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Long noncoding RNAs APOA1-AS, IFNG-AS1, RMRP and their related biomolecules in Egyptian patients with relapsing-remitting multiple sclerosis: Relation to disease activity and patient disability

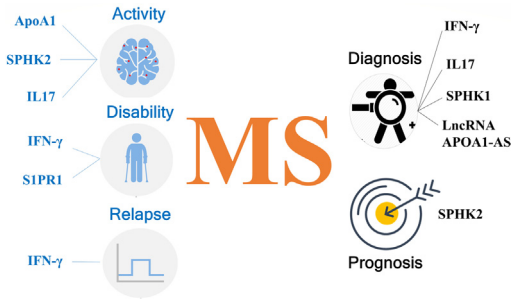
Heba R. Ghaiad [a,*], Alaa N. Elmazny [b], Mohammed M. Nooh [a], Maha M. El-Sawalhi [a], Amira A. Shaheen [a]

[a] Biochemistry Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt
[b] Neurology Department, Faculty of Medicine, Cairo University, Cairo, Egypt

HIGHLIGHTS

- LncRNA APOA1-AS and IFNG-AS1 expression is upregulated in RRMS patients.
- ApoA1 levels, SPHK2 expression, and IL17 levels are higher during MS relapses.
- S1PR1 expression and IFN-γ levels were linked to EDSS.
- Only IFN-γ levels was associated with relapse rate in RRMS.
- An excellent diagnostic power for IFN-γ, IL17, SPHK1 and APOA1-AS was found.

GRAPHICAL ABSTRACT



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ABSTRACT

Lately, long noncoding (lnc) RNAs are increasingly appreciated for their involvement in multiple sclerosis (MS). In inflammation and autoimmunity, a role of apoprotein A1 (ApoA1), mediated by sphingosine 1-phosphate receptors (S1PRs), was reported. However, the epigenetic mechanisms regulating these biomolecules and their role in MS remains elusive. This case control study investigated the role of ApoA1, sphingosine kinase 1 and 2 (SPHK1 & 2), S1PR1 & 5, interferon-γ (IFN-γ) and interleukin 17 (IL17) in MS, beside three lncRNA: APOA1-AS, IFNG-AS1, and RMRP. Expression of SPHKs, S1PRs, and lncRNAs were measured in 72 relapsing-remitting MS patients (37 during relapse and 35 in remission) and 28 controls. Plasma levels of ApoA1, IFN-γ and IL17 were determined. The impact of these parameters on MS activity, relapse rate and patient disability was assessed. APOA1-AS, IFNG-AS1, SPHK1 & 2, and S1PR5 were upregulated in RRMS patients. Differences in ApoA1, SPHK2, and IL17 were observed between relapse and remission. Importantly, ApoA1, SPHK2, and IL17 were related to activity, while S1PR1 and IFN-γ were linked to disability, though, only IFN-γ was associated with relapse rate. Finally, an excellent diagnostic power of IFN-γ, IL17, SPHK1 and APOA1-AS was demonstrated, whereas SPHK2 showed promising prognostic power in predicting relapses.

Introduction

Multiple sclerosis (MS) is a complex autoimmune disease driven mainly by self-reactive T helper (Th) cells with characteristic foci of inflammation and demyelination in brain, spinal cord and

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* Corresponding author at: Biochemistry Department, Faculty of Pharmacy, Cairo University, Kasr el Ainy st., 11562 Cairo, Egypt.
E-mail address: Heba.r.ghaiad@gmail.com (H.R. Ghaiad).

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optic nerve [1]. In most cases, MS follows a relapsing-remitting course (RRMS) with subacute episodes of neurological symptoms followed by recovery. By time, some patients with RRMS shift into secondary progressive form of the disease [1]. To date, biochemical markers that might provide objective criteria to confirm or rule out diagnosis of MS are lacking, making MS a difficult disease to diagnose and treat. Moreover, the clinical progression of MS is highly variable, and predicting prognosis is quite challenging. Hence, enhanced understanding of MS pathogenesis and progression would aid in the early detection and the optimal disease management [2].

Although brain is one of the most lipid-rich organs in the body, the derangements of lipid metabolism in MS have not yet been properly investigated [3]. Based on the chronic inflammatory character of MS and the anti-inflammatory role of high-density lipoproteins (HDLs), it remains necessary to know how lipoproteins metabolism influences MS activity and progression [4]. Thus, a better understanding of HDL in MS may help to explain the variability of MS course and may unveil new therapeutic targets tailored specifically for MS patients [4]. Apoprotein A1 (ApoA1), a major protein component of HDL, is a constitutive anti-inflammatory molecule in many biological processes [5], where it is believed to block interactions of macrophages with T-cells, resulting in reduction of Th1 and Th17 cytokines [6]. The main proinflammatory T-cell populations associated with MS, are the Th1 that secretes interferon-gamma (IFN-γ) and tumor necrosis factor α (TNF- α) in addition to the Th17 that secretes interleukin (IL)17, IL21, and IL22 [7]. ApoA1 also plays a pivotal role in healing and neuronal regeneration [8]. Therefore, studying the control of ApoA1 expression during periods of inflammation could provide important information about the mechanisms of HDL regulation and its role in MS pathogenesis.

Lately, long noncoding (lnc) RNAs are increasingly appreciated for their involvement in epigenetic regulation of MS pathogenesis [9]. An endogenously expressed antisense lncRNA molecule, APOA1-AS, was identified as a negative transcriptional regulator of ApoA1 both *in vivo* and *in vitro* [10]. Thus, studying ApoA1 and APOA1-AS might provide valuable insights into the role of HDL in MS activity and progression. Two other lncRNAs were also addressed in this study, the first being lncRNA IFNG-AS1, that is specifically expressed by the Th1 subset and is necessary for active transcription and expression of IFN-γ in Th1 cells [11]. Another cell-intrinsic cue contributing to production of Th17 cytokines during both homeostasis and inflammation is lncRNA RMRP. RMRP is the RNA component of the mitochondrial RNA-processing endoribonuclease (RNase MRP), which is essential for regulating a subset of critical genes implicated specifically in the Th17 effector program [12].

