EUROMEDITERRANEAN BIOMEDICAL JOURNAL
2023,18 (23) 108-114
(FORMERLY: CAPSULA EBURNEA)

Original article

MICRORNA-21 AS NOVEL BIOMARKER FOR PANCREATIC BETA CELLS STRESS AND/OR DEATH IN PATIENTS WITH DIABETES MELLITUS TYPE 1

Hussein Chaseb Awad [1], Khwam R. Hussein [2], Hasan Abd Ali Khudhair [2]

1. College of Health and Medical Technology/Al-Basrah, Southern Technical University, Ministry of Higher Education and Scientific Research, Iraq
2. Al-Nasiriyah Technical Institute, Southern Technical University, Ministry of Higher Education and Scientific Research, Iraq

ARTICLE INFO

Article history:
Received 17 Jun 2023
Accepted 23 Jul 2023
Published 16 Sep 2023

Keywords:
T1D, beta cells, stress, death, vitamin D, miRNAs.

ABSTRACT

The research aimed to detect the roles of vitamin D (VD) and micro-ribonucleic acid (miRNA)-21-5p as potential predictors and diagnostic biomarkers for type 1 diabetes (T1D) and to determine the degree of association and predictability of them on beta (β) cells stress/death. A case-control study included three study groups: a T1D group that comprised 35 newly onset T1D patients, a first-degree relatives (FDRs) group that included a total of 35 FDRs of T1D patients, and a healthy control (HC) group that included a total of 20 subjects. All study subjects were evaluated for their serum connecting (C) peptide and VD, as well as the expression folds of serum miRNA-21-5p. The findings revealed significantly lower levels of C. peptide and VD among T1D and FDRs subjects than the HC group, whereas the folds of miRNA-21-5p were significantly higher in T1D subjects than FDRs and HC subjects. Furthermore, the level of C. peptide had a valuable positive association with VD within T1D and FDRs groups, whereas the folds of miRNA-21-5p have a valuable positive association with C. peptide and VD in FDRs and T1D groups, respectively. Vitamin D had exhibited a significantly decreased level among T1D and FDRs, and positively correlated with residual β-cells function, which indicated the possible utility of its low level as a β-cells stress/death predictive and diagnostic biomarker. Micro-RNA-21-5p indicated the possible utility of its high folds expression as β-cells stress/death predictive and diagnostic biomarker and the high level of miRNA-21-5p might play a vital role in T1D prevention.

© EuroMediterranean Biomedical Journal 2023

1. Introduction

Type 1 diabetes mellitus is an auto-immune condition represented by pancreatic β-cells loss due to an autoimmune attack, which is manifested by the incidence of the auto-antibodies (Abs) against pancreas gland antigens [1].
At the time of diagnosis, individuals with T1D are estimated to have lost approximately 80%–90% of the insulin synthesis β-cells function and/or mass. To conserve β-cells from destruction, many immune modulatory drugs have been used and have led to only lower β-cells function preservation without full recovery from T1D [2].

During the preclinical and early clinical stages of T1D, there are few selected alternative candidate intrinsic paths in which some determinants might be involved in the stress and death of the low proliferative and regenerative human β-cells [3]. Of these are the C. peptide/insulin ratio, the expression of variable levels of certain miRNAs molecules, and many others. Connecting peptide level is widely accepted as a biomarker for endogenous insulin excretion and function of pancreatic β-cells [4], which are used to evaluate the destruction of β-cells by autoimmune processes [5]. Lowest or depleted C. peptide concentrations are indicators of T1D progression after diagnosis, and the lowest C. peptide level is generally detected after ~one year of T1D onset. However, most previous studies conclude that any remaining activity of β-cells may reduce T1D complications [6].

* Corresponding author: Hasan Abd Ali Khudhair, *hasanabdali89@stu.edu.iq*
DOI: 10.3269/1970-5492.2023.18.23
All rights reserved. ISSN: 2279-7165 - Available on-line at www.embj.org

In the context of decreased glucose tolerance in individuals with VD deficiency and the presence of 1,25-dihydroxyvitamin D_3 in islet pancreatic and immune cells, the principal cells that are involved in the pathophysiology of both types of diabetes mellitus (DM), it was speculated to be the potential roles of VD in the pathophysiology and preventing strategies of both types of diabetes. In human and animal models, a deficiency of VD affects insulin production capacity and increases the risks of T2D [7]. By increasing insulin receptor expression or activation the peroxisome proliferator-activated receptor (PPAR), VD may be improve insulin sensitivity [8]. The occurrence of T1D in non-obese diabetic mice may be delayed through immunomodulation by using pharmacological doses of 1,25-dihydroxyvitamin D_3 or its analogs, but to our knowledge, no human research is available on this issue [7].

Micro-RNAs are small RNA molecules with non-coding properties that have a crucial involvement in post-transcriptional gene expression regulation. They are endogenous, highly conserved, and typically consist of 21–23 nucleotides [9].

They regulate multiple cellular processes, including differentiation, growth, metabolism, catabolism, and apoptosis [10]. Previous research reported many miRNAs molecules are differently present in T1D samples. These reports were conducted on human or animal models with T1D using cultured cells, body fluids, and solid tissue specimens, in which multiple techniques were used for determining the expression of genes [11].

Novel biomarkers identification is consistent with pancreatic cells autoimmunity detection at the prodromal stages of T1D [12]. Micro-RNAs are considered potential warranted biomarkers due to the facility to detect them in peripheral blood samples [13]. Consequently, the current study was conducted to determine which miRNAs could serve as a new T1D biomarker. More than two research studies show that miRNA-21 is statistically higher in the serum of T1D subjects [14]. On the other hand, other previous studies revealed a decreased level of miRNA-21-5p in the bloodstream [15]. Micro-RNAs were also detected in exosome specimens that were derived from blood because it has been verified that exosomes act as carriers for miRNAs during interactions between cells in *vitro* [16]. Another research reported that the amount of exosomal miRNA-21-5p in serum was higher in newly diagnosed T1D patients than the HC, indicating that miRNA-21-5p in serum exosomes could serve as a candidate T1D biomarker [15].

