Type 1 Diabetes in Children with Genetic Risk may potentially be predicted very early with a blood miRNA

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Progression to clinical type 1 diabetes is monitored through the appearance of islet autoantibodies against pancreatic β-cell antigens, and most children with two or more

autoantibodies progress to disease (1). However, autoantibodies indicate already active islet autoimmunity, and by that time, loss of immune tolerance may have reached a point of no return. Thus, there is an urgent need for biomarkers that would predict the disease before the appearance of islet autoantibodies and provide a longer window for intervention. New biomarkers might help identify optimal sets of subjects for clinical trials or a subgroup of patients who may more likely benefit from a given therapy.

MicroRNAs (miRNAs) secreted in extracellular vesicles have been detected in blood and may have biomarker potential (2). Several studies have shown the usefulness of miRNAs as biomarkers for many diseases (reviewed in (3)). Aberrant miRNA expression has been observed in sera of type 1 diabetes patients (reviewed in (4)). However, most miRNA studies have analyzed samples at or after the onset of clinical type 1 diabetes.

Here we analyzed whether we can detect changes in miRNA levels before and during islet autoimmunity in whole blood samples from children with HLA-conferred risk of type 1 diabetes participating in the Type 1 Diabetes Prediction and Prevention (DIPP) study (5). Children from the DIPP study with high HLA-conferred risk were followed up, and whole blood samples were collected at multiple time points. Case-control matching was based on HLA-DQB1 genotype, date and place of birth, and sex, similarly as described earlier (5). To study genome-wide miRNA profiles before the clinical presentation of type 1 diabetes, we first performed miRNA-sequencing (miRNA-seq) on 87 longitudinal samples collected from four multiple autoantibody-positive cases and their matched autoantibody negative controls (**Fig. 1A: miRNA-seq discovery cohort**) using the Illumina HiSeq 2500 platform. A linear mixed-effects model for each miRNA was used to test differential expression between cases and controls. The most significantly upregulated miRNA in cases was hsa-miR-6868-3p ($p < 0.001$), which has not been earlier associated with type 1 diabetes. Interestingly, hsa-miR-6868-3p was upregulated already before seroconversion (**Fig. 1B**). We confirmed the finding ($p < 0.001$) on these and ten

66 additional case-control pairs using miRNA-seq of two time-points before seroconversion (**Fig.**
67 **1A: miRNA-seq validation cohort; Fig. 1C**).
68 We further confirmed the miRNA upregulation by TaqMan qRT-PCR assay in samples collected
69 from 29 case-control pairs, of which 14 were included in the miRNA-seq analysis (**Fig. 1A:**
70 **TaqMan validation)**. A strong correlation (r=0.75) was observed between the sequencing and
71 TaqMan results for hsa-miR-6868-3p expression. Convincingly, the TaqMan data showed
72 higher hsa-miR-6868-3p expression in cases than controls ($p < 0.001$) across the time points
73 (**Fig. 1D**), recapitulating the miRNA-seq result.

74 Given the early upregulation of hsa-miR-6868-3p, we tested whether the miRNA can classify the
75 29 cases from controls already before seroconversion, using the average expression before
76 seroconversion for a given child. The ΔCt expression values were adjusted for individual HLA
77 type and TaqMan plates using a linear model. The area under the receiver operating
78 characteristic curve (AUROC) was 0.76 (**Fig. 1E**), suggesting that the miRNA may indeed
79 potentially serve as a screening biomarker for the stratification of children at increased genetic
80 risk for type 1 diabetes.

81 Besides blood, breast and brain, the miRNA is expressed in the pancreas (**Fig. 1F**), suggesting
82 an interesting possibility that its upregulation in blood samples of case children may potentially
83 originate from the pancreas and circulate through blood under inflammatory conditions. It is
84 important to note that this miRNA may also come from blood lymphocytes, given its expression
85 in these cells (**Fig. 1G**).

86 The ROC analysis implied that hsa-miR-6868-3p may serve as a screening biomarker for the
87 stratification of children at risk of islet autoimmunity. However, the small cohort size of 58 study
88 subjects is a limitation of the study, and the finding should be validated in whole-blood samples
89 of an independent, preferably larger cohort. For a screening biomarker, high sensitivity is
90 preferred over high specificity (i.e., false negatives are of more concern than false positives).

