ORIGINAL ARTICLE

Effects of GLP-1 Receptor Polymorphisms on Adolescent Obesity

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Received: 04 Jul. 2022; Accepted: 23 Mar. 2023

Abstract- Obesity is becoming a concerning disease in developing countries. Like other multifactorial diseases, genetics plays a substantial role in the development of this disease. We tried to investigate genetic variations (mutation/polymorphism) of GLP-1R gene in children diagnosed with obesity and to identify their possible connections with obesity and other conditions. Genomic DNA was extracted from 162 overweight/obese patients and 100 controls. Later, full exon sequencing and association studies were carried out. Three polymorphisms and one mutation were detected in the fourth and fifth exons of the GLP-1R gene. Some variations were detected in three cases from which 1/3 had non-alcoholic fatty liver disease (NAFLD) but none showed insulin resistance (IR). There were also statistically meaningful results for 'Odds Ratio' among different genotypes and allele frequencies in groups with NAFLD and/or IR. In addition, there was an increase in risk for NAFLD and a decrease in risk for IR. In the homozygous group, also the prospect of IR was double declined. Patients with the A allele of this polymorphism showed a drop in risk for IR as well. GLP-1R polymorphisms could influence obesity and diabetes and thus the functional analysis of the GLP-1R polymorphisms is benevolent.
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Acta Med Iran 2023;61(4):238-246.

Keywords: Glucagon-like peptide-1; Glucagon-like peptide-1 (GLP-1) receptor; Single-nucleotide polymorphism; Obesity development

Introduction

With increasing the westernization of diet and lifestyle, obesity is becoming a concerning disease in developing countries (1). Also, in developed countries, there has been a considerable number of obese children (2). Like other multifactorial diseases, genetics plays a substantial role in the development of obesity (3).

Obesity has been implicated in the development of some chronic diseases, e.g., type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD) (4,5). Impaired secretion of insulin and insulin resistance is the cornerstone of T2DM pathogenesis. The affected patients are prone to develop retinopathy, nephropathy, neuropathy, and cardiovascular diseases, which owes to the fact that T2DM can lead to multiple organ failure (6). The deposition of aberrant adipose tissue in the hepatocytes is the fundamental process of developing NAFLD, which leads to hepatocyte injury. Of interest, T2DM patients have a noticeably increased risk of NAFLD development (7). Although the pathology of NAFLD is still under investigation, insulin resistance has a well-established role in its pathogenesis (5). Since some patients with NAFLD are young, this might explain the genetic background of this disease (8).

It is well-established that the increased level of

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glucagon-like peptide-1 (GLP-1) can induce insulin release from pancreatic β-cells (9). In response to food intake, GLP-1 is released from the L cells of the intestinal mucosa (10). Regarding the positive impact of GLP-1R on insulin regulation, stimulating this axis has shown promising results in reducing the complications of T2DM and NAFLD (11-13). However, different response rates among patients have given rise to the notion that genetic factors have a notable effect on this pharmacological approach (14). Previous reports have demonstrated that patients with T2DM might show single-nucleotide polymorphisms (SNPs) in the GLP-1R gene; however, they are limited (15,16). Indeed, GLP-R1 polymorphisms can change the pharmacological effects of GLP-1-based medication. To the best of our knowledge, this study, for the first time, aims to investigate the genetic variations of GLP-1R in Turkish obese patients. Furthermore, this study intends to find the potential associations between the genetic variations of GLP-R1 with obesity and developing T2DM and NAFLD in the Turkish population.

Materials and Methods

Study population

The study population consisted of 162 obese children (86 males, 76 females). With a mean age of 12.8±2.1 years, their age range was 11 to 16. The children, who were diagnosed with diabetes mellitus, Cushing syndrome, growth hormone deficiency, hypothyroidism, familial hypercholesterolemia, and hypertension, with a history of corticosteroid use, were not included in this study.

Physical examinations and anthropometric measurements of all adolescents were performed. Height was measured in stocking feet to the nearest 0.5 cm using a stadiometer. Bodyweight was recorded using calibrated scales in light clothing to the nearest 0.1 of a kg. Height and body weight measurements were taken twice, and the mean of two readings was calculated. The body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). Obesity was defined as BMI exceeding the 95th percentile for the patient's age and sex (17).