Another point of interest is sphingosine 1-phosphate (S1P), a bioactive lysophospholipid constituting an active part of HDL composition. S1P is generated in the central nervous system (CNS) by the phosphorylation of sphingosine by sphingosine kinase 1 and 2 (SPHK1 and 2) [13]. Upon release, S1P activates a family of G protein-coupled receptors (S1PR1-5) to induce different cell responses [14]. The "inside-out" signaling by S1P plays a role in many inflammatory and autoimmune disorders including MS. Increased S1P levels were observed in cerebrospinal fluid (CSF) of RRMS patients during relapses and increased CSF levels of S1P was associated with disability in MS patients [15].

In this context, the aim of this study was designed to investigate the epigenetic machinery regulating ApoA1, IFN-γ and IL17 levels by measuring the expression levels of three related lncRNAs; APOA1-AS, IFNG-AS1, and RMRP. Additionally, gene expression of SPHK1 & 2 and S1PR1 & 5 was measured. Furthermore, the impact of these parameters on MS activity, future progression and patient disability was also evaluated. Finally, the potential use of these biomarkers as novel non-invasive diagnostic and prognostic tools for MS was also assessed.

Subjects and methods

Participants

This study included 100 participants, 28 healthy controls and 72 MS patients recruited from Kasr Al-Ainy Multiple Sclerosis Research Unit (KAMSU) at Cairo University Hospitals, Egypt, between October 2017 and March 2018. A confirmed clinical diagnosis was performed by a neurologist according to the 2010 revision of the McDonald criteria [16]. In this study, 37 RRMS patients were in relapse (acute or worsening of a neurologic deficit that lasts at least 24 h and separated from a previous attack by at least 30 days in absence of fever and infection) [16]. Patients with relapses were assessed within 7 days from onset and samples were obtained before methylprednisolone therapy. Whereas, 35 RRMS patients were in clinical remission (relapse-free for at least 90 days before sample collection) [16]. Exclusionary criteria included: pregnancy, current or recent inflammatory or infectious diseases, familial hypercholesterolemia, lipid-lowering drugs or steroid intake one month prior to enrollment. As regards the controls, 28 age and sex-matched healthy individuals volunteered to participate in the study without any diagnostic criteria of MS and free of neurological and autoimmune diseases. All participants gave written informed consent. The study protocol was approved by the Research Ethics Committee for experimental and clinical studies at the Faculty of Pharmacy, Cairo University, Cairo, Egypt (approval number: BC 1956) and conformed to the 1975 Helsinki declaration, revised in 2008.

Prediction of future progression in MS course can be inspected in terms of increased annualized relapse rate (ARR) [17]. ARR is the number of confirmed relapses experienced by the patient in one year, herein ARR in the past 2 years was calculated for each patient [18]. Meanwhile, the Expanded Disability Status Scale (EDSS) was used to evaluate neurological disability and assess clinical severity [19]. Accordingly, both relapse and remission groups were sub-divided according to their:

1. ARR, into two groups: low ARR group (<1) and high ARR group ($\geq$1).
2. EDSS score, into ambulatory patients (EDSS < 6) and assisted or non-ambulatory group (EDSS $\geq$ 6).

Sample collection and biochemical measurements

Whole venous blood samples were collected into vacuette collection tubes (Greiner Bio-One, Frickenhausen, Germany) containing ethylene diamine tetraacetic acid (EDTA). The samples were centrifuged at 3000*g* for 15 min and the buffy coats were collected and instantly used for total RNA extraction. The separated plasma was aliquoted and stored at −20 °C.

Plasma IL17 and IFN-γ were measured by Quantikine HS ELISA (R&D Systems, Minneapolis, USA) and expressed as picograms per milliliter. Postprandial plasma levels of ApoA1 were measured using commercial sandwich ELISA kit (NOVA, Beijing, China) and expressed as nanogram per milliliter, whereas total cholesterol and triglycerides were measured by enzymatic spectrophotometric methods, while HDL-cholesterol was determined by precipitant method using commercially available kits. Finally, low-density lipoprotein (LDL)-cholesterol concentrations were estimated by Friedewald's formula [20].

Total RNA isolation and qRT-PCR

Long noncoding RNA expression levels of APOA1-AS, IFNG-AS1, and RMRP together with gene expression levels of SPHK1, SPHK2, S1PR1, and S1PR5, were assessed using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

Total RNA was extracted from the buffy coats using TRIzol Plus RNA Purification Kit (Invitrogen Life Technologies, Carlsbad, USA). The RNA concentration and quality were assessed using Q5000 UV–Vis Spectrophotometer nanodrop (Quawell, San Jose, USA).

Complementary DNA (cDNA) was synthesized from total RNA recovered on the same day using Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany). Gene expression was measured using StepOne Real-Time PCR System (Thermo Fisher Scientific, San Jose, USA) and Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, San Jose, USA). The primers used were pre-designed using NCBI primer Blast and verified by in-silico PCR tool of the University of California, Santa Cruz (UCSC) genome browser, and eventually custom-made by Invitrogen (Carlsbad, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping reference gene. The primers were listed in Table 1. The cycle threshold (C_T) values were normalized using GAPDH as endogenous control, then they were represented relative to the healthy control values, where the changes in target expression were calculated using $\Delta\Delta C_T$ method and presented as fold change ($FC = 2^{-\Delta\Delta CT}$).

Statistical analysis

All measured parameters were subjected to normality testing using Shapiro–Wilk normality test. Experimental data were depicted as mean and standard error of the mean (SEM) or median, interquartile range (IQR) and range whenever appropriate. Categorical data were represented by frequency and percentage. Continuous, normally distributed datasets were analyzed for significance using unpaired Student's two-tailed t-tests and ANOVA with Tukey's post hoc test; for non-parametric data, Mann Whitney or Kruskal Wallis test with Dunn's post hoc test were used. Categorical data were compared by Chi-square test or Fischer exact test. For receiver operating characteristic (ROC) analysis, area under the curve (AUC), Youden's index and optimal cut-off values were calculated. MS group was compared to healthy controls to assess the diagnostic power of our parameters, whereas relapse values were compared to remission values as the reference standard to assess the prognostic power of measured parameters. In all cases, probabilities of less than 0.05 ($p < 0.05$) were considered statistically significant, with a 95% confidence interval. All data were statistically analyzed using GraphPad Prism 7.0 (GraphPad Software).