The current model reaches a higher sensitivity of 0.86 at the specificity of 0.66. It remains to be seen whether combining hsa-miR-6868-3p with other miRNAs or mRNAs will improve the performance of the predictive model. It will also be interesting to determine whether the miRNA expression correlates with the time from seroconversion to clinical disease.

Declarations

Data availability

Analyzed count data from miRNA-seq of the discovery cohort (87 samples from four case-control pairs) and the validation cohort (56 samples from fourteen pairs) can be accessed from the ArrayExpress database (https://www.ebi.ac.uk/arrayexpress/) using the accession codes, E-MTAB-10959 and E-MTAB-10968, respectively. Other data generated during the current study are available from the corresponding author on a reasonable request.

Funding

This research was supported by the Academy of Finland including InFLAMES, SYMMyS, and personalized medicine programs (grant numbers: 337530, 292482, 250114, 292482, 294337, 292335, 319280 and 314444 329277, 331790, 296801, 304995, 310561, 314443, and 329278); Juvenile Diabetes Research Foundation, including grants 1-SRA-2016-342-M-R and 1-SRA-2019-732-M-B; the Diabetes Research Foundation (Diabetestutkimussäätiö); the Novo Nordisk Foundation; and the Finnish Cancer Foundation; the Sigrid Jusélius Foundation; Pediatric Research Foundation; Turku University Hospital Special Governmental Grants; and the Special Research Funds for University Hospitals in Finland. It was also supported by grants from the European Research Council ERC (677943); European Union's Horizon 2020 research and innovation programme (955321); and the European Foundation for the study of Diabetes. Biocenter Finland and ELIXIR Finland also support our research.

Authors' contributions

TS and UUK designed experiments, analyzed the data, prepared the figures, and wrote the manuscript. OR designed experiments, analyzed data, and wrote the manuscript. AL, HK, MV-M, MN, JM, and TH contributed to the design of the study and analysis. HH, JI, RV, JT and MK were responsible for the DIPP cohort. RV and MK were responsible for the islet autoantibody analyses. All authors contributed to the final version of the manuscript. RL initiated, designed and supervised the study. LLE participated in the design of the study and analysis, and supervised the study. RL and LLE are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Acknowledgements

The authors are grateful to the families of the DIPP study participants and the study group. We also thank the personnel of the HLA laboratory at the University of Turku and the islet autoantibody laboratory at the University of Oulu. Marjo Hakkarainen and Sarita Heinonen are acknowledged for their skilful assistance in the laboratory. NGS sequencing was performed at the Finnish Functional Genomics Centre (FFGC), Turku, part of the Biocenter Finland network. We acknowledge the Finnish Centre for Scientific Computing (CSC) for data analysis servers.

Duality of Interest

The authors declare that there is no conflict of interest.