Diagnosis of NAFLD was made based on increased echogenicity via ultrasound compatible with fatty infiltration of the liver with or without elevated alanine aminotransferase (ALT) levels. NAFLD grading by ultrasonography was done according to previous literature (18). Obese patients were divided into two groups: patients with NAFLD and patients without NAFLD.

The control group for the NAFLD part was obese patients without NAFLD, who consisted of 32 individuals (12 males, 20 females, mean age 12.40±0.35 years, range 11-16 years). They were admitted to the child outpatient clinic of our hospital for screening tests. None of the control group had a history of chronic disease. Anthropometric values and abdominal ultrasonography of the controls were in the normal range. Insulin and glucose levels, lipid profiles, and liver function tests were in normal ranges.

Peripheral blood samples were collected from 162 patients with obesity. Also, 100 samples for the control group were collected. All of these individuals were 11-16 years old. A 2 ml of peripheral blood sample was taken from each child in the study. DNA was isolated from taken blood samples using the *High Pure PZR Template Preparation Kit* (*Roche).* The concentration and purity of the obtained DNA were measured using *ND-1000 Spectrophotometer Nanodrop* (ng/ml).

Polymerase chain reaction (PCR) was performed to extract DNA from samples. Specific primers were designed for these regions to replicate the exon 2, 4, and 5 of the GLP-1R gene in the PCR. The NCBI Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design the primers. Later they were synthesized in lyophilized forms in *Metabion Gmbh* (*Germany*) company (Table 1).

Table 1. GLP-1R gene, exon 2, 4 and 5 primers

Exon 2	Forward	5'-catcaaagtcctccagaagttg-3'
	Reverse	5'- cagaatttggggctttgaggtt-3'
Exon 4	Forward	5'-cagtgaagtgctccaagacatg-3'
	Reverse	5'-cagcgtatatgtcaggggagg-3'
Exon 5	Forward	5'-ctgcttcattcctctatctggg-3'
	Reverse	5'-tgtattcacctctctggccttg-3'

The followed PCR program and used protocol are summarized in table 2.

After the routine control of amplified regions with agarose gel (Figure 1), Sanger sequencing was performed for these PCR products. The first purification [Exo Sap-IT (GML, Wollerau Switzerland)] was applied

to remove dNTPs and primers from the environment. 2 µl of Exosap was added to 2 µl of the PCR product. The temperature for enzyme incubation was 37º C for 30 minutes. Deactivation was set at 80º C for 15 minutes. This is followed by the second amplification process (cycle sequencing PCR) using the protocols specified in the kit. Each sample underwent the same amplification protocol. Afterward, the products were purified for the second time using the kit (Zymo Research, ZRDNA Sequencing Clean-Up Kit). The purified PCR products were transferred to appropriate wells. They were sequenced in capillary electrophoresis equipment (Applied Biosystem 3130 Genetic Analyzer) and analyzed according to the "NC_000006 reference sequence" (SeqScape v2.6; Applied Biosystems, USA). Target variation in the GLP-1R gene (exon 2, 4, and 5) was examined with SPSS 15 statistical program (SPSS Inc., Chicago, IL, USA), and mean ± standard deviation was calculated.

Table 2. Used PCR program

Effect	Temperature	Time	Number of cycles
Denaturation	95 ^{0}C	15 minutes	1 cycle
Denaturation	95 ^{0}C	30 seconds	
Connection	57 ^{0}C	45 seconds	35 cycles
Elongation	72 0C	45 seconds	
Final elongation	72 0C	7 minutes	1 cycle

