Original Article

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Serum Galectin-9 and TIM-3 Expression in Relapse-Remitting Multiple Sclerosis: Assessment of Immune Checkpoint Biomarkers

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Abstract:

Objective: An immune checkpoint molecule, T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), is an important effector of T cell exhaustion and tolerance by interacting with its ligand, Galectin-9 (Gal-9). This study investigated whether serum TIM-3/Gal-9 levels could reflect immune regulatory dysfunction in relapsing-remitting multiple sclerosis (RRMS).

Material and Methods: Serum TIM-3 and Gal-9 were evaluated intwo main groups of the study (RRMS patients and control groups) with enzyme-linked immunosorbent assay (ELISA). For the evaluation of diagnostic utility, statistical studies were performed using the Mann-Whitney U test, Spearman's correlation, and receiver operating characteristic (ROC) curve analysis.

Results: TIM-3 levels were substantially higher in patients than in controls. Gal-9 levels revealed no significant difference, but were markedly heterogeneous in patients. There was a significant positive correlation between study markers in both groups. ROC analysis revealed that TIM-3 had modest diagnostic efficacy, but Gal-9 lacked discriminatory value. A subgroup analysis revealed no significant difference in biomarker levels between individuals on disease-modifying medications and those not on treatment.

Conclusion: This study represents the first evidence that serum TIM-3 is considerably higher in RRMS, indicating immune checkpoint dysregulation, which is fundamental to MS pathogenesis. Although Gal-9 did not have direct diagnostic value, its strong, significant correlation with TIM-3 suggests dysregulation of immune tolerance mechanisms. These findings require validation in large cohorts with clinical correlation, but identify TIM-3 as a candidate immunological exhaustion biomarker, and Gal-9/TIM-3 axis as a potential therapeutic target.

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Keywords: Autoimmune, Gal-9, multiple sclerosis, RRMS, TIM-3

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disorder of the central nervous system, affecting approximately 2.8 million individuals globally, with a steadily increasing incidence in recent decades[1]. It is distinguished by inflammation, progressive neurodegeneration, demyelination, and axonal injury, which causes significant disability and reduced quality of life[2]. MS is a complex autoimmune disease, where T- cell responses against myelin Ag cause neuroinflammation and tissue destruction[3].

The MS pathogenesis involves self-reactive

T-cells escaping peripheral tolerance pathways and infiltrating the central nervous system (CNS), initiating demyelination[4]. Immune checkpoint pathways maintain peripheral tolerance and prevent autoimmune pathogenesis[5]. Among these regulatory pathways, the T-cell Immunoglobulin and Mucin-domain containing Protein 3 has emerged as an essential modulator of T-cell exhaustion and immune responses[6].

T-cell Immunoglobulin and Mucin-domain containing Protein 3 is an inhibitory transmembrane receptor, expressed on regulatory T-cells, T-helper 1, and also several other immune subsets[7], which transmit negative signals to restrain the T-cells and prevent excessive immune response[8]. Its principal ligand, (Gal-9), is a β-galactoside-binding lectin involved in cell adhesion, apoptosis, and immunoregulation[9]. TIM-3 / Gal-9 interaction induces T-cell exhaustion and supports peripheral immune tolerance[10]. In multiple sclerosis, activation of glial cells leads to increased production of Gal-9, which can promote pro-inflammatory responses in microglia and has been linked to secondary disease progression. Dysregulation or functional anomalies of T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) have been reported in autoimmune conditions, such as rheumatoid arthritis and multiple sclerosis. The Gal-9/ TIM-3 axis is thought to play a regulatory role in immune responses that protect myelin, partly through modulation of major histocompatibility complex class II (MHC-II) expression, a marker of functionally protective immune activity in multiple sclerosis[11-13].

Reliable biomarkers for MS identification, disease progression, and treatment responses remain a major clinical task. Lately, diagnostic methods have become dependent chiefly on clinical symptoms, cerebrospinal fluid investigation, and magnetic resonance imaging (MRI), which might not fully reflect illness progression or predict therapeutic consequences[14]. Current trends emphasize the importance of discovering biomarkers that can detect MS through its preclinical phase. Serum biomarkers offer important advantages due to their non-invasive nature, cost-effectiveness, and suitability for serial monitoring[15].