Also, pancreatic cellular damage may be detected by the presence of extracellular vesicles miRNA-21-5p. According to many previous studies, miRNA-21-5p rises after the administration of inflammatory mediators like, interferon (IFN)-γ, interleukin (IL)-1β, and tumor necrosis factor (TNF)-α, which is speculated to be the pro-inflammatory properties [17]. According to the above-cited studies, the role of miRNA-21-5p in the pathogenicity of T1D remained controversial among newly onset T1D or their FDRs. Thereof, the recent research aimed to study the roles of VD and miRNA-21-5p as potential predictors and/or diagnostic biomarkers for T1D and to determine their predictive accuracy and the degree of relationship to pancreatic cell stress and death (relative to the serum C-peptide). This may lead to a new strategy of earlier diagnosis and immune-modulating therapy to preserve the remaining β-cells of newly onset T1D or their FDRs.

2. Materials and Methods

Study design/subjects

Case-control research was carried out at the Thi-Qar Specialized Diabetes, Endocrine, and Metabolism Center, Thi-Qar governorate, Iraq, during August and December-2022. This study includes three study groups: a T1D group comprised of 35 newly onset T1D patients (20 females and 15 males) with a range of ages between 3-18 years, a group of FDRs which includes a total of 35 FDRs of T1D patients (20 women and 15 men) between the ages of 10 and 40 years, and the HC group including a total of 20 subjects with the percentage of females and males 12/8, and their ages ranged between 5-20 years.

Exclusion and inclusion criteria

For the T1D group, subjects with one of the following criterion were omitted from the recent research: subjects with non-T1D, patients who have been receiving treatment with corticosteroids for the past four weeks, the existence of any other chronic or autoimmune disease, current surgery (within the previous six months), under any biological agent, current transfusions of blood (within the previous six months), presence of any diabetes complications (nephropathy, neuropathy, and retinopathy), uncontrolled hyperglycemia, patients who had been diagnosed with T1D for more than one year, and absence of FDRs. The patients with the following criterion had been enrolled in recent research: recent diagnosis of T1D (less than one year), the patients have FDRs, absence of any type of diabetes complications, patients with controlled hyperglycemia, and have not agreed with any of the exclusion criterion listed above.

For the FDRs group, the subjects that met at least one of the following criteria had been omitted from this group: the occurrence of any chronic or auto-immune condition, FDRs under corticosteroid therapy throughout the past four weeks, current transfusions of blood (within the previous six months), taking any biological agents and current surgery (within the previous six months).

For the HC subjects, the same omitted criteria of the FDRs subjects had been applied for this group of subjects. Additionally, individuals with any infection had been excluded. This group of individuals do not have a history of DM in their families.

Sera collection

By puncturing a vein, 3-5 milliliters (ml) of blood were obtained. The collected samples completed the coagulation process at room temperature in gel vacuum tubes (China), then serum was separated by centrifuging the samples at 3600 rounds/minute (min) for ten min. Each sample of serum had been separated into two aliquots; one of them was mixed with Trizol [400 microliters (µl) serum + 600 µl Trizol (Thermo Scientific, USA)] which was used for miRNA extraction, and the other one was stored at $^{-}$80 ºC until their need for C-peptide and VD assays.

Human connecting peptide and vitamin D assays

Serum C. peptide and VD were detected and titrated in picograms (pg)/ml and nanogram (ng)/ml, respectively, using a human C-peptide enzyme-linked immune-sorbent assay (ELISA) kit (SunLong Biotech, China, catalogue number: SL0531Hu), and a human VD ELISA Kit (SunLong Biotech, China, Catalogue number: SL1831Hu), respectively, which was based on sandwich-ELISA as the method, and the assays protocol had been performed according to the manual manufacturer's recommendations at Thi-Qar Health Department, Imam Al-Hussein Teaching Hospital.

Molecular detection of miRNA-21-5p

The molecular assay was executed in a Specialist Laboratory center in Baghdad and all assay procedures were performed in reference to the manual recommendations. Total serum RNA had been extracted using Trizol reagent method (Thermo Scientific, USA). The isolated RNA was used for the synthesis of the miRNA-21-5p and RNU43 (housekeeping gene) complementary deoxyribonucleic acid (cDNA) molecules using miRNA-21-5p-reverse transcriptase (RT) and RNU43-RT primers (RT step) (**Table 1**) by Thermal Cycler program (BioRad, USA). Quantus Fluorometer *(*Promega, USA) (Fluorescence method) was performed to measure the level of isolated cDNA molecule to determine the quality of samples for the next applications, a cDNA level of 5-7 ng/µl was applicable. Real time-quantitative polymerase chain reaction (qPCR) was performed for quantification of miRNA-21-5p and RNU43 using miRNA-21-5p-forward (F), RNU43-F, and universal reverse primers (**Table 1**) by Mic-qPCR cycler (Bio-molecular System, Australia) (**Figures 1, 2**). Relative quantification of gene expression was performed using Pfaffi Method [18]. By using real-time cycler software, the threshold cycle (Ct) was determined for the samples, and selected gene data expressions were standardized according to housekeeping. Data analysis was performed using the ΔΔCt methods, and a fold change in gene expression was used to express the results: the difference in Ct values (ΔCt) between each target gene and the gene for housekeeping was calculated according to the following equation [19].

$$\Delta CT = CT_{gene} - CT_{housekeeping\ gene} \text{ [20]}$$

$$\Delta\Delta CT = \Delta CT_{Treated\ or\ Control} - \text{Average } \Delta CT_{Control}$$

Statistical analysis

For data presentation and analysis, SPSS (version 24) was used. The frequencies, relative frequencies, and means had been obtained as descriptive methods. The associations among parameters were obtained using the chi-square statistical method. Differences had been considered statistically valuable when the P-value was <0.05.

Primer Name	Sequence (5′———3′)	Temp.	Company	Country
miRNA-21-5p-RT*	GTTGGCTCTGGTGCAGGGTCCGAGGT ATTCGC ACCAGAGCCAACTCAACA	60 °C	Macrogen	South Korea
miRNA-21-5p-F1*	GTTTGGTAGCTTATCAGACTGA			
RNU43-RT*	GTTGGCTCTGGTGCAGGGTCCGAGGT ATTCGC ACCAGAGCCAACAATCAG	55 °C	Macrogen	South Korea
RNU43-F*	GTGAACTTATTGACGGGCG			
Universal Reverse*	GTGCAGGGTCCGAGGT			
Temp: temperature, **miRNA:** micro-ribonucleic acid, and **RT:** reverse transcriptase.				

