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Diagnostic and Prognostic Value of miRNA 33 and miRNA122 in Metabolic Syndrome

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ABSTRACT

The metabolic syndrome (MetS) concept gathers in a single entity a set of metabolic abnormalities that have in common a close relationship with ectopic deposit of lipids, insulin resistance, and chronic low-grade inflammation. The aim of present study is to investigate whether patients with metabolic disorder have distinct expression of (miRNA122 and miRNA-33) when compared with different disease groups in addition to control group from the people whose apparent healthy. A case-control study was conducted on the study to identify the expression of (miRNA-122 and miRNA-33) by real time PCR, and the association of its susceptibility to Mets. There was a highly significant variation in the level of miR-33 among study groups ($P< 0.001$) particularly in group of central obesity, hypertension and diabetes group followed by central obesity and hypertensive group then by central obesity group and finally by control group. There was significant variation in level of miR-122 among study groups ($p< 0.001$). The highest level was seen in group of central obesity, hypertension and diabetes groups followed by central obesity and hypertensive group then by central obesity group and finally by control group In conclusion miR-33, along with miR-122 could be used to diagnose / prognostic biomarker with high sensitivity and specificity for metabolic disorders and cardiovascular disease.

KEYWORDS: Metabolic syndrome, microRNA, Diagnostic, Prognostic, Iraq

INTRODUCTION

In several cases, chronically exposures for positively caloric balances consider the driving forces to occurrence and advancement of metabolic syndrome (MetS). The mainly trait for diagnosing of MetS involved atherogenic dyslipidemia, hyperglycemia, central adiposity, and arterial hypertension [1, 2] Many other states concerned to metabolically derangement which does not act as a part in MetS detecting scheme. In MetS, cognitive impairment, atherogenesis, and diabetes (type 2) are the main long-term complications [3, 4, 5]. Biomarkers in metabolic syndrome reach a remarkable observation in last decades due to progressing updates for literatures of molecular scientific at a field. In addition, a number of pro-inflammatory markers play a role in diagnosis of several biomolecular factors in sera or tissue as the microRNAs [6]. Among many different states, miRNA recently recognize as a regulator to metabolism the glucose and lipid through initiation cardiovascular and metabolic diseases [7]. These microRNAs have a role in disorders of lipid metabolites to have greatly and globally public health events. Fatty liver and dyslipidemia are major manifestations of these disorders. Recently, the miR-33 that is a regulator gene included in efflux of cholesterol and fatty acid oxidation, has been considered as a good therapeutic target for these disorders [8] that it is important for cholesterol regulation [9]. Also liver-enriched miR-122 (now identified as miR-122-5p) was the first miRNA to be recognized functionally associated with a metabolic phenotype, and in particularly to regulate cholesterol and lipid metabolism [10, 11]. According to such information, the goal of current study to investigate whether the patients of metabolic disorder were having distinct expression of miRNA122 and miRNA-33 using qPCR technique.

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MATERIALS AND METHODS

Patients and Control: A case-control study was conducted on the following study groups during the period from November 2019 to September 2020. The patients were grouped to 3 categories (first: obese patients group; second: obese and hypertension patients group; and the third: obese, hypertension and diabetes mellitus patients group. each of these group include (30) patients mixed of males and females with age range 18-66 years old. This study was carried out at Al-Diwaniyah Teaching Hospital. The patients were diagnosed clinically by physician as having at least three of the five metabolic disorder criteria factors to be diagnosed with metabolic syndrome. Direct interview was performed with the patients to report main data related to age, sex, family size, residence, family history of obese, hypertension, diabetes, heart disease and others. In addition, the apparently healthy individuals represented as control group. The study performed under the regulation and according to ethics of Al-Diwaniyah Teaching Hospital.

Collection of blood samples: 5 ml of venous blood was drained form each participant into K3-EDTA anticoagulated tube and stored at -20°C for RNA will extract, and the data were obtained along with clinical parameters.

Total RNA were extracted from whole blood samples by using (TRIzol® reagent kit) and done according to company instructions. The Primers of miRNA33 and miRNA122 were designed based on NCBI data, and provided by the company of Macrogen / Korea (Table 1).

