURYTM RNA Isolation Kit-Biofluids and RT-qPCR was performed with ExiLENT SYBR® Green Master Mix according to the manufacturer's instructions (Exiqon A/S, Vedbaek, Denmark). All RT-qPCR assays were performed in a LightCycler480® Real-Time PCR System in 384 well plates (Roche, Hvidovre, Denmark). Quality control was done on a subset of samples (n=10) with synthetic spike-in RNAs, as a quality control of the RNA isolation and cDNA synthesis. A detailed description of the RNA isolation and quality control procedure is outlined in [9]. Assessment of haemolysis was evaluated by comparing the levels of hsa-miR-451a (miRNA highly expressed in erythrocytes) with hsa-miR-23a-3p (stable in plasma/serum and not affected by haemolysis) as described by Blondal et al. [35].

Each plasma sample was run on a selected assay containing a panel of 179 predefined human serum/plasma miRNAs (V3; Exiqon), including miRNAs known to be involved in immunological and metabolic diseases. The 100 miRNAs with complete data that were quantified in all samples in the miRNA expression profiling, were employed for the normalization of data using global mean method, since this approach has been shown to be the most stable normalizer according to Mestdagh et al. [36]. The formula used to calculate the normalized Cp values is:

$$\Delta Cp = \text{average Cp (all samples)} - \text{assay Cp (sample)}$$

4.6 Validation of target genes and pathway analysis

The validated targets for the eight candidate miRNAs found in this study were retrieved using the validated target module (VTM) in miRWalk 2.0 [14] which is based on experimentally verified miRNA interactions retrieved from automated text mining in PubMed. The pathways associated with the validate target genes were retrieved based on PantherDB annotations [15].

4.7 Statistical analysis

All statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC, USA). The miRNA levels with complete data were included for analysis and were managed on a base-2 logarithmic scale (log2). The influence of diabetes duration on circulating levels of miRNAs were analyzed by a linear mixed model for repeated measurements including the covariates for sex and

age at the visit (to account for the potential age development) and with unstructured covariance matrix. P-values for the differentially expressed miRNAs associated with disease duration, were further adjusted according to the Hochberg approach ($p<0.05$). For antibodies and cytokines, the Spearman correlation analysis used the measured values, and included values below the detection limit as a value below all other, and was performed to determine the relationship between variables and was considered significant with a corresponding $p\leq0.05$.

Ethics approval and consent to participate: 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.

Author Contributions: N.S, L.B.N, H.B.M and F.P designed the study; P.H performed the statistical analysis; A.H.M and S.K performed the bioinformatics analyses; S.F was involved in collection and characterization of the cohort; all authors were involved in drafting the manuscript, approving the final draft, and agree to be accountable for the work; all authors read and approved the final manuscript.

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