Table 1
Primer sequences used in qRT-PCR.

Gene	Primer Sequence
APOA1-AS	Forward 5′ ATG CTG GTC ACT TCA GTC CC 3′
	Reverse 5′ AGG GGA TTG GTT ATG AGG CT 3′
IFNG-AS1	Forward 5′ ACA GAA CCA TCA GAC CGC AG 3′
	Reverse 5′ CTC ACT TTG GCT GCC TGG TA 3′
RMRP	Forward 5′ GTC CGC CAA GAA GCG TAT CC 3′
	Reverse 5′ CAC TGC CTG CGT AAC TAG AGG 3′
SPHK1	Forward 5′ ATC TAA CTC GAG GTG CTC GC 3′
	Reverse 5′ AGT AGG GAC GCG TTT GTC AG 3′
SPHK2	Forward 5′ GGC CTT TGT TAC GCG TGT TAG 3′
	Reverse 5′ TGG GCC TGT CTC ATC CAT TG 3′
S1PR1	Forward 5′ GGG AGC AAT AAC TTC CGC CT 3′
	Reverse 5′ AAG ACC GTG GTG CAG AAG AG 3′
S1PR5	Forward 5′ CCA CCT TCA CCC CGT ATC C 3′
	Reverse 5′ GCA GGG AGT TCT CCG AAC TTT T 3′
GAPDH	Forward 5′ ACC TTG TGT CCC TCA ATA TGG T 3′
	Reverse 5′ GTA CTC AGC GCC AGC ATC G 3′

APOA1-AS: Apoprotein A1 antisense transcript; IFNG-AS1: Interferon-γ antisense transcript 1; RMRP: RNA component of mitochondria RNA processing endoribonuclease; SPHK1: Sphingosine Kinase 1; SPHK2: Sphingosine Kinase 2; S1PR1: Sphingosine 1-phosphate receptor 1; S1PR5: Sphingosine 1-phosphate receptor 5; GAPDH: Glyceraldehyde3-phosphate dehydrogenase.

Results

The demographics and clinical characteristics of the patients and healthy controls are summarized in table 2.

Apoprotein A1 and APOA1-AS in MS and their relation to disease activity, relapse rate, and patient disability

As shown in Fig. 1A, RRMS patients showed significantly lower levels of plasma ApoA1 and HDL-cholesterol along with higher LDL-cholesterol compared to control values (Fig. 1A). Whereas, their APOA1-AS expression was dramatically upregulated reaching six fold of healthy control values (Fig. 1B).

On considering disease activity, plasma ApoA1 levels were significantly lower in patients during active relapses than in remission, while its plasma level in remission was comparable to that of healthy control values (Fig. 2A). Meanwhile, plasma HDL-cholesterol was significantly lower in patients during relapses and in remission than control values. However, plasma LDL-cholesterol levels were significantly higher in both relapse and remission groups compared to controls (Fig. 2A). APOA1-AS was significantly upregulated in patients during relapse and in remission when compared to healthy controls (Fig. 2B).

By stratifying both relapse and remission groups according to ARR, only ApoA1 levels were significantly lower in patients during relapse with high ARR (≥1) compared to those in remission with high ARR (≥1) (Table 3). Again, based on EDSS as a measure of disability, ApoA1 levels were significantly lower in ambulatory patients (EDSS < 6) particularly during relapse than those with the same disability level in remission (Table 4).

Interferon-γ and interleukin 17 in MS and their relation to disease activity, relapse rate, and patient disability

In MS patients, both plasma IFN-γ levels and IFNG-AS1 expression were markedly increased reaching about 3.5 and 5 fold of the control values (Fig. 1A and B). Likewise, plasma IL17 concentrations in patients were nearly 2.5 fold of healthy control levels, however, RMRP expression showed insignificant increase (Fig. 1A and B).

Regarding their impact on disease activity, plasma IFN-γ and IL17, as well as the expression of IFNG-AS1, were significantly higher in patients during relapse and in remission than controls. However, only plasma levels of IL17 were significantly higher in patients during relapse than those in remission (Fig. 2A and B).

Based on ARR values, patients with high ARR (≥1) showed lower IFN-γ levels than those with low ARR (<1). At low ARR

Table 2
Demographics and clinical characteristics of study populations.

		Controls	Relapse	Remission	*P value*
		n = 28	n = 37	n = 35	
Age (y)					
	Range	28–44	20–52	23–48	0.0655
	Median (IQR)	39 (6)	33.8 (14.3)	35.5 (13.3)	
Sex; female/male, n (ratio)		19/9 (2.1)	24/13 (1.9)	27/8 (3.4)	0.4335
Consanguinity; n (%)		4 (14)	4 (10.8)	7 (20)	0.547
Age at onset (y)					
	Range	–	14–43	15–47	0.5937
	Median (IQR)	–	25.5 (15.8)	25 (13)	
Onset; n (%)		–			
	EOMS	–	6 (16)	1 (3)	0.0558
	AOMS	–	31 (84)	34 (97)	
Symptoms at onset; n, (%)		–			
	Sensory	–	8 (22)	6 (17)	0.4795
	Motor	–	15 (40)	14 (40)	
	Visual	–	10 (27)	8 (23)	
	Brain stem	–	4 (11)	4 (11)	
	Cerebellar	–	0 (0)	3 (9)	
EDSS					
	Range	–	1–7.5	0.5–6.5	0.3297
	Median (IQR)	–	2.5 (3.3)	3 (3.3)	
Illness Duration (y)					
	Range	–	0.5–17	1–15	0.0319
	Median (IQR)	–	3.5 (5)	6 (8.5)	
Relapses in last 2 years					
	Range	–	0–8	0–8	0.7437
	Median (IQR)	–	2 (2)	2 (2)	
ARR in last 2 years					
	Range	–	0 – 4	0 – 4	0.7172
	Median (IQR)	–	0.5 (1)	0.5 (1)	
Treatment; n (%)					
	Interferon-β	–	22 (59.5)	19 (54.3)	0.7717
	Fingolimod	–	3 (8.1)	2 (5.7)	
	Azathioprine	–	12 (32.4)	14 (40)	

n: number; y: year; IQR: Interquartile range; EOMS: Early-onset multiple sclerosis; AOMS: Adult-onset multiple sclerosis.