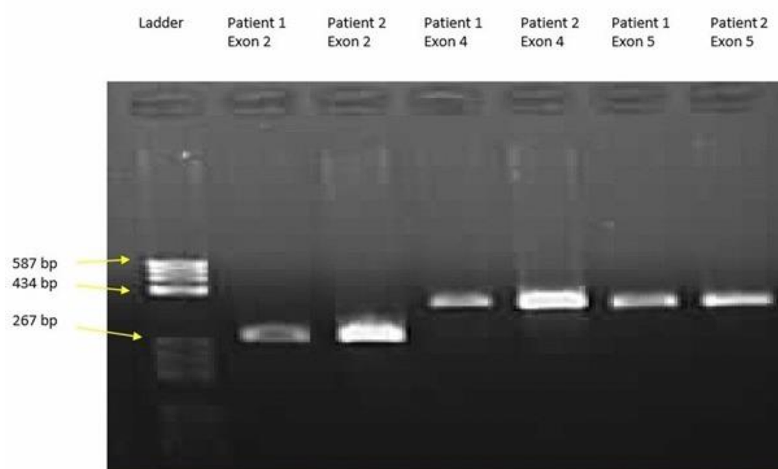

Figure 1. Agarose gel image of amplification products of GLP-1R gene exon 2 (280bp), 4 (383bp) and 5 (380bp). (DNA marker: O'Rang Ruler 100bp DNA Ladder)

Statistical analyses

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 15.0. Data were expressed as mean±standard errors of the mean. In group comparisons, the chi-square test and if the expected value was under 5, Fisher's exact chi-square test was used. Comparing averages of more than two groups, the Kruskal-Wallis test, for two groups student's T-test and in case the variable was under 30, the Mann-Whitney U test was applied. $P<0.05$ was considered significant. Besides, taking *Odds Ratio* (*OR*) values' advantages into consideration (19), OR values were used to quantify the relationship between an independent variable and risk assessment.

Results

The study included 162 obese children whose average age was 12.8±2.1 years. A total of 86 patients (53.1%) were male, and 76 (46.9%) were females. 37 (25.8%) of these children had insulin resistance according to HOMA-IR value. In 61 (37.6%) of them, NAFLD was observed.

Detected mutations and polymorphisms

In our study, sequence analysis was performed for the GLP-1R gene, e.g., exon 2, 4, 5, and exon-intron junctions. Subsequently, one polymorphism in exon 5,

two polymorphisms in exon 4 (Figure 2), and one mutation in exon 4 (Figure 3) were detected.

rs6918287 (G>A) polymorphism

This SNP was detected in 10 (6.2%) patients in heterozygous form and 1 patient (0.6%) in homozygous form. This polymorphism is present in exon 4 of the GLP-1R gene. Allele frequencies of this SNP were 96.3% for the G allele (normal allele) and 3.7% for the A allele. No significant association was identified between genotypes distribution according to gender. Also, no difference was detected when we compared genotypes based on factors associated with obesity (height, weight, and body mass index) (Table 3).

No association was found between this SNP and NAFLD/insulin resistance (Table 4, 5).

Furthermore, the occurrence of insulin resistance is two times higher in patients with heterozygous genotypes (Table 5). No connection was found between the allele frequencies with insulin resistance (Table 6).

However, in risk calculations, the rate of NAFLD in individuals with allele A was estimated to increase about 1.8 times (Table 7).

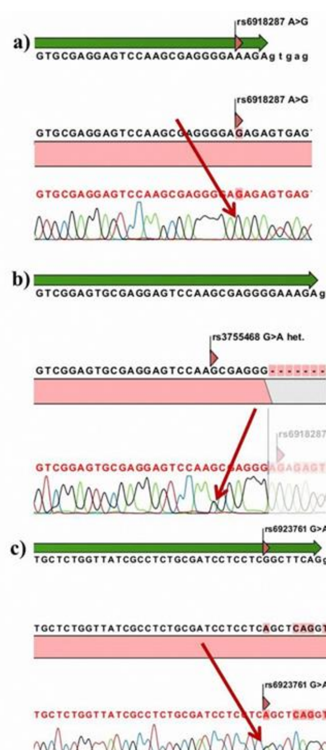

Figure 2. GLP-1R gene, sequence analysis of detected polymorphisms in exon 4 and 5: a) rs6918287 homozygous; b) rs3765468 heterozygous; c) rs6923761 heterozygous