*The cDNA sequences of the miRNA gene were obtained from the National Center for Biotechnology Information Gen-Bank database. Primers for real-time qPCR were designed with a melting temperature of 58°C-62°C using primer Premier 3 software, the length of the primers ranging from 18 to 23 nucleotides, and the length of the PCR amplicon ranged between 75 and 150 base pairs.

Table 1. The study primers

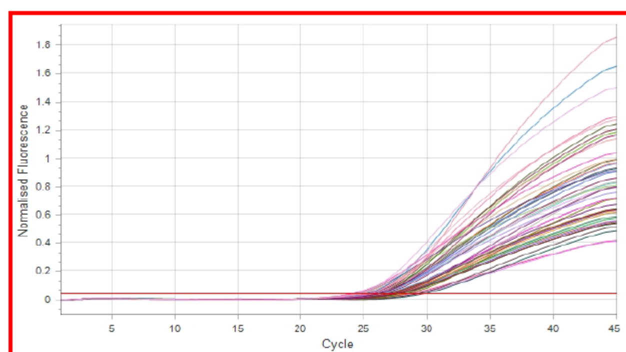

Figure 1. Micro-RNA-21-5p real time polymerase chain reaction step

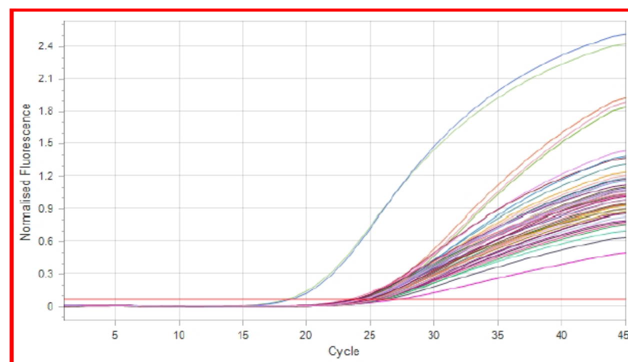

Figure 2. RNU43 real time polymerase chain reaction step

3. Results

The current research had been performed during the time between August 2022 and December 2022 and had three study groups which are: the T1D group comprised of 35 newly onset T1D patients (20 females and 15 males) with an age range from 3-18 years, FDRs group that included a total of 35 FDRs of T1D patients (15 males and 20 females) with a range of age within 10-40 years, and HC group that included a total of 20 subjects with the percentage of females and males 12/8, and their ages ranged between 5-20 years.

Figure 3-A shows that the T1D and FDRs groups had a significantly (p<0.05) elevated below normal C. peptide level (57% and 60%, respectively) in comparison with the HC group (50%). Nevertheless, there were no statistical differences (p>0.05) in the frequency percent of C. peptide concentration between T1D and FDRs subjects. Regarding the C. peptide mean titers (Figure 3-B), the low mean titers were reported in the T1D group (264.3 pg/ml) and FDRs (238.4 pg/ml) compared to HC (285.9 pg/ml) with statistically significant differences (P<0.05). The differences in mean values among T1D and FDRs groups were not significant (p>0.05).

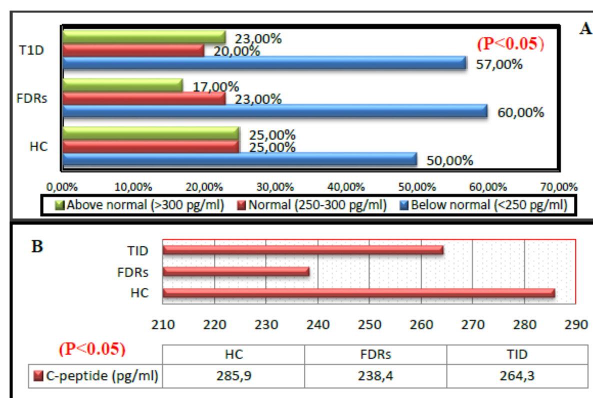

Figure 3. The results of connecting peptide in all study groups, (A): frequency (%), (B): mean titer, (FDRs, first degree relatives; TID, type 1 diabetes; HC, health control; pg, picograms; ml, milliliter; C, connecting).

Figure 4-A shows that the majority of T1D and FDRs group subjects had below-normal level serum VD (82.86% for both) compared with HC (60%) with a statistically significance difference (p<0.05). Non-valuable differences (p>0.05) were documented among subjects of T1D and FDRs groups regarded to frequency percent of VD level. Concerning the mean titers of VD (Figure 4-B), the lowest mean titer had been reported in the T1D patients (2.1 ng/ml) and FDRs group (1.9 ng/ml) than HC group (3.3 ng/ml) with a statistically significant difference (p<0.05). There was no statistically significant difference (p>0.05) in mean values among the T1D and FDR groups.

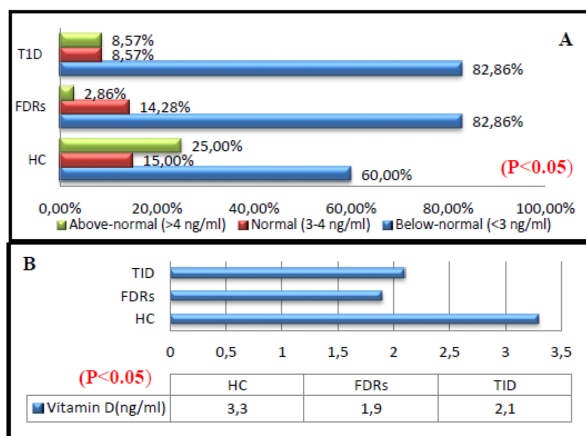

Figure 4. The results of vitamin D in all study groups, (A): frequency (%), (B): mean titer, (FDRs, first degree relatives; TID, type 1 diabetes; HC, health control; ng, nanogram; ml, milliliter).