Table (1): Primers applied for detection of miRNA33 and miRNA122

Primer		Sequence
RT primer (specific) hsa-miR-33		GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGACTGCAAT
miR-33 qPCR primer	F	AACACGCGTGCATTGTAGTT
	R	GTCGTATCCAGTGCAGGGT
RT primer (specific) hsa-miR-122		GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGACCAAACA
miR-122 qPCR primer	F	AACCGGTGGAGTGTGACAAT
	R	GTCGTATCCAGTGCAGGGT
GAPDH qPCR primer	F	TCAGCCGCATCTTCTTTTGC
	R	TTAAAAGCAGCCCTGGTGAC

The Nanodrop (Thermoscientific, USA) was used to check the concentration and purity of extracted RNA.

DNase I Treatment: Extracted RNAs were treated with DNase I enzyme according to manufacturer instruction (Promega company, USA). The cDNA synthesis: According to manufacturer instructions, cDNAs of miRNA33 and miRNA122 as well as GAPDH gene were synthesized in Thermocycler under specific conditions (Figure 1). Mastermix preparation: The qPCR mastermix was prepared following the instructions of RealMODTM Green SF 2X qPCR kit uses the SYBER Green dye. Thermocycler was performed following the designed protocol. Data analysis: All qPCR data were analyzed using ΔCT Method [12].

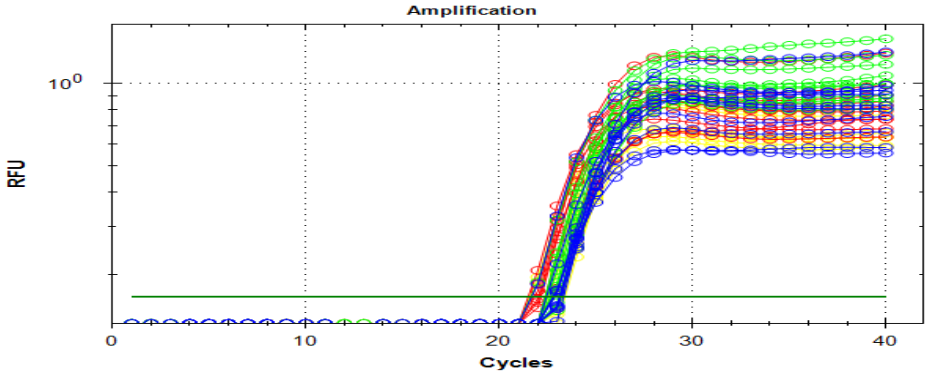

Figure 1: The qPCR amplification plots for GAPDH that showed the no variations for qPCR threshold cycle number between groups.

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Where red plots (group central obesity patients), the blue plots (group central obesity and hypertension patients), the green plots (group central obesity and hypertension and DM patients), and the yellow plots (group healthy control).

RESULTS AND DISCUSION

The comparison of miR-33 and miR-122 among study groups (Table 2, Figures 2, 3, 4, 5), a highly significant variation in the level of miR-33 were showed among study groups ($p< 0.001$). The highest level was seen in a group of central obesity; hypertension and diabetes groups followed by central obesity and hypertensive group then by central obesity group and finally by control group (Figure 2).

There was also highly significant variation in the level of miR-122 among study groups ($p< 0.001$); the highest level was seen in group of central obesity, hypertension and diabetes groups followed by central obesity and hypertensive group then by central obesity group and finally by control group (Figure 3).

Table 2: Comparison of miR-33 and miR-122 among study groups

Characteristic	Control *n* = 48	Central obesity *n* = 30	Central obesity and hypertension *n* = 30	Central obesity, hypertension and diabetes *n* = 30	*p*
miR-33					
Median (IQR)	1.00 (0.79) D	22.67 (19.80) C	25.99 (15.54) B	30.42 (8.95) A	<0.001 K HS
Range	0.26 -4.67	2.69 -91.93	7.22 -151.27	21.95 -132.87	
miR-122					
Median (IQR)	0.98 (1.04) D	24.60 (21.26) C	38.90 (22.45) B	46.45 (14.80) A	<0.001 K HS
Range	0.18 -6.12	4.73 -233.07	15.03 -75.25	24.38 -72.88	

n: number of cases; K: Kruskal Wallis test; HS: highly significant at $p \leq 0.01$; Capital letters A, B, C and D were used to indicate level of significance following Mann Whitney U test between every two groups.