(<1), patients during relapse showed significantly higher IFN-γ levels than those in remission, whereas, at high ARR (≥1), patients in remission had lower plasma IL17 levels than those during relapse (Table 3). According to EDSS scores, assisted or non-ambulatory patients (EDSS ≥ 6) during relapse and in remission showed significantly lower IFN-γ concentrations than ambulatory (EDSS < 6) patients. On the other hand, plasma IL17 levels were significantly higher in patients with low and high EDSS during relapse than the corresponding patients in remission (Table 4).

Sphingosine kinases and sphingosine 1-phosphate receptors in MS and their relation to disease activity, relapse rate, and patient disability

RRMS patients showed a dramatic upregulation of SPHK1 and SPHK2 expressions reaching about 26 and 7 fold of healthy control values respectively. Moreover, patients showed a 2-fold increase in S1PR5 expression compared to control levels, without any significant change in S1PR1 expression (Fig. 1C).

In terms of disease activity, SPHK1 & 2, as well as S1PR5 expressions, were significantly upregulated in both relapse and remission groups compared to control values. Only patients during relapse showed significantly higher expression of S1PR1 compared to healthy controls. Noticeably, SPHK2 expression was significantly lower in patients during relapse than those in remission (Fig. 2C).

ARR stratification showed that patients during relapse with low ARR (<1) had significantly downregulated expression of SPHK1, S1PR1 and 5, compared to corresponding patients in remission (Table 3). EDSS stratification showed a significantly lower expression level of SPHK2 and S1PR1 in ambulatory patients (EDSS < 6) during relapse, compared to ambulatory patients (EDSS < 6) in remission. In relapse group, the assisted or non-ambulatory patients (EDSS ≥ 6) had significantly higher S1PR1 expression than ambulatory patients (EDSS < 6) (Table 4).

Diagnostic potential of the studied parameters

ROC curve analysis showed a rather good diagnostic performance for APOA1-AS followed by ApoA1; where the AUCs were 0.82 and 0.74 respectively. Interestingly, the diagnostic power of SPHK1 was superior to SPHK2, S1PR1, and S1PR5, with an AUC value of 0.89. An excellent diagnostic performance was noticed for IFN-γ (AUC = 0.97) and IL17 (AUC = 0.91) (Fig. 3 and Table 5).

Prognostic potential of the studied parameters

Both ApoA1 and APOA1-AS were comparable in their prognostic performance to discriminate RRMS patients during active relapses from those in remission; ApoA1 had an AUC of 0.71 and APOA1-AS had an AUC of 0.71. Similarly, both IFNG-AS1 and IL17 exhibited good discriminative powers with AUC of 0.75 for IFNG-AS1 and AUC of 0.72 for IL17. However, the best prognostic potential was given by SPHK2 with an AUC value of 0.83 (Fig. 3 and Table 5).

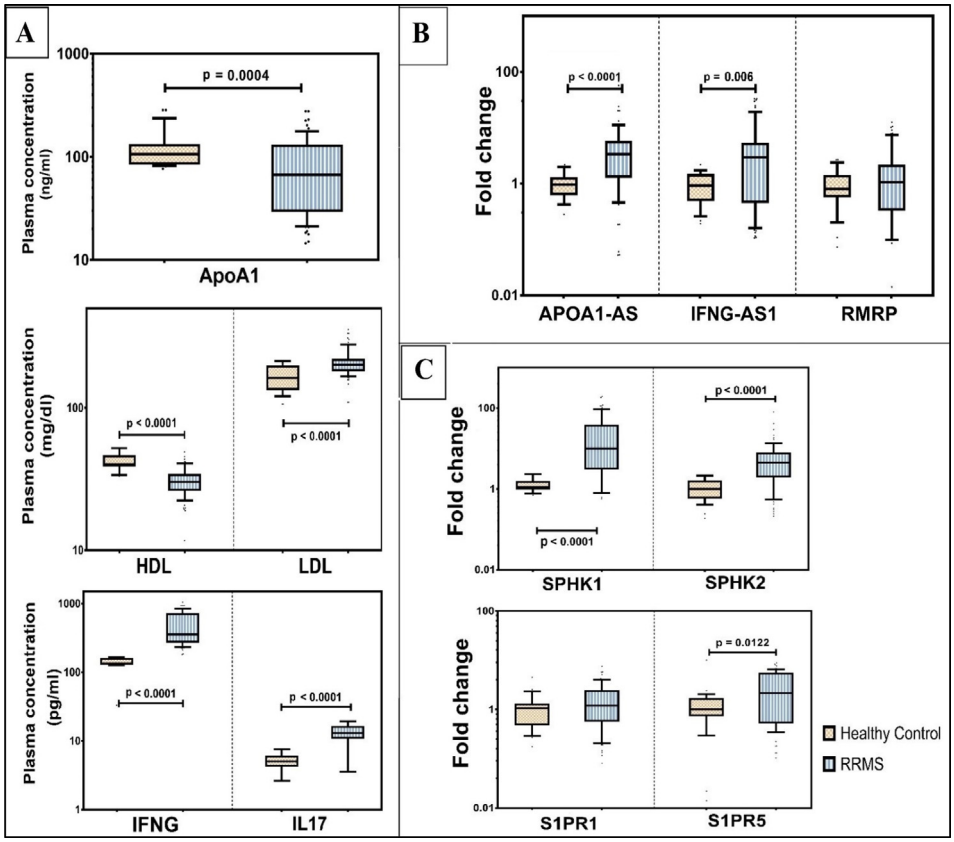

Fig. 1. Biochemical measurements in RRMS (n = 72) and healthy controls (n = 28) (A) Plasma levels of ApoA1, HDL-cholesterol, LDL-cholesterol, IFN-γ and IL17 (B) Long noncoding RNAs expressions (Fold Change). (C) Expression of sphingosine kinases (SPHKs) and sphingosine 1-phosphate receptors (S1PRs) (Fold Change). Box plots show the median as a band inside each box, while boxes and whiskers delineate 25–75th and 10–90th percentiles, respectively. Dots outside the whiskers indicate outliers. Significant *p-values* are indicated on graph at $p < 0.05$.