The results in Figure 5-A show that most of the T1D subjects (51.8%) were with high folds of miRNA-21-5p compared to the FDRs and HC subjects (28.6 and 40%, respectively) with statistical differences (p<0.05). However, no statistically significant difference (p>0.05) was noticed between the FDRs group and HC group for this parameter. The mean titer for serum miRNA-21–5p folds was statistically (p<0.05) elevated in the T1D patients (2.42 folds) compared to the FDRs subjects (*2* folds) and HC group (1.6 folds). In comparing the mean values of the FDRs and HC subjects, there were no statistical differences among them (p>0.05) (Figure 5-B).

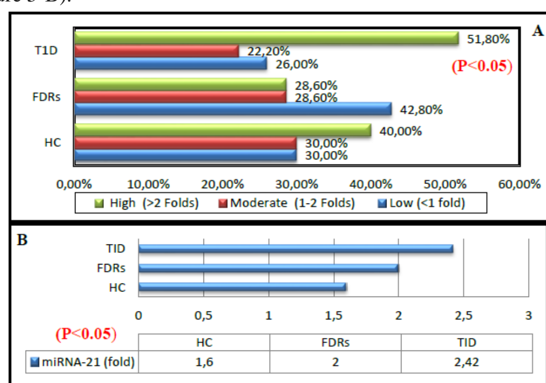

Figure 5. The results of micro-RNA-21-5p in all study groups, (A): frequency (%), (B): mean titer, (FDRs, first degree relatives; TID, type 1 diabetes; HC, health control; miRNA, micro-ribonucleic acid).

Table (2) shows the correlation between the C. peptide and VD levels. The percentage of below-normal VD concentration had been statistically elevated (p<0.05) in T1D patients and FDRs subjects with below-normal C. peptide levels (90% and 90.5%, respectively) in comparison with T1D and FDRs subjects with above-normal C. peptide levels (50% and 33.3%, respectively), whereas the difference was not statistically valuable (p>0.05) in VD serum level among HC individuals with below normal, normal, above normal C. peptide concentration. For mean titer of VD level, the findings within the same table illustrated a significantly (p<0.05) lower VD mean titer among T1D and FDRs subjects with below normal C. peptide level (1.6 ng/ml, and 1.5 ng/ml, respectively) in comparison with T1D and FDRs subjects with above normal C. peptide level (3.3 ng/ml and 3.4 ng/ml, respectively), while the HC subjects revealed non-significant differences (p>0.05) in VD concentration among individuals with below normal, normal, above normal C. peptide concentration.

Biomarkers			Vitamin D (ng/ml)								P. value
			Below N (<3)		Normal (3-4)		Above N (>4)		Total		
			FR (%)	Mean	FR (%)	Mean	FR (%)	Mean	FR (%)	Mean	
Connecting peptide (pg/ml)	T1D	Below N (n=20)	**18(90)**	1.3	1(5)	3.1	1(5)	4.8	20(100)	**1.6**	<0.05
		Normal (n=7)	7(100)	2.3	0(0)	0	0(0)	0	7(100)	2.3	
		Above N (n=8)	**4(50)**	2.1	2(25)	3.4	2(25)	5.7	8(100)	**3.3**	
		Total (n=35)	29(82.86)	1.6	3(8.57)	3.3	3(8.57)	5.4	35(100)	2.1	
	FDRs	Below N (n=21)	**19(90.5)**	1.4	2(9.5)	3.2	0(0)	0	21(100)	**1.5**	<0.05
		Normal (n=8)	8(100)	1.9	0(0)	0	0(0)	0	8(100)	1.9	
		Above N (n=6)	**2(33.3)**	2.8	3(50)	3.5	1(16.67)	4.2	6(100)	**3.4**	
		Total (n=35)	29(82.86)	1.6	5(14.28)	3.4	1(2.86)	4.2	35(100)	1.9	
	HC	Below N (n=10)	7(70)	2	1(10)	3.1	2(20)	9.7	10(100)	3.6	>0.05
		Normal (n=5)	2(40)	1.3	1(20)	3.1	2(40)	4.7	5(100)	3	
		Above N (n=5)	3(60)	2	1(20)	3.2	1(20)	5.2	5(100)	2.9	
		Total (n=20)	12(60)	1.9	3(15)	3.1	5(25)	6.8	20(100)	3.3	

pg, picogram; **ml**, milliliter; **FDRs**, first degree relatives; **T1D**, type 1 diabetes; **HC**, healthy control; **N**, normal; **n**, number; **ng**, nanogram; **FR**, frequency; **%**, percent; **below N**, <250 pg/ml; **normal**, 250-300 pg/ml; **above normal**, >300 pg/ml.

Table 2. The correlation between connecting peptide and vitamin D levels

Table (3) showed the correlation of the serum C. peptide with serum miRNA-21-5p. The differences in frequency percent of low miRNA-21-5p folds were significantly (P<0.05) higher in FDRs individuals with below-normal C. peptide concentration [4/8 (50%)] than FDRs subjects with normal and/or above normal C. peptide level [2/6 (33.3%)]. Additionally, the mean value of miRNA-21-5p, was statistically (p<0.05) low in FDRs subjects with below normal C. peptide level (1.7 folds) compared to subjects with normal and/or above normal C. peptide level (2.5 folds). There were no statistical differences (p>0.05) in the correlation among serum C. peptide and miRNA-21-5p of T1D and HC groups for both frequency percent and means values.