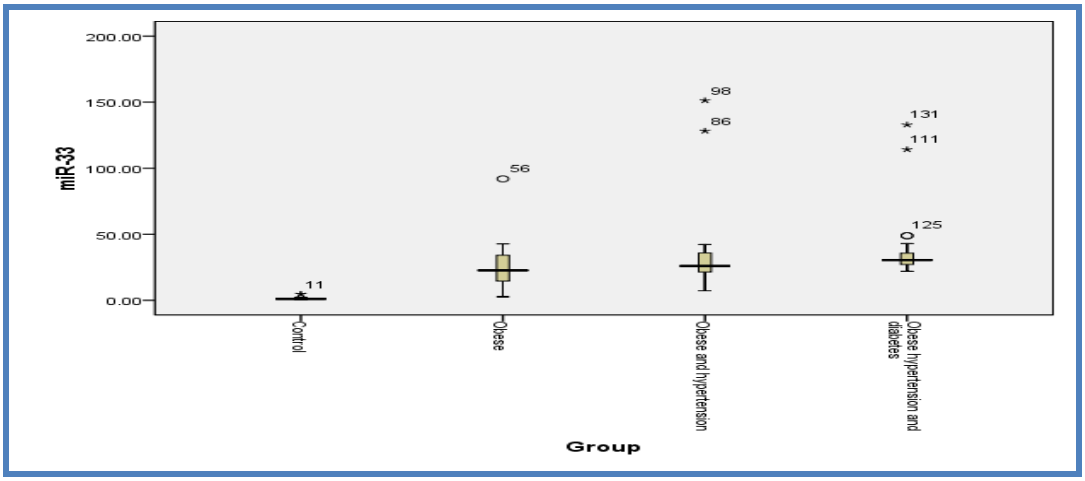

Figure 2: Box plot showing comparison of miR-33 expression level among study groups

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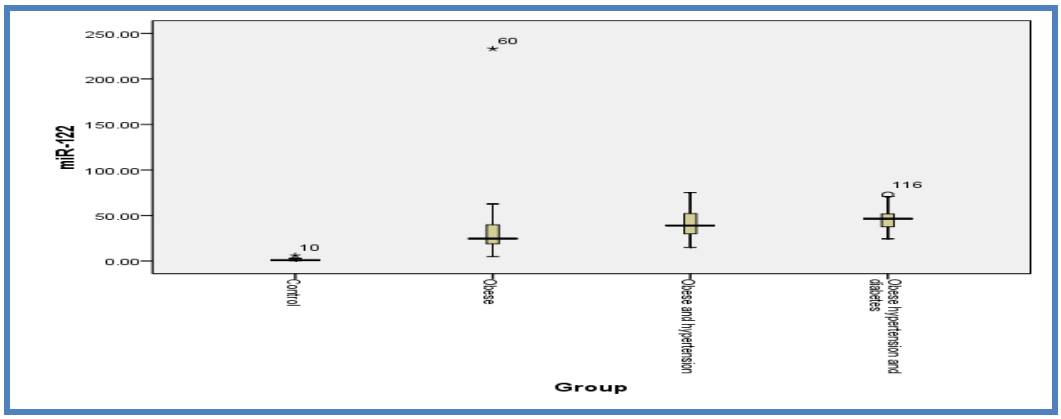

Figure 3: Box plot showing comparison of miR-122 expression level among study groups