Discussion

The current study reported the differential expression of three lncRNAs: APOA1-AS, IFNG-AS1, and RMRP besides the differential gene expression of SPHK1, SPHK2, S1PR1 and S1PR5 in a cohort of MS patients. We aim to objectively elucidate a framework for the involvement of three intermingled axes in MS pathogenesis along with their possible links to MS activity, relapse rate, and patient disability.

ApoA1, the main constitutive protein in HDL, was reported to be neuroprotective in experimental autoimmune encephalomyelitis (EAE) [21]. Recently, lncRNA APOA1-AS, an endogenous regulator of ApoA1 biogenesis, has gained much interest. In this report, MS patients showed a marked upregulation in APOA1-AS along with lower levels of ApoA1 and HDL-cholesterol, together with higher LDL-cholesterol. APOA1-AS recruits histone-modifying enzymes, known to epigenetically repress ApoA1 promoter, thus reducing ApoA1 transcription [10]. To date, no study has characterized APOA1-AS role in MS or any other neurodegenerative diseases. However, decreased levels of ApoA1 were demonstrated in MS [21], and other neurodegenerative diseases [22].

HDL exhibits anti-inflammatory effects through managing cholesterol efflux [4]. RRMS patients displayed a distorted lipoprotein profile in which HDL was functionally and structurally modified at ApoA1 site compromising its anti-inflammatory capacity [4]. In our study, plasma ApoA1 was lower during relapse than in remission. In fact, reduction of HDL-cholesterol and ApoA1 had been linked to increased activity in other autoimmune diseases [23]. To the best of our knowledge, we are the first to describe divergences in ApoA1 reflecting MS activity.

Accumulation of permanent neurological deficits following relapses promote MS progression, therefore ARR has been used to predict future MS progression [17]. In this report, MS patients during relapse with low ARR showed lower ApoA1 concentration than those in remission, suggesting association between ApoA1 levels and MS progression. Such finding agrees with a previous report in which ApoA1-deficient mice experienced exacerbated EAE upon induction, which was attributed to increased inflammatory cytokines including: TNF-α and IL23 [21]. The link between reduced ApoA1 and both MS activity and future progression seemed to be attributed to the loss of the neuro-regenerative power of ApoA1 [8].

The EDSS is the most commonly used scale for measuring severity of disability among MS patients [24]. Herein, ambulating patients of relapse group showed lower ApoA1 concentrations than those in remission, confirming ApoA1 power to discriminate MS relapses from remission especially at lower EDSS. In fact, ApoA1 has been used as a biomarker for some neurodegenerative diseases [25]. Herein, even though APOA1-AS had shown greater diagnostic ability than ApoA1, both were comparable in predicting MS activity.

Concurrent upregulation of IFN-γ and IFNG-AS1 was observed in different autoimmune diseases [11]. In this study, IFNG-AS1 upregulation and higher IFN-γ levels were found in RRMS patients. Several studies reported elevated IFN-γ in MS patients [26]. In fact, the role of IFN-γ in MS has been an enigmatic paradox, since some