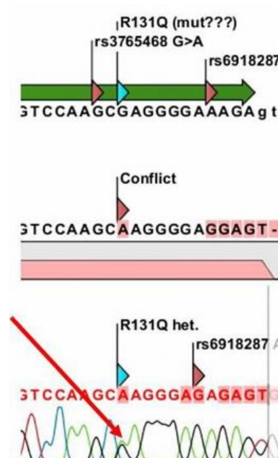

Figure 3. GLP-1R gene, detected heterozygous R131Q mutation in exon 4

rs3765468 (G>A) polymorphism

This polymorphism was in exon 4 and was heterozygous in 32 patients (19.7%) and homozygous in 3 patients (1.9%). The G allele frequency was 88.2%, and the A allele frequency was 11.8%. No significant association was found between genotype distributions according to gender. No connection was observed when comparing the factors associated with obesity within the homozygous and heterozygous groups (Table 3). Although no significant associations were identified among genotype distribution and NAFLD/insulin resistance, NAFLD incidence increases up to 3-fold in homozygous genotypes (Table 4). Moreover, the occurrence of insulin resistance is approximately 1.8 times higher in heterozygous genotypes (Table 5). No differences were observed in patient allele frequencies associated with NAFLD/insulin resistance (Table 6, Table 7).

Table 3. The relationship between genotypes and obesity variations.

SNP	Obesity varieties	GG	GA	AA	*P*
	Height (cm)	161.00	154.55	156.94	0.572
rs6918287	Weight (Kg)	72.00	66.14	73.15	0.624
	BMI (Kg/m^2)	27.00	27.25	29.46	0.381
	Height (cm)	157.04	156.41	151.67	0.529
rs3765468	Weight (Kg)	72.44	74.41	65.67	0.665
	BMI (Kg/m^2)	29.15	29.97	28.58	0.776
	Height (cm)	156.54	157.35	156.35	0.866
rs6923761	Weight (Kg)	73.12	72.85	69.20	0.585
	BMI (Kg/m^2)	29.87	28.77	27.76	0.043

Table 4. The relationship between genotypes and NAFLD

SNP		Without NAFLD		With NAFLD		All		*P*	OR	95.0% C.I.		*P*
		n	%	n	%	n	%			Min.	Max.	
	GG	93	92.1	58	95.1	151	93.2		1.000	-	-	0.870
rs6918287	AG	7	6.9	3	4.9	10	6.2	641	1.455	0.362	5.852	0.597
	AA	1	1.0	0	0	1	6		-	-	-	-
	GG	78	77.2	49	80.3	127	78.4		1.000	-	-	0.459
rs3765468	AG	22	21.8	10	16.4	32	19.8	0.431	1.382	0.604	3.165	0.444
	AA	1	1.0	2	3.3	3	1.9		0.314	0.028	30557	0.35
	GG	53	52.5	38	62.3	91	56.2		1.000	-	-	0.254
rs6923761	AG	41	40.6	17	27.9	58	35.8	0.250	1.729	0.857	3.490	0.126
	AA	7	6.9	6	9.8	13	8.0		-	0.260	2.688	0.764

Table 5. The relationship between genotypes and insulin resistance

SNP		Without Insulin Resistance		With Insulin Resistance		All		*P*	OR	95.0% C.I.		*P*
		n	%	n	%	n	%			Min.	Max.	
	GG	99	93.4	33	89.2	132	92.3		-	-	-	0.591
rs6918287	AG	6	5.7	4	10.8	10	7	0.486	2.00	0.532	7.525	0.305
	AA	1	9.0	0	0	1	7		-	-	-	-
	GG	84	79.2	26	70.3	110	76.9		-	-	-	0.364
rs3765468	AG	19	17.9	11	29.7	30	21.0	0.206	1.870	0.789	4.434	0.155
	AA	3	2.8	0	0	3	2.1		-	-	-	-
	GG	56	52.8	24	64.9	80	55.9		1.000	-	-	0.442
rs6923761	AG	41	38.7	11	29.7	52	36.4	0.437	0.626	0.276	1.421	0.263
	AA	9	8.5	2	5.4	11	7.7		0.519	0.104	2.581	0.423

Table 6. The relationship between allele frequencies and insulin resistance

SNP		Without insulin resistance		With insulin resistance		All		*P*	OR	95.0% C.I.	
		n	%	n	%	n	%			Min.	Max.
rs6918287	G	204	74.5	70	25.5	274	100	0.378	1.457	0.426	4.988
	A	8	66.7	4	33.3	12	100				
rs3765468	G	187	74.8	63	25.2	250	100	0.308	1.306	0.608	2.805
	A	25	69.4	11	30.6	36	100				
rs6923761	G	153	72.2	59	27.8	212	100	0.130	0.659	0.347	1.252
	A	59	79.7	15	20.3	74	100				