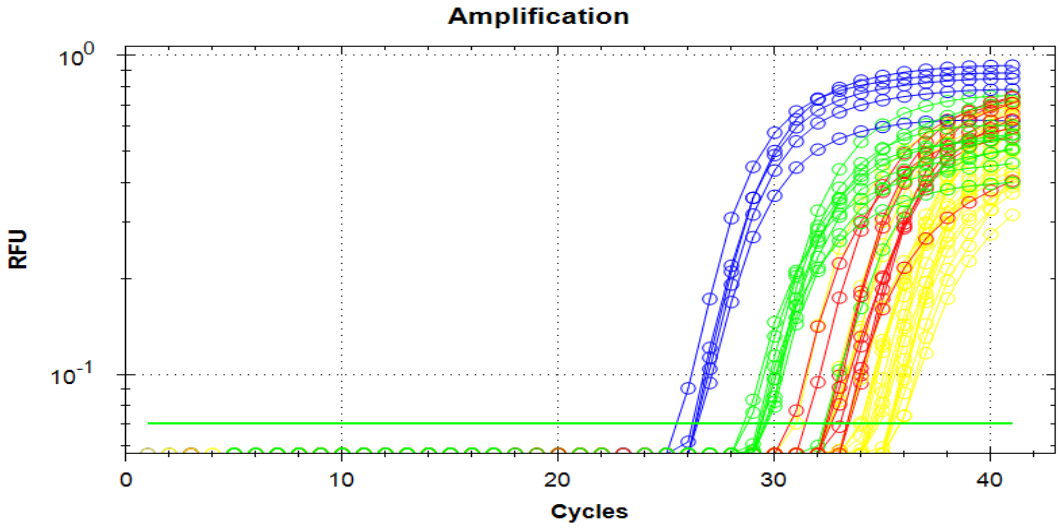

Figure (4): The qPCR amplification plots for miRNA-33 that showed the differences in qPCR threshold cycle numbers between groups.

Where, the red plots (group central obesity patients), the blue plots (group central obesity and hypertension patients), the green plots (group central obesity and hypertension patients and DM patients), and the yellow plots (group of healthy control).

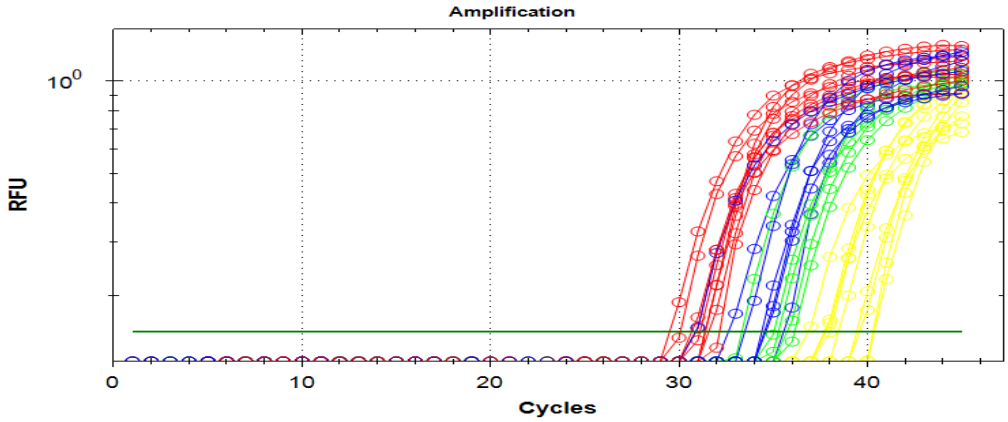

Figure 5: The qPCR amplification plots for miRNA-122 that showed the differences in qPCR threshold cycle numbers between groups.

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Where, the red plots (group central obesity patients), the blue plots (group central obesity and hypertension patients), the green plots (group central obesity and hypertension patients and DM patients patients), and the yellow plots (group healthy control). Growing evidences suggested faulty regulation of lipid metabolism which promoting to metabolic diseases. Our findings revealed gradual increases in miRNA expression (33, 122) in conjunction with the development of the stage of metabolic disorder. This difference in levels of gene expression from the normal level in the control group has indications that may be predictive or may be diagnostic indications. However, here it is necessary not to confuse the role of miRNA in regulatory function or as a biomarker, but with its increase, consider a sign of importance value must explain. The circulating miRNA in mammalians packaged inside lipid or lipoprotein complexes [13, 14]. Researchers showed an association with protein [15] makes miRNAs resistance for the activity of ribonuclease. miRNAs secret insides exosome might play different fates in comparison to this related with protein or HDL [16, 17]. Some miRNAs tend preferentially to excreted through tissue to be more prone to exist in circulation [18]. However, variation in concentrations of miRNA is not necessarily reflecting deregulation in expression of miRNAs inside a cell. Hence, miRNAs using as a biomarker must not confound with regulatory functions inside cell; though recent evidences reported that extracellular miRNAs found in bodily fluids having distinctly and physiologically functions [19, 20, 21]. Mainly functions and existence of miRNAs are still elusive. The miRNAs roles in obesity and diabetes, recently studied. Ortega et al. [22] established the foundation to motivate further global investigations of miRNAs as circulating biomarkers for obesity in humans.