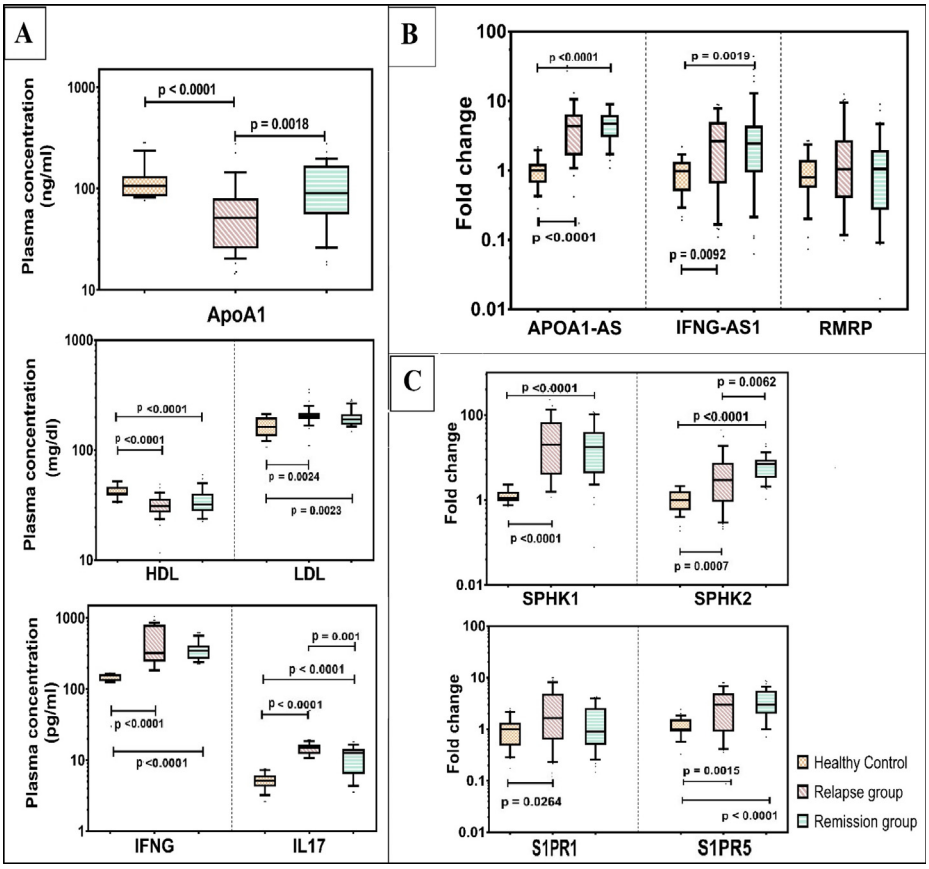

Fig. 2. Biochemical measurements in relapse group (n = 37), remission group (n = 35) and healthy controls (n = 28) (A) Plasma levels of ApoA1, HDL-cholesterol, LDL-cholesterol, IFN-γ and IL17 (B) Long noncoding RNAs expressions (Fold Change). (C) Expression of sphingosine kinases (SPHKs) and sphingosine 1-phosphate receptors (S1PRs) (Fold Change). Box plots show the median as a band inside each box, while boxes and whiskers delineate 25–75th and 10–90th percentiles, respectively. Dots outside the whiskers indicate outliers. Significant *p-values* are indicated on graph at $p < 0.05$.

Table 3
Biochemical measurements in RRMS patients during relapse and in remission according to annualized relapse rates (ARR).

		Relapse		Remission	
		<1 n = 19	≥ 1 n = 18	<1 n = 19	≥ 1 n = 16
Plasma level of					
	ApoA1 (ng/ml)	57.3 ± 11.5	76.2 ± 15.5	85.5 ± 16.2	124.9 ± 15.3 [xy]
	HDL (mg/dl)	31.6 ± 1.9	32.1 ± 1.6	28.8 ± 1.6	30.1 ± 1.7
	LDL (mg/dl)	190.5 ± 6.1	222 ± 15.9	198.5 ± 10.8	211.9 ± 12.1
	IL17 (pg/ml)	13.8 ± 0.7	14.7 ± 0.6	11.8 ± 1	9.8 ± 1.1 [y]
	IFN-γ (pg/ml)	638.9 ± 68.3	285.4 ± 29.9 [x]	471 ± 27.4 [x]	307.2 ± 14.2 [yz]
lncRNA expression (FC)					
	APOA1-AS	4.8 ± 1.8	3.6 ± 0.8	3.4 ± 0.6	2.9 ± 0.6
	IFNG-AS1	2.9 ± 1	3.7 ± 1.2	3.2 ± 0.8	2.05 ± 0.5
	RMRP	5 ± 1.8	6.9 ± 3.3	5.4 ± 4.2	2.4 ± 0.7
Gene expression (FC)					
	SPHK1	12.1 ± 5.5	26.8 ± 9.8	23.4 ± 5 [x]	18.1 ± 6
	SPHK2	5 ± 1	7.1 ± 2.7	5.7 ± 1.1	6.8 ± 1.2
	S1PR1	1 ± 0.3	2 ± 0.5	2.4 ± 0.5 [x]	3.2 ± 0.7
	S1PR5	1.4 ± 0.5	2.7 ± 0.7	5 ± 1.3 [x]	2.9 ± 0.8

Data are represented as mean ± SEM.[x] is significantly different from relapse patient with low ARR.[y] is significantly different from patients during relapse with high ARR. [z] is significantly different from patients in remission with low ARR at $p < 0.05$.

studies confirmed a prominent proinflammatory role, while others showed a protective function in MS [27]. Increased production of IFN-γ in CNS has been suggested to increase expression of class II antigens and enhance myelin antigen presentation to sensitized T cells, which can initiate MS or exacerbate present symptoms [28].

Th17 cells are involved in triggering and maintaining tissue damage in chronic neuroinflammation [29]. Herein, IL17 increased in MS patients, which is consistent with previous reports [30]. IL17 can induce glial activation, IL6, and IL1β expression, as well as nitric oxide release from astrocytes and microglia, that can inflect

Table 4
Biochemical measurements in RRMS patients during relapse and in remission according to expanded disability status scale (EDSS).

		Relapse		Remission	
		< 6 n = 20	n = 17	< 6 n = 21	n = 14
Plasma level of					
	ApoA1 (ng/ml)	62.1 ± 9.6	78.3 ± 24.8	101.1 ± 12.8 [a]	121 ± 25.5
	HDL (mg/dl)	31.8 ± 1.5	32 ± 2.3	29.9 ± 1.9	32.1 ± 1.9
	LDL (mg/dl)	206.9 ± 9.9	223.5 ± 17.5	205.6 ± 9.2	213.7 ± 19.9
	IL17 (pg/ml)	14.5 ± 0.6	13.3 ± 0.5	10.7 ± 1.3 [a]	9 ± 0.5 [b]
	IFN-γ (pg/ml)	529.6 ± 87.3	246.1 ± 7.8 [a]	359.3 ± 32.4	267.6 ± 9 [c]
lncRNA expression (FC)					
	APOA1-AS	3.6 ± 1	5.9 ± 1.9	8.5 ± 3.1	2.6 ± 0.6
	IFNG-AS1	2.1 ± 0.5	8 ± 2.1	2.9 ± 0.7	4.4 ± 1
	RMRP	7.2 ± 2.4	1.9 ± 0.8	2 ± 0.6	1.4 ± 0.5
Gene expression (FC)					
	SPHK1	101.4 ± 52.6	60.7 ± 14.2	33.2 ± 7.5	26.3 ± 10.1
	SPHK2	8.1 ± 3.9	7.1 ± 4.5	20.6 ± 7.8 [a]	14.05 ± 7.3
	S1PR1	1 ± 0.1	2.5 ± 0.5 [a]	2.2 ± 0.4 [a]	1.5 ± 0.4
	S1PR5	2.8 ± 0.5	2.8 ± 0.7	2.3 ± 0.5	1.9 ± 0.4

Data are represented as mean ± SEM.
[a] is significantly different from relapse patient with EDSS < 6.
[b] is significantly different from patients during relapse with EDSS ≥ 6.
[c] is significantly different from patients in remission with EDSS < 6 at $p < 0.05$.

direct toxicity to myelin and exposed axons [1]. RMRP, a critical regulator of Th17 function, was identified in tissue cultures and animal models of autoimmunity, where decreased RMRP levels caused a reduction in IL17 [31]. Herein, RMRP expression was assessed in peripheral blood of MS patients for the first time and showed statistically insignificant increased levels.