Table 7. The relationship between allele frequencies and NAFLD

SNP		Without NAFLD		With NAFLD		All		*P*	OR	95.0% C.I.	
		n	%	n	%	n	%			Min.	Max.
rs6918287	**G**	193	61.9	119	38.1	312	100	0.546	1.850	0.491	6.969
	A	9	75.0	3	25.0	12	100				
rs3765468	**G**	178	62.2	108	37.8	286	100	0.532	1.040	0.516	2.097
	A	24	63.2	14	36.8	38	100				
rs6923761	**G**	147	61.3	93	38.8	240	100	0.290	1.200	0.714	2.017
	A	55	65.5	29	34.5	84	100				

rs6923761 (G>A) polymorphism

This SNP was identified in exon 5 and was heterozygous in 58 cases (36.0%) and homozygous in 13 cases (8%). The G allele frequency was 74%, and the A allele frequency was 26%. No difference between the distribution of this genotype and gender was detected. In patients with homozygous genotypes, the average BMI was significantly lower (*P*=0.043) (Table 3). A more detailed statistical analysis (Mann Whitney U test) was applied to confirm these results, but no association was observed (Table 4). No association was found between this SNP with NAFLD/insulin resistance. However, the prevalence of NAFLD increased to 1.7-fold (Table 4), and the incidence of insulin resistance decreased 1.6 times in heterozygous genotypes (Table 5). Confirming this finding, the occurrence of insulin resistance was approximately two times less in individuals with homozygous genotype (Table 5). In patients with the A allele, the incidence of insulin resistance was reduced by 1.5 times. However, no significant association was observed between allele frequencies and NAFLD/insulin resistance.

R131Q variation

The variation, R131Q exchange (c.392 G>A), was identified as a mutation in the literature. In this mutation, a transition of G to A occurs in position 392 of complementary DNA and consequently results in the substitution of glycine for arginine at residue 131. R131Q variation was found in 3 cases (1.85%). This variation is present in exon 4 of the GLP-1R gene. All of three patients with this variation were male. One-third of these individuals (33.3%) were affected with NAFLD; however, none of them had insulin resistance. R131Q variation was analyzed in 100 healthy control samples (200 alleles) and detected in two healthy individuals. Moreover, in silico modeling programs (*Mutation Taster, PolyPhen2*) were used for this variation, and it has been indicated that this variation has a benign character.

Discussion

Although there have been remarkable advances in the treatment of patients with insulin resistance, insulin resistance, obesity, and their subsequent diseases, e.g., NAFLD, have remained troublesome diseases worldwide (20). Indeed, a better understanding of insulin resistance biology and its genetics can pave the road for introducing new approaches for patients with T2DM and NAFLD. GLP-R1-based therapy has been investigated in patients with insulin resistance complications (13,21). However, the response rate of this approach varies among the affected patients. This different response rate might be stemmed from the diverse genetical differences of the patients (14,22). Therefore, this study has aimed to demonstrate the role of GLP-1R's SNP in developing insulin resistance and NAFLD.

GLP-1 is an essential incretin, and its interaction with GLP-1R substantially regulates glucose homeostasis (23). The genetic variation of GLP-1R might have a considerable role in the downstream pathways of this complex. The full sequence of coding exons of the GLP-R1 gene has shed light on the genetic variations in the GLP-R1 gene. Also, exons 2, 4, and 5 have been designated as hot spots (19). Our study has confirmed two polymorphisms in exon 4 (rs6918287 G>A and rs3765468 G>A), one polymorphism in exon 5 (rs6923761 G>A), and one variation in exon 4 (R131Q, c.392 G>A). In terms of allele frequencies of rs6918287 polymorphism, our study has shown no statically significant association between this polymorphism and NAFLD/insulin resistance. In the patient with the homozygous mutant genotype of rs6918287 polymorphism, fatty liver was present. Comparing the allele frequencies of this SNP in accordance with fatty liver, the risk of the entitled disease is1.8 fold higher in