The over expressed of miR-33a detected in this study may be due to increasing SREBP1, SREBP2 and FASN expression. SREBP1 or SREBP2 lowered fatty acid level resulting in decreasing the expression of SREBP genes need to biosynthesis of lipid [23]. Hence, TG can be affected by miR-33a through SREBPs as well as FASN and this was in agreement with the result of other studies [24, 25, 26]. MiR-33a, regulator key of lipid metabolism encoded within SREBP2, reported to have therapeutic role in cardiovascular diseases [26]. Goedeke et al. reported that miR-33 play a role in metabolism of fatty acid [27]. We reported novel roles to miR-33a in metabolism of lipid and accumulation of TG in liver. Clinical elevation of serum miR-33a considers dangerous agent in dyslipidemi. Due to this, miR-33a can be served as marker in correction of metabolic disorder and we can considered that Mets specific miRNA, and its function is decrease cholesterol transport/export. miR-33a locates in the 16^{th} intron of SREBP-2 and SREBP-1 can regulate the gene that controlling the uptakes and synthesis of cholesterol [28, 29]. SREBF can activate expression more than 30 genes that synthesizing or up taking fatty acids, phospholipids, cholesterol, and triglycerides along with the NADPH cofactor need for synthesizing these molecules [30]. ATP-binding cassette (ABC) A1 and ABCG1 were well studied as cholesterol transporters in different types of cells [31, 32]. However, the decreasing of ABCA1 enhances the intracellular cholesterol excesses in macrophage leading for inflammatory processes with apoptosis of cells that eventually causing atherosclerosis [33]. As well as, SREBF signaling pathways regulating different processes in cells such as cell cycle progression and phagocytosis [34]. Many reports confirmed the role of cholesterol in apoptosis, and the role of SREBP/miR-33 in cycle progression and growing of cells [35, 36]. Hence, abnormalities in miR-33a can affect level of cholesterol and contribute in tumorigenesis [37]. In mice, short-terms anti-miR-33 causes a significant reduction in atherosclerosis; while, long-terms cause a significant increase in circulating TG [38], and up regulating expression of miR-33 in liver [39]. Also, other studies reported regulatory roles of lipid metabolism in transporting as well as in insulin signaling pathways [7, 40, 41]. Additionally, inhibition of miR-33 confirmed to attenuate the progression of atherosclerosis [7, 41]. Recently study in mice was demonstrated the role of miR-33 in obesity exhibition, insulin resistance and increasing intake of food suggest the complex role of miR-33 [43]. Therefore, we can consider miRNA-33 as of a prognostic value for metabolic syndrome, and according to that, the previously mentioned data could explain the significant association between miRNA-33 and metabolic syndrome in the present study. Thus, miRNA33 can consider as a prognostic indication for metabolic disease, and miRNAs may differ significantly among diseased and control patients. In previously reports, studies have shown that miR-122 is unregulated due to metabolic disorders in liver as it included in lipid and cholesterol regulation [44, 10].