In this investigation, insignificant changes in IFN-γ, IFNG-AS, and RMRP were observed between relapse and remission groups, however, MS patients during relapses exhibited higher IL17 level, suggesting possible relation between IL17 and MS activity. A concurrent increase in Th17 cells and IL17 levels in CSF and serum of MS patients during relapse was previously reported [30]. Interestingly, our results revealed elevated IL17 in patients during relapses with both low and high EDSS scores. Such findings confirm the involvement of IL17 in relapses irrespective to severity of disability. Likewise, at high ARR, IL17 was elevated in relapse compared to remission, suggesting IL17 as a potential predictor for relapses especially in MS patients with high ARR.

In our study, MS patients during relapse and in remission with higher EDSS showed lower IFN-γ. Similarly, patients with high ARR exhibited lower IFN-γ than those with low ARR, revealing an association between IFN-γ and both EDSS and ARR. Contradictory functions of INF-γ was demonstrated in EAE [32]. Moreover, a stage-specific role for IFN-γ in EAE and MS was reported [27]. Therefore, further studies are needed to clearly track IFN-γ over MS course. In our study, ROC curve analysis showed excellent diagnostic power of IFN-γ and IL17 for MS.

Herein, SPHK1, SPHK2, and S1PR5 were upregulated in MS together with insignificant S1PR1 upregulation. Importantly, SPHK1 is highly enriched in nerve terminals, where it stimulates oligodendrocyte progenitor survival [33]. Despite the absence of reports describing SPHKs expression in blood of MS patients, our findings are in accordance with SPHK1 upregulation found on reactive astrocytes and macrophages isolated from MS lesions, in addition to marked increase in SPHK1 expression and functionality in activated rat astrocytes [34]. SPHK2, on the other hand, regulates histone acetylation, hence epigenetically controls the expression of genes involved in pro- or anti-inflammatory pathways depending on the surrounding environment [35]. Therefore, we speculate that SPHK2 upregulation observed herein is related to the altered inflammatory milieu and apoptotic oligodendrocyte loss in MS patients.

Human S1PRs expression in the CNS is quite obscure, especially in pathological conditions [36]. In fact, reports demonstrated S1PRs expression in rodents' or human brain cells [36], yet data about S1PRs expression in blood of MS patients are still lacking. In this study, the insignificant S1PR1 upregulation agrees with a study reporting S1PR1 upregulation in active and inactive MS lesions [36]. Modulating S1PR5 in human oligodendrocytes is essential for survival [37], thus, S1PR5 upregulation observed herein might represent a compensatory mechanism related to failed remyelination accompanying MS, however further studies are required to validate such assumption.

Herein, MS patients during relapse exhibited lower SPHK2 expression than those in remission. Based on EDSS, only ambulatory patients during relapse showed lower SPHK2 expression. Such findings raise a possibility that downregulation of SPHK2 might be involved in MS relapses especially in patients with low EDSS.

Even though S1PR1 was insignificantly upregulated in MS patients, it was significantly upregulated in relapse group particularly in assisted or non-ambulatory patients, suggesting that S1PR1 expression can be a useful predictor for future worsening of disability during relapses. Additionally, based on ARR, patients in remission with low ARR showed upregulated SPHK1, S1PR1, and S1PR5. Such observation complies with the role of S1P in augmenting repair processes [38] and supporting remyelination in MS [39]. On conducting ROC analysis, SPHK1 showed good diagnostic power, whereas SPHK2 showed sufficient prognostic ability. Such differences in discriminative power could be related to the difference in their intracellular location [40]. Our data may suggest a crucial role for SPHK1 in MS pathogenesis while the role of SPHK2 might emerge during active MS.

Finally, this report has some limitations, firstly, being a single-center study, which necessitates comparable multi-centered studies to obtain more data and compare outcomes. Another caveat is the relatively small sample size in some subgroups. Further studies with larger multi-centered cohorts are recommended. Additionally, reports investigating longitudinal samples should be considered to monitor progression within MS patients.