Biomarkers			Micro-RNA-21-5p (Folds)*								P. value
			Low (<1)		Moderate (1-2)		High (>2)		Total		
			FR (%)	Mean	FR (%)	Mean	FR (%)	Mean	FR (%)	Mean	
C. peptide (pg/ml)	T1D	Below N (n=16)	6 (37.5)	0.47	2 (12.5)	1.2	8 (50)	3.6	16 (100)	2.1	>0.05
		N-A (n=11)	1 (9.1)	0.88	4 (36.4)	1.7	6 (54.5)	3.8	11(100)	2.8	
		Total (n=27)	7 (26)	0.5	6 (22.2)	1.5	14 (51.8)	3.7	27 (100)	2.4	
	FDRs	Below N (n=8)	**4 (50)**	0.6	3 (37.5)	1.5	1 (12.5)	6.4	8 (100)	**1.7**	<0.05
		N-A (n=6)	**2 (33.3)**	0.23	1 (16.7)	1.3	3 (50)	4.3	6 (100)	**2.5**	
		Total (n=14)	6 (42.8)	0.46	4 (28.6)	1.5	4 (28.6)	4.9	14 (100)	2	
	HC	Below N (n=5)	1 (20)	0.04	3 (60)	1.8	1 (20)	2.3	5 (100)	1.6	>0.05
		N-A (n=5)	2 (40)	0.6	0 (0)	0	3 (60)	2.3	5 (100)	1.6	
		Total (n=10)	3 (30)	0.4	3 (30)	1.8	4 (40)	2.4	10 (100)	1.6	

C, connecting; **pg**, picogram; **ml**, milliliter; **T1D**, type 1 diabetes; **HC**, healthy control; **FDRs**, first degree relatives; **N**, normal (250-300 pg/ml); **n**, number; **A**, above normal (>300 pg/ml); **RNA**, ribonucleic acid; **FR**, frequency; **%**, percent; **below N**, <250 pg/ml.

*Out of 90 subjects, only 51 subjects were evaluated for serum miRNA-21-5p expression due high cost of this assay and the current research not being supported financially.

Table 3. The correlation between connecting peptide and micro-RNA-21-5p

Table (4) illustrates the relationship between the serum miRNA-21-5p and

serum VD levels. The frequency percent of below normal VD level among T1D subjects with low serum miRNA-21-5p folds was elevated (100%) with valuable difference ($p<0.05$) as compared with T1D patients with moderate and/or high miRNA-21-5p folds (80%), whereas the FDRs and HC groups subjects revealed non-significant differences ($p>0.05$) in frequency percent of VD level between subjects with low and moderate/high miRNA-21-5p folds. The mean titer of serum VD was significantly decreased in T1D with low miRNA-21-5p fold (1.4 ng/ml) in comparison with subjects with moderate and/or high miRNA-21-5p (2.2 ng/ml), however, there were no statistical differences ($p>0.05$) demonstrated in mean titer of serum VD between subjects with low and moderate/high miRNA-21-5p folds within FDRs and HC groups.

Biomarkers			Vitamin D (ng/ml)								P. value
			Below N (<3)		Normal (3-4)		Above N (>4)		Total		
			FR (%)	Mean	FR (%)	Mean	FR (%)	Mean	FR (%)	Mean	
MiRNA-21-5p (Folds)*	T1D	Low (n=7)	7 (100)	1.4	0 (0)	0	0 (0)	0	7 (100)	1.4	<0.05
		M-H (n=20)	16 (80)	1.7	2 (10)	3.5	2 (10)	4.5	20 (100)	2.2	
		Total (n=27)	23 (85.2)	1.6	2 (7.4)	3.5	2 (7.4)	4.6	27 (100)	2	
	FDRs	Low (n=6)	4 (66.6)	1.1	1 (16.7)	3.5	1 (16.7)	4.2	6 (100)	2	>0.05
		M-H (n=8)	7 (87.5)	2.1	1 (12.5)	3.6	0 (0)	0	8 (100)	2.3	
		Total (n=14)	11 (78.5)	1.7	2 (14.3)	3.5	1 (7.1)	4.2	14 (100)	2.2	
	HC	Low (n=3)	2 (66.7)	1.9	0 (0)	0	1 (33.3)	5.3	3 (100)	3	>0.05
		M-H (n=7)	5 (71.4)	1.9	1 (14.3)	3.1	1 (14.3)	5	7 (100)	2.5	
		Total (n=10)	7 (70)	1.9	1 (10)	3.1	2 (20)	5.1	10 (100)	2.7	

ng, nanogram; **ml**, milliliter; **FDRs**, first degree relatives; **T1D**, type 1 diabetes; **HC**, healthy control; **N**, normal; **n**, number; **M**, moderate (1-2 folds); **H**, high (>2 folds); **miRNA**, micro-ribonucleic acid; **FR**, frequency; **%**, percent; **low**, <1 fold.

*Out of 90 subjects, only 51 subjects were evaluated for serum miRNA-21-5p expression due high cost of this assay and the current research not being supported financially.

Table 4. The correlation between micro-RNA-21 and vitamin D

4. Discussion

Type 1 diabetes is a chronic immune-reactive condition that occurs due to the damage of pancreatic β-cells that produce insulin by the T-cell-mediated immunological response. Islet autoimmunity is typically found in subjects who have a genetic susceptibility to the disease, and genetic factors assume a valuable role in the T1D pathophysiology. These processes vary greatly between individuals; therefore, a variety of endotypes have been suggested [21]. The diagnosis of T1D is frequently delayed, often after decreasing C. peptide levels. The newest and most efficient markers might ease this issue and assist in early pre-clinical detection. Serum levels of C. peptide were used to measure the degree of pancreatic β-cells damage due to autoimmunity [22].

This study indicated that the amount of C. peptide had decreased in the T1D group in comparison with the HC subjects (Figure 3), which is highly consistent with the evidence above. Very few investigations had explored the gradual depletion in the C. peptide level through the phases of T1D until reaching an undetectable level within years after onset. Many studies have shown that the levels of C. peptides follow this pattern [22, 23] in which they observed that C. peptide levels decreased gradually within the pre-diabetic period but accelerated within the clinical period. In line with the last mentioned two studies, a recent study showed a significantly lower C. peptide level among the FDRs group (as pre-diabetes) compared with the HC group (Figure 3).

The role of VD in the β-cells function could be mediated by the circulating 1,25-dihydroxyvitamin D binding to the VD receptor on β-cells. Alternatively, VD could be active due to 25-hydroxyvitamin D is activated via 1-alpha-hydroxylase, an enzyme present in β–cells. By increasing insulin receptor expression or PPAR activation, VD could directly improve insulin sensitivity [24].