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Elevation of miR-12 can be related positively with the stage of metabolic syndrome suggesting the miRNAs role in insulin sensitivity and adiposity [45]. Ortega et al. [22] reported that the level of miR-122 can increase in serum of patients who having a moderate obesity; however, there is decreasing in morbidity of obese patients. In contrast, other study confirmed that the miR-122 can elevate robustly due to obesity exhibiting the tendency for increasing the severity of condition. Additionally, our findings showed direct relationship between the concentration of serum miR-122 and Mets developed in adult patients, which disagreement with that reported by Ortega *et al*. [22]. This disparity could be related partially to differences in definition of obesity in comparison to the normal glucose metabolic condition. In previously detected findings, it confirmed that miR-122 is necessary in many actions like cholesterol, triglyceride, and fatty acid metabolism as well as in terminal stage of target regulation in hepatocytes [44, 46, 47, 48, 49]. Besides, miR-122 can standout because its involvement in CVD due to its ability for regulating genes of lipid metabolism, considering that, the Mets are specific to target genes (HMGCR, ALDO, G6PC, AMPKα1), and functions (↑Cholesterol synthesis). Nevertheless, experimentally performed studies, the findings revealed that vascular complication is low, and that. miR-122 concentration was increased in patients having acute coronary syndrome and hyperlipidemia [50, 51]. In adition, the role of statins in decreasing the concentration of miR-122 was explained [52]. Negative relationship between miR-122 and cardiovascular outcomes was reported [53, 54]. As well as, evaluation of miR-122-5p can act as an indicator for adverting the metabolic health status independently to obesity [55]. The miR-122 can express initially in liver and appear the effect on metabolism of hepatic fatty-acid and cholesterol [44, 56]. Subsequently, it showed that miR-122 targeted by antisense inhibitor can result in reducing the plasma cholesterol as it act in a compensatory mechanism for reducing the level of hepatic lipid and LDL levels but not HDL [11, 46]. The findings of present study provided the evidences that confirmed the circulating miRNAs in patients with metabolic disorders. A part from detecting the involvement of aberrant circulating miR-33/122 under a metabolic disorders, we showed that miR-33 andmiR-122 microRNAs can act as crucial regulators for cholesterol/lipids; and may consider as therapeutic targets for treating of atherosclerosis and glucose regulation. Most importantly, other studies demonstrated that miR-122 was positively associated with increased odds of insulin resistance and developing into DM in humans, indicating its potential diagnostic value. present findings suggest that circulating miR-33 and miR-122 may act as a promising biomarker of obesity ,HP and DM. In addition, there can be conflicting observations regarding changes in miRNA levels. These disparities can be attributed to differences in sample size, time of sampling, miRNA quantification methods, and miRNA normalization parameters. In order to calculate the cutoff values of miR-33 and miR-122 for the Metabolic syndrome as diagnostic tests or tests, ROC curve analysis was carried out and the results were showed (Table 3, Figures 3 and 4).

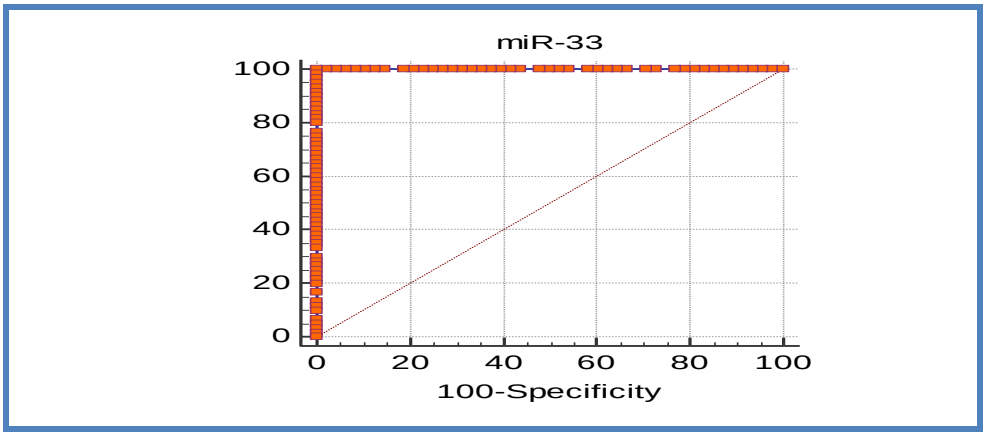

Figure 3: Receiver operator characteristic curve to find the best miR-33 cutoff value that can predict diagnosis of metabolic syndrome