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Figure Legend

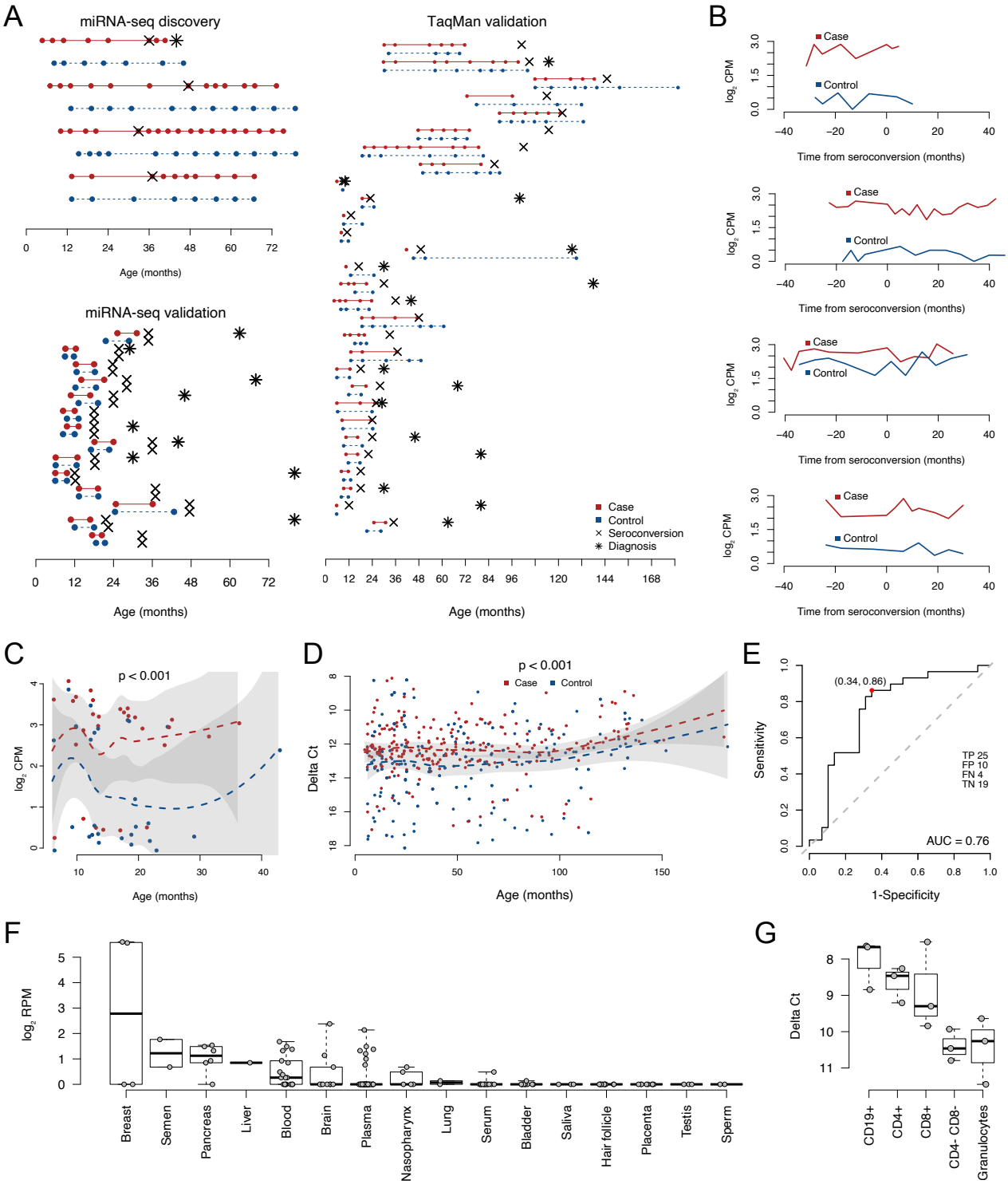

Figure 1. hsa-miR-6868-3p as early marker for type 1 diabetes

(**A**) The samples from the miRNA-seq discovery cohort (top left), the miRNA-seq validation cohort (bottom left), and the TaqMan validation cohort (right). Each line is an individual, and each dot is a PAXgene sample. (**B**) The line plots showing the expression profiles of hsa-miR-6868-3p for the four case-control pairs of the discovery cohort. The plots are seroconversion centered. CPM stands for counts per million. (**C**) Line plots showing the average longitudinal case-control profiles of hsa-miR-6868-3p in the validation cohort. Each red and blue dot shows a case or a control sample, respectively. The dashed lines show average expression, and the grey area shows a 95% confidence interval. (**D**) TaqMan expression of hsa-miR-6868-3p over time in 29 case-control pairs. The p value shown at the top was obtained from the linear mixed-effects model. (**E**) Receiver operating characteristic (ROC) analysis showing the performance of hsa-miR-6868-3p in differentiating the DIPP cases from controls in samples before the appearance of islet autoantibodies. The delta Ct values were adjusted using linear regression with HLA-type (DR3/DR4, DR3/DR3, DR4/DR4, DR3/other, DR4/other) and TaqMan plate as explanatory variables. The resulting residuals were used to normalize the possible effects of HLA and plate. True positives (TP), true negatives (TN), false positives (FP), and false negatives (FN) as predicted by the model are shown on the plot. (F) The expression profile of hsa-miR-6868-3p across different tissues. The data was taken from miRmine database (https://guanfiles.dcmb.med.umich.edu/mirmine/index.html). (G) Expression of hsa-miR-6868-3p in blood cells of healthy donors (n=3). Each dot represents an individual.