The previous studies on VD deficiency in T1D children have been limited. One study from Italy [25] and a second study from Sweden, [26], revealed that the patients with T1D had a lower mean level of VD at the time of diagnosis compared to the control group. Another study conducted on Australian adolescents with T1D indicated that 43% of them were VD-deficient [27]. The current study resembles all studies mentioned above, where the lowest level of VD was recorded in the T1D patients than in the HC with significant differences (Figure 4). The impact of VD depletion on the immune system cells and the expression of the VD receptor on β-cells is often taken to support a mechanistic involvement of VD in the development of T1D. The findings of the current study revealed a significantly lower VD concentration in the FDRs individuals compared to the HC control (Figure 4). In agreement with this finding, another study showed that VD concentrations were greatest for those with normoglycemia compared to those with prediabetes and diabetes [28]. Extracellular miRNAs can be detected in the bloodstream, which makes them an ideal potential biomarker. Therefore, the newest T1D biomarkers are necessary to complete the data obtained from auto-Abs existence with other genetic and environmental risk factors [29]. Micro-RNAs regulate gene expression post-transcriptionally and contribute to cellular processes like differentiation, proliferation, and apoptosis. The role of microRNAs as non-invasive markers was studied in a variety of diseases and pathologic processes because they can be detected in cell-free circulation (i.e., serum and plasma) [30]. Micro-RNA-21-5p is an important novel biomarker that can help in the diagnosis of islet autoimmunity at an earlier stage and more accurately predict the onset of T1D [31].

In agreement with these results, this study's results (Figure 5) showed significantly higher miRNA-21-5p folds in T1D patients compared to the HC and FDR groups. Coinciding with these findings, other previous studies reported a higher expression of miRNA-21-5p in conditions characterized by impaired immunological responses, such as T1D [32, 33, 34]. Previous evidence indicates that miRNAs are also important in the immune systems functions, and β-cells metabolism, death, and proliferation, which play a key role in T1D pathogenesis. Micro-RNA-21-5p expressions were reported to be induced in MIN6 cells and human pancreatic islets in response to inflammatory mediators like IL-1β and TNF [35], indicating that these miRNAs may be involved in the destruction of β-cells mediated by cytokines. In addition, miRNA-21-5p expression was elevated in NOD mouse islets during the onset of prediabetic insulitis, possibly as a protective response since miRNAs-21-5p knockdown in MIN-6 cells enhances programmed cell death [14]. Along the same lines, the current study showed elevated folds of miRNAs-21-5p among FDRs as compared with HC groups (Figure 5), as well as the folds of miRNA-21-5p were positively associated with C. peptide level among FDRs groups (Table 3).

The results of the study suggest that miRNA-21-5p in human serum is an essential biomarker for T1D diagnosis and prediction. The earliest detection of its abnormal expression helps in T1D detection, T1D intervention, and early detection of pre-clinical diabetes individuals.

Dysregulation of metabolic processes that are controlled by insulin is associated with VD depletion, diabetes, and obesity. Insulin resistance and pancreatic β-cells dysfunction are correlated with low VD levels [36, 37]. Vitamin D activity is consistent with insulin release (phase 1) and insulin secretion (phase 2) from islet β-cells in *vitro* and VD-deficient models [38]. In line with these studies, recent research showed (Table 2) a significant positive association between VD and C. peptide levels among T1D and their FDRs.

Similar to the recent study findings, other studies have found that increasing VD may contribute to increasing T regulatory cell and C. peptide concentrations (insulin secretion biomarker) [39, 40], where VD

was positively correlated with stimulated C. peptide concentrations among newly onset T1D subjects [39].

There is proof of evidence indicating that VD can affect the amount of microRNAs by changing enzyme expression that contributes to the synthesis of microRNAs and inducing the transcription of microRNAs both directly and indirectly [41]. Studies revealed that circulating levels of miRNA-21-5p were correlated with the residual β-cells function [42] and miRNA-21-5p regulation associated with VD deficiency [43]. Consistent with these findings, the current investigation revealed a significant positive correlation between the concentration of miRNA-21-5p folds and the concentration of VD in T1D patients (Table 4).

Previous studies suggest that VD deficiency-associated miRNA-21 regulation may involve in the pathophysiology of chronic metabolic and inflammatory diseases like DM, lipid metabolism issues, periodontitis, hypertension, and other glucometabolic diseases [44]. In another study, VD therapy in Wistar mice with kidney ischemia-reperfusion damage led to a statistical rise in the level of miRNA-21 [45], whereas Rendina *et al*. [44] has shown that correcting the VD depletion did not affect the levels of circulating miRNA-21. Nevertheless, future study is required to completely clarify the genetic processes that result in the regulation of miRNAs and to fully understand the intricate interactions that take place between miRNAs and VD- associated targets.

Some of the patients and/or their guardians who visited the Diabetes and Endocrinology Specialist Center refused the sampling process, whereas some patients refused the sampling process of their FDRs. This issue may affect the final results of the current study due to the restrictions imposed by the patients and/or guardians. A further limitation was the kits' sensitivities and specificities that were used for biomarkers identification and quantification due to there being some variance among the producing companies. Finally, due to the high cost of molecular assay and the current research not being supported financially, only 51 subjects were evaluated for serum miRNA-21-5p expression.

5. Conclusions

Vitamin D had exhibited a significantly decreased level among T1D and FDRs and positively correlated with residual β-cells function (relative to the serum C-peptide), which indicated the utility of its low level as a β-cells stress and/or death predictive and diagnostic biomarker, and the VD deficiency is a risk factors to T1D, thereof VD supplementation is required to decrease the incidence of T1D.

Micro-RNA-21-5p revealed a significantly higher folds expression among T1D and positively correlated with both residual β-cells function (relative to the serum C-peptide) and VD level in FDRs and T1D, respectively, which indicated the possible utility of its high folds expression as β-cells stress and/or death predictive and diagnostic biomarker and the higher expression of miRNA-21-5p may be an important factor in the preventing T1D.

These findings indicated an essential role for this miRNA in understanding the pathogenicity of disease and reflects endogenous β-cells stress and/or death which may be used as a predictor, diagnostic, and therapeutic target during the pre-clinical and initial stages of T1D.