individuals with A (mutant) allele. In the very same patient, insulin resistance was not present, but the results of the analyses in cases with heterozygous genotype demonstrated a 2-fold increase in insulin resistance. However, there were no reliable results about allele frequencies. Considering the genotype distribution of rs3765468 polymorphism in groups with fatty liver and insulin resistance, a 3-fold rise of fatty liver risk in patients with homozygous mutant genotype was observed. In the very same group of homozygous mutants, the risk of insulin resistance tent to increase 1.8 times. No previous database on this matter was found and the probable negative association between this SNP and metabolic syndrome is added to the literature. Digging through previous works on rs6923761 polymorphism, no differences were found in its genotypes in accordance with diabetes (24,25). However, another study revealed a slight change in the response to insulin secretion in cases with this particular SNP, after getting GLP-1 (26). In our study insulin resistance showed a 1.6 times reduction in cases with heterozygous genotype. This decrease was 2-fold in the ones with homozygous genotype. In the individuals with A (mutant) allele insulin resistance is eroded 1.5 times. Moreover, individuals with heterozygous genotype had a 1.7-fold higher risk for the fatty liver but no supporting value was present in the homozygous group. Reconciling rs6923761 polymorphism with obesity variables, an average reduction in BMI was proposed in the mutant group (25). A similar result was obtained in the analysis of our homozygous mutant genotype, but further statistical analyses demonstrated no association between BMI and this SNP's genotypes.

The summary of the analysis's results alongside their odds Ratios is listed below (Table 8). These results demonstrated that except for the polymorphisms, in our research a variation in exon 4 was identified (R131Q c.392 G>A). This mutation was observed in 3 male cases in heterozygous form. Tokuyama however, detected this variation in 36 patients with type 2 diabetes (27 wild, 9 heterozygous, and 0 homozygous) and reported R131Q as a mutation (16). In our research, R131Q variation was analyzed in 100 healthy control samples (200 alleles) and detected in two normal individuals. Moreover, in silico modeling programs (*Mutation Taster, PolyPhen2*) were used for this variation and they indicated that this variation has a benign character. As a result of our studies, R131Q seems to be a polymorphism rather than a mutation.

Limitations of this study, however, were as follows; GLP-1R gene's all exons and also introns should have been analyzed and also protein studies were needed. The patient and control groups could also be higher in number. The GLP-1R gene should also have been studied functionally using other molecular biology techniques (27). Moreover, the primers described for our project were not efficient for the whole length of the exons therefore using more than one pair of primers in future designing might be useful. The association between rs6923761 G>A polymorphism and insulin resistance has been studied in some investigations (28-30).

Table 8. The analysis's results about the relationship between the allele frequencies and NAFLD and insulin resistance (Odds Ratios)

Allele Frequencies	NAFLD	Insuline Resistance
rs6918287	X	AG OR=2.00
rs3765468	AA OR=0.314	AG OR=1.870
rs6923761	AG OR=1.729	AG OR=0.626
rs6918287	A OR=1.850	X
rs3765468	X	X
rs6923761	X	A OR=0.659

Altogether, the aim of our study was to investigate genetic variations (mutation/polymorphism) of GLP-1R gene in children diagnosed with obesity and to identify their possible connections with obesity and other conditions. For this purpose, 162 patients and 100 controls were examined and 3 polymorphisms and 1 variation were found. Individuals with homozygous and heterozygous genotypes for polymorphisms/mutations were compared in terms of NAFLD and insulin resistance. According to statistical analysis, it was observed that the existence of polymorphisms and mutation may increase the risk of fatty liver and insulin resistance. For the variable of obesity, however, no significant difference was obtained. Moreover, our study

indicated that R131Q is more probable to be a polymorphism rather than a mutation. GLP-1R's gene consists of 13 exons and due to its expression in a lot of organs; it seems like a logical approach to sequence its whole gene. Therefore, in the contribution of our work, we plan to sequence intronic regions and the entire exons. Besides, a comparison of these data with GLP-1's blood values may be informative. The polymorphism and mutation found in this article can be investigated in different ethnic groups and larger populations. Further consideration of metabolic diseases other than obesity, NAFLD, and T2DM and their connections with GLP-1R is expected to provide great benefits.

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