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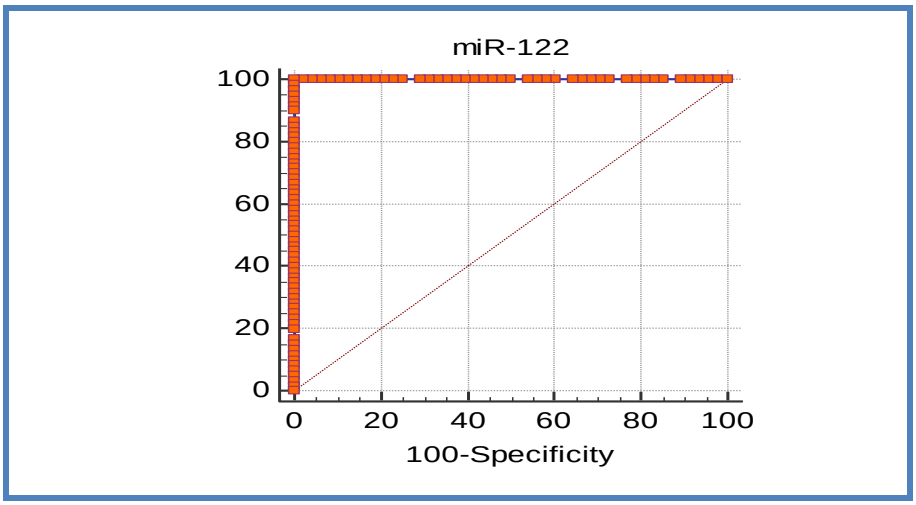

Figure 4: Receiver operator characteristic curve to find the best miR-122 cutoff value that can predict diagnosis of metabolic syndrome

The diagnostic role of miR-33, miR-122 in metabolic syndrome was showed (Figures 3, 4; Table 2). The cutoff value of miR-33 was >4.67 fold change with 100 % sensitivity, 100 % specificity and 100 % accuracy level. The cutoff value of miR-122 was >6.12 fold change with 100 % sensitivity, 100 % specificity and 100 % accuracy level. MiR-33, along with miR-122, could be used to diagnose/prognostic biomarker with high sensitivity and specificity for metabolic disorders and cardiovascular disease this may be due to it's tissue specific, this in agreement with [57]. Therefore, in present results, we found that the circulating miRNAs might be used as a suitable biomarker for the detection, prognostic and fallow up of many metabolic disorders like hypertension, obesity and that associated with abnormal glucose metabolism, including DM and its secondary complications [58].

Table 3: Sensitivity and specificity of miR-33 and miR-122 level in metabolic syndrome patients

Characteristic	miR-33	miR-122
Cutoff	>4.67	>6.12
AUC (95 % CI)	1.000 (0.966 to 1.000)	1.000 (0.966 to 1.000)
Accuracy %	100	100
P-value	< 0.001 HS	< 0.001 HS
Sensitivity %	100	100
Specificity %	100	100

AUC: area under the curve; CI: confidence interval; HS: highly significant at $p \leq 0.01$

This reinforces the concept that increased circulating miR-122 might flag a problem with liver metabolisms and require further laboratory and clinical analyses going beyond the normal routine. One of the advantages of using these RNAs as diagnostic indicators for metabolic diseases is because they are fast, sensitive, compared to traditional methods that require time and effort to link all the criteria for metabolic disorder.

CONCLUSION

The Comparison of miR-33 and miR-122 among study groups the present study show highly significant variation in the level of miR-33 and miR-122 among study groups ($p < 0.001$). The levels of miR-33 and miR-122 were expressed significantly in serum samples from Mets patients with and without treatment compared with control subjects. We successfully combined these microRNAs using ROC analysis, which allowed for

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more specific and sensitive discrimination. In addition, ROC analysis supported good diagnosis accuracy of these miRNAs. The sensitivity, specificity of miR-33, miR-122 and may be conceders as 100 %, 100 %, for both. Therefore using both of these two miRNA as diagnostic and prognostic biomarker gives more accuracy and precise results, more than single one.

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