6. Abbreviations

µl, microliter; **Abs,** antibodies; **C,** connecting; **cDNA,** complementary deoxyribonucleic acid; **Ct,** threshold cycle; **DM,** diabetes mellitus; **ELISA,** enzyme linked immune-sorbent assay; **F,** forward; **FDRs,** first degree relatives; **HC,** healthy control; **IFN,** interferon; **IL,** interleukin; **min,** minute; **miRNA,** micro-ribonucleic acid; **ml,** milliliter; **ng,** nanogram; **pg,** picograms; **qPCR,** quantitative polymerase chain reaction; **RT,** reverse transcriptase; **T1D,** Diabetes type 1; **TNF,** tumor necrosis factor; **VD,** vitamin D; **β,** beta.

References

1. Baynes HW. Classification, pathophysiology, diagnosis and management of diabetes mellitus. J diabetes metab. 2015 May 1;6(5):1-9.
2. Syed F, Evans-Molina C. Nucleic acid biomarkers of β cell stress and death in type 1 diabetes. Current opinion in endocrinology, diabetes, and obesity. 2016 Aug;23(4):312.
3. Atkinson MA, Bluestone JA, Eisenbarth GS, Hebrok M, Herold KC, Accili D, Pietropaolo M, Arvan PR, Von Herrath M, Markel DS, Rhodes CJ. How does type 1 diabetes develop? The notion of homicide or β-cell suicide revisited (Diabetes (2011) 60,(1370-1379)). Diabetes. 2012 Jun 1;61(6):1648.
4. Anastasiou IA, Eleftheriadou I, Tentolouris A, Koliaki C, Kosta OA, Tentolouris N. The effect of oxidative stress and antioxidant therapies on pancreatic β-cell dysfunction: results from in vitro and in vivo studies. Current medicinal chemistry. 2021 Feb 1;28(7):1328-46.
5. Lebastchi J, Herold KC. Immunologic and metabolic biomarkers of β-cell destruction in the diagnosis of type 1 diabetes. Cold Spring Harbor perspectives in medicine. 2012 Jun 1;2(6):a007708.
6. Wang L, Lovejoy NF, Faustman DL. Persistence of prolonged C-peptide production in type 1 diabetes as measured with an ultrasensitive C-peptide assay. Diabetes care. 2012 Mar 1;35(3):465-70.
7. Mathieu C, Gysemans C. Vitamin D and diabetes. Av diabetol. 2006 Jul;22(3):187-93.
8. Mitri J, Pittas AG. Vitamin D and diabetes. Endocrinology and Metabolism Clinics. 2014 Mar 1;43(1):205-32.
9. Ross JS, Carlson JA, Brock G. miRNA: the new gene silencer. American journal of clinical pathology. 2007 Nov 1;128(5):830-6.
10. Carrington JC, Ambros V. Role of microRNAs in plant and animal development. Science. 2003 Jul 18;301(5631):336-8.
11. Butz H, Kinga N, Racz K, Patocs A. Circulating miRNAs as biomarkers for endocrine disorders. Journal of endocrinological investigation. 2016 Jan;39:1-0.
12. Mathieu C, Lahesmaa R, Bonifacio E, Achenbach P, Tree T. Immunological biomarkers for the development and progression of type 1 diabetes. Diabetologia. 2018 Nov;61:2252-8.
13. Huang X, Liang M, Dittmar R, Wang L. Extracellular microRNAs in urologic malignancies: chances and challenges. International journal of molecular sciences. 2013 Jul 16;14(7):14785-99.
14. Roggli E, Britan A, Gattesco S, Lin-Marq N, Abderrahmani A, Meda P, Regazzi R. Involvement of microRNAs in the cytotoxic effects exerted by proinflammatory cytokines on pancreatic β-cells. Diabetes. 2010 Apr 1;59(4):978-86.
15. Sims EK. Microrna 21 targets B Cell Lymphoma 2 (Bcl2) Mrna to increase beta cell apoptosis and exosomal Microrna 21 could serve as a biomarker of developing Type 1 Diabetes Mellitus. M.S. Thesis. 2018, Translational Science, Indiana University.
16. Nielsen LB, Wang C, Sørensen K, Bang-Berthelsen CH, Hansen L, Andersen ML, Hougaard P, Juul A, Zhang CY, Pociot F, Mortensen HB. Circulating levels of microRNA from children with newly diagnosed type 1 diabetes and healthy controls: evidence that miR-25 associates to residual beta-cell function and glycaemic control during disease progression. Experimental diabetes research. 2012 Oct;2012.