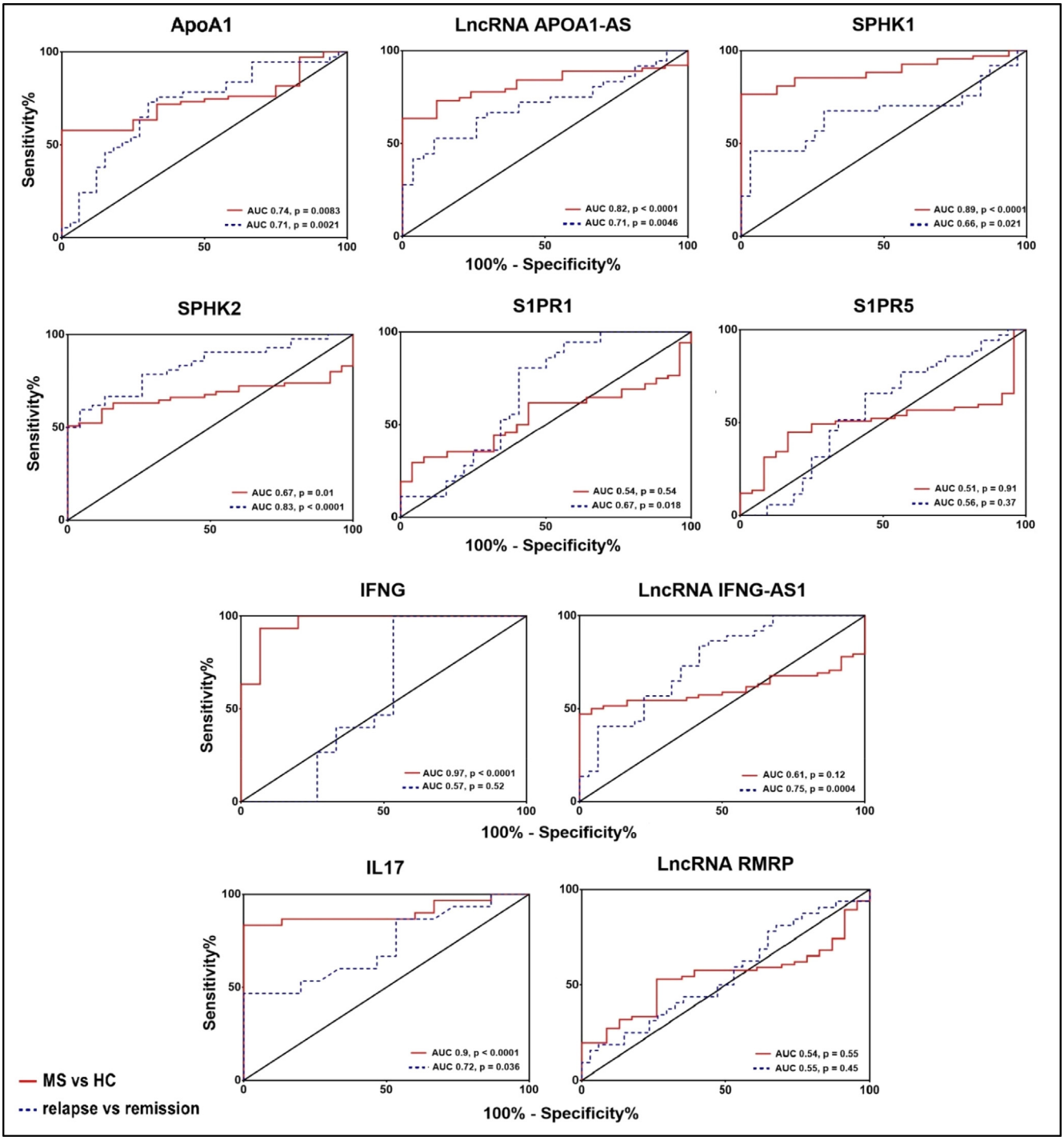

Fig. 3. Receiver operating characteristic (ROC) curves showing diagnostic power (in solid lines) in differentiating MS patients (n = 72) from healthy controls (n = 28) and prognostic power (in dotted lines) in differentiating MS patients during relapse (n = 37) from those in remission (n = 35) of measured parameters.

Conclusions

In summary, this study demonstrated upregulation of lncRNAs APOA1-AS and IFNG-AS1 but not RMRP along with overexpression of SPHK1, SPHK2 and S1PR5 genes in blood of Egyptian MS patients. Moreover, significant differences in ApoA1, SPHK2, and IL17 were found between patients during relapse and those in remission. Our findings also linked ApoA1, SPHK2, and IL17 to MS activity, and both S1PR1 and IFN-γ to patient disability. However, only IFN-γ was associated with relapse rate and consequently future progression in MS. Finally, our study provides evidence for excellent diagnostic power of IFN-γ, IL17, SPHK1 and lncRNA APOA1-AS in differentiating MS patients, whereas SPHK2 showed the highest prognostic power in predicting MS patients in relapses.

Compliance with Ethics Requirements

All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institu-

Table 5
Diagnostic and prognostic values of measured parameters in predicting MS patients and MS patients in relapse.

Biomarker		AUC	*p-value*	Cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
ApoA1	MS vs. HC	0.74	0.0083	<76.3 ng/ml	57.8	100	100	48
	relapse vs. remission	0.71	0.0021	<70.2 ng/ml	73	70	72	71
LncRNA APOA1-AS	MS vs. HC	0.82	<0.0001	>2.2 fold	63.5	100	100	51.6
	relapse vs. remission	0.71	0.0046	<1.7 fold	52.8	88.9	83.4	64.1
IFNG	MS vs. HC	0.97	<0.0001	>205.5 pg/ml	93.3	93.3	97.3	84.4
	relapse vs. remission	0.57	0.5203	<677.3 ng/ml	100	46.7	66.5	100
LncRNA IFNG-AS1	MS vs. HC	0.61	0.1262	>2.3 fold	100	47	82.9	100
	relapse vs. remission	0.75	0.0004	<3.4 fold	83.8	58.1	67.9	77.2
IL17	MS vs. HC	0.9	<0.0001	>8.4 ng/ml	83.3	100	100	70
	relapse vs. remission	0.72	0.0362	<10.4 ng/ml	46.7	100	100	64
LncRNA RMRP	MS vs. HC	0.54	0.5549	>0.9 fold	53	73.9	83.9	38
	relapse vs. remission	0.56	0.45	>0.3 fold	81.3	32.4	56	62.1
SPHK1	MS vs. HC	0.89	<0.0001	>2.5 fold	76.5	100	100	62.3
	relapse vs. remission	0.66	0.021	<3.1 fold	46	96.7	93.7	62.9
SPHK2	MS vs. HC	0.67	0.0109	>2.2 fold	50.8	100	100	44.2
	relapse vs. remission	0.83	<0.0001	<1.8 fold	59.5	95.7	93.6	69.1
S1PR1	MS vs. HC	0.54	0.5441	<0.3 fold	29.4	96	95	34.6
	relapse vs. remission	0.67	0.0177	<1.2 fold	80.6	59.4	67.7	74.3
S1PR5	MS vs. HC	0.51	0.9139	<0.6 fold	44.8	83.3	87.3	37
	relapse vs. remission	0.56	0.3728	<1.3 fold	65.7	56.3	61.4	60.8

MS: Multiple sclerosis; HC: Healthy controls; AUC: Area under the curve; PPV: Positive predictive value; NPV: Negative predictive value.

tional and national) and with the Helsinki Declaration if 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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