17. Angelescu MA, Andronic O, Dima SO, Popescu I, Meivar-Levy I, Ferber S, Lixandru D. miRNAs as Biomarkers in Diabetes: Moving towards Precision Medicine. International Journal of Molecular Sciences. 2022 Oct 25;23(21):12843.
18. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. Biotechnology letters. 2004 Mar;26:509-15.
19. Younus N, Altaee M, Sharba ZA. Correlation of MicroRNAs-122a Gene Expression with Diabetic for Iraqi Patients. Journal of Applied Sciences and Nanotechnology. 2021 Sep 1;1(3):64-72.
20. Artemaki PI, Papanota AM, Karousi P, Liacos C, Kontos CK, Patseas D, Papadimitriou MA, Eleutherakis-Papaiakovou E, Avgeris M, Kanellias N, Bagratuni T. The Clinical Significance of a Novel microRNA Signature in Multiple Myeloma. Blood. 2019 Nov 13;134:5529.
21. Zajec A, Trebušak Podkrajšek K, Tesovnik T, Šket R, Čugalj Kern B, Jenko Bizjan B, Šmigoc Schweiger D, Battelino T, Kovač J. Pathogenesis of type 1 diabetes: established facts and new insights. Genes. 2022 Apr 16;13(4):706.
22. Sheet MM, Khudhair HA. Beta-cell Death and/or Stress Biomarkers in Diabetes Mellitus Type. Al-Kufa University Journal for Biology. 2019 Feb 24;11(1).
23. Sosenko JM, Palmer JP, Rafkin-Mervis L, Krischer JP, Cuthbertson D, Matheson D, Skyler JS. Glucose and C-peptide changes in the perionset period of type 1 diabetes in the Diabetes Prevention Trial–Type 1. Diabetes Care. 2008 Nov 1;31(11):2188-92.
24. Infante M, Ricordi C, Sanchez J, Clare-Salzler MJ, Padilla N, Fuenmayor V, Chavez C, Alvarez A, Baidal D, Alejandro R, Caprio M. Influence of vitamin D on islet autoimmunity and beta-cell function in type 1 diabetes. Nutrients. 2019 Sep 11;11(9):2185.
25. Bruno G, Runzo C, Cavallo-Perin P, Merletti F, Rivetti M, Pinach S, Novelli G, Trovati M, Cerutti F, Pagano G, Piedmont Study Group for Diabetes Epidemiology. Incidence of type 1 and type 2 diabetes in adults aged 30–49 years: the population-based registry in the province of Turin, Italy. Diabetes care. 2005 Nov 1;28(11):2613-9.
26. Littorin B, Blom P, Schölin A, Arnqvist HJ, Blohme G, Bolinder J, Ekbom-Schnell A, Eriksson JW, Gudbjörnsdottir S, Nyström L, Östman J. Lower levels of plasma 25-hydroxyvitamin D among young adults at diagnosis of autoimmune type 1 diabetes compared with control subjects: results from the nationwide Diabetes Incidence Study in Sweden (DISS). Diabetologia. 2006 Dec;49:2847-52.
27. Burger RA, Sill MW, Monk BJ, Greer BE, Sorosky JI. Phase II trial of bevacizumab in persistent or recurrent epithelial ovarian cancer or primary peritoneal cancer: a Gynecologic Oncology Group Study. Journal of clinical oncology. 2007 Nov 20;25(33):5165-71.
28. McCarthy K, Laird E, O'Halloran AM, Walsh C, Healy M, Fitzpatrick AL, Walsh JB, Hernández B, Fallon P, Molloy AM, Kenny RA. Association between vitamin D deficiency and the risk of prevalent type 2 diabetes and incident prediabetes: A prospective cohort study using data from The Irish Longitudinal Study on Ageing (TILDA). Eclinicalmedicine. 2022 Nov 1;53:101654.
29. Bonifacio E. Predicting type 1 diabetes using biomarkers. Diabetes care. 2015 Jun 1;38(6):989-96.
30. Van den Berg MM, Krauskopf J, Ramaekers JG, Kleinjans JC, Prickaerts J, Briedé JJ. Circulating microRNAs as potential biomarkers for psychiatric and neurodegenerative disorders. Progress in neurobiology. 2020 Feb 1;185:101732.
31. Guay C, Regazzi R. Circulating microRNAs as novel biomarkers for diabetes mellitus. Nature Reviews Endocrinology. 2013 Sep;9(9):513-21.
32. Pauley KM, Cha S, Chan EK. MicroRNA in autoimmunity and autoimmune diseases. Journal of autoimmunity. 2009 May 1;32(3-4):189-94.
33. Sheedy FJ. Turning 21: induction of miR-21 as a key switch in the inflammatory response. Frontiers in immunology. 2015 Jan 29;6:19.
34. Churov AV, Oleinik EK, Knip M. MicroRNAs in rheumatoid arthritis: altered expression and diagnostic potential. Autoimmunity reviews. 2015 Nov 1;14(11):1029-37.
35. Assmann TS, Recamonde-Mendoza M, De Souza BM, Crispim D. MicroRNA expression profiles and type 1 diabetes mellitus: systematic review and bioinformatic analysis. Endocrine connections. 2017 Nov;6(8):773.
36. von Hurst PR, Stonehouse W, Coad J. Vitamin D supplementation reduces insulin resistance in South Asian women living in New Zealand who are insulin resistant and vitamin D deficient–a randomised, placebo-controlled trial. British Journal of Nutrition. 2010 Feb;103(4):549-55.
37. Kabadi SM, Lee BK, Liu L. Joint effects of obesity and vitamin D insufficiency on insulin resistance and type 2 diabetes: results from the NHANES 2001–2006. Diabetes care. 2012 Oct 1;35(10):2048-54.
38. John AN, Jiang FX. An overview of type 2 diabetes and importance of vitamin D3-vitamin D receptor interaction in pancreatic β-cells. Journal of Diabetes and its Complications. 2018 Apr 1;32(4):429-43.
39. Aljabri KS, Bokhari SA, Khan MJ. Glycemic changes after vitamin D supplementation in patients with type 1 diabetes mellitus and vitamin D deficiency. Annals of Saudi medicine. 2010 Nov;30(6):454-8.
40. Bogdanou D, Penna-Martinez M, Filmann N, Chung TL, Moran-Auth Y, Wehrle J, Cappel C, Huenecke S, Herrmann E, Koehl U, Badenhoop K. T-lymphocyte and glycemic status after vitamin D treatment in type 1 diabetes: A randomized controlled trial with sequential crossover. Diabetes/Metabolism Research and Reviews. 2017 Mar;33(3):e2865.
41. Beckett C, Taylor H. Human growth and development. Sage; 2019 Mar 18.
42. Liu Y, Ma M, Yu J, Ping F, Zhang H, Li W, Xu L, Li Y. Decreased serum microRNA-21, microRNA-25, microRNA-146a, and microRNA-181a in autoimmune diabetes: potential biomarkers for diagnosis and possible involvement in pathogenesis. International Journal of Endocrinology. 2019 Sep 9;2019: 8406438.
43. Watanabe K, Narumi T, Watanabe T, Otaki Y, Takahashi T, Aono T, Goto J, Toshima T, Sugai T, Wanezaki M, Kutsuzawa D. The association between microRNA-21 and hypertension-induced cardiac remodeling. PLoS One. 2020 Feb 10;15(2):e0226053.
44. Rendina D, D′ Elia L, Abate V, Rebellato A, Buondonno I, Succoio M, Martinelli F, Muscariello R, De Filippo G, D′ Amelio P, Fallo F. Vitamin D Status, Cardiovascular Risk Profile, and miRNA-21 Levels in Hypertensive Patients: Results of the HYPODD Study. Nutrients. 2022 Jun 28;14(13):2683.
45. Golmohammadi MG, Banaei S, Nejati K, Chinifroush-Asl MM. Vitamin D3 and erythropoietin protect against renal ischemia-reperfusion injury via heat shock protein 70 and microRNA-21 expression. Scientific Reports. 2020 Dec 1;10(1):20906.