Considering the relevance of immune exhaustion pathways in autoimmune diseases with limited understanding of TIM-3/Gal-9 axis in MS, we hypothesized that their serum concentrations can effectively distinguish between healthy controls and MS patients. Also, we proposed that these biomarkers could be associated with disease characteristics, such as activity, duration, and disability progression, thereby providing potential value for clinical management. This study compared serum Gal-9 and TIM-3 levels between relapsing -remitting multiple sclerosis (RRMS) patients and healthy individuals, evaluated their diagnostic potential, assessed correlations with clinical parameters, and investigated sex-specific differences in biomarker expression.

Material and Methods

Study participants and design

This case-control observational investigation was

conducted from July to October 2025. Following the Declaration of Helsinki principle. A pilot study and literature review were used to calculate the sample size. Based on a moderate effect size (Cohen's d=0.6), a statistical significance level of α=0.05, and a statistical power of 80.0%, a sample size of 45 participants per group was determined. To accommodate an expected 10.0% dropout rate, each group had 50 participants.

Inclusion and exclusion criteria

Inclusion criteria: Study groups were categorized into two groups: the RRMS patients group consisted of 50 individuals with a confirmed diagnosis of RRMS according to the 2017 McDonald Criteria, aged between 20 and 50 years, with a disease duration of at least six months, all patients were in the remission phase, defined as at least 30 days since the last relapse with no history of malignancy (within the past five years) and no with immunodeficiency disorders, The control group included 50 healthy volunteers, aged between 27 and 38 years, matched as closely as possible to the patient group for sex and age distribution, with no history of autoimmune, neurological, or chronic inflammatory diseases, and no acute infections within the four weeks preceding enrollment.

Exclusion criteria: Participants were excluded if they had acute infections or had received vaccinations within the previous four weeks, were pregnant or lactating, had a current or recent history of malignancy (within the past five years), were diagnosed with immunodeficiency disorders, or had used immunosuppressive medications, excluding MS disease-modifying therapies.

Blood sample collection and processing

A venous blood sample (5 ml) was collected via venipuncture from each participant in this study, then placed in a specific gel tube for serum separation after standing at room temperature for 15 minutes. Samples were centrifuged at 400 × g for 15 minutes for separation, all serum samples were stored at –20 °C and analyzed within one month to avoid repeated freeze–thaw in order to assessment of the immunological markers (TIM-3 and Gal-9) using a commercially available Human ELISA kit (Bioassay Technology Laboratory, China. Cat. No E7264Hu, E29988Hu respectively), according to the manufacturer's recommended protocol.

Ethical approval

The study protocol was approved by the Medical Research Bioethical Committee of the College of Medicine, University of Karbala (No: 25-78, Date 1/8/2025), in collaboration with the Iraqi Ministry of Health.

Quantification of TIM-3 and Gal-9 levels

Gal-9/TIM-3 ELISA kits, based on the principles of the sandwich ELISA technique, were used for quantitative detection of the study markers in patient/control serum samples.

Statistical analysis:

The data were analyzed using the Statistical Package for the Social Sciences (SPSS) software, version 25 (Chicago, IL, USA). Normality of data distribution was assessed by applying the Shapiro-Wilk test. Normal data distribution was expressed as a mean, while non-normal data distribution was expressed as a median. The Chi-square was used to analyze categorical variables, which were reported as numbers and percentages. For detection correlation, the Spearman correlation test was employed. ROC curve was utilized to determine the diagnostic performance of the studied biomarkers. Non-parametric statistical methods, including the Mann-Whitney U test, were applied to compare marker levels in groups. The statistically significant value was less than 0.05.

Results

The demographic analysis reveals significant variances and similarities between the research groups. There are no significant differences in age between patients and controls ("p-value"=0.6221), mean ages were comparable (33.2 vs 33.3 years), but the patient group had higher age variability ("S.D."=8.2 vs 2.9) and a considerably broader age range (20-50 years vs 27-38 years). Sex distribution showed females making up the majority in both groups (70.0% in controls, 80.0% in patients, "p-value"=0.500).

Age distribution differed significantly between groups ("p-value"=0.001), with patients more evenly distributed across age categories 20-30 (44.0%), 31-40 (34.0%), 41-50 (22.0%), while controls concentrated between 31 and 40 (78.0%), with no participants older than 40 (Table 1).

Immunological marker analysis:

Gallevels showed no significant difference between patients and controls (362.43 vs 332.80, "p-value"=0.33); however, greater variability in patients (51.27-2541.07) compared to controls (231.84-1165.93) was seen. In contrast, TIM-3 levels were significantly elevated in patients compared to controls (623.03 vs 511.45, "p-value"=0.026), with a wide range (0.00-4616.06) (Table 2).

Table 2 Serum level of TIM-3 and Gal-9 in RRMS patients and healthy controls

Variables	Study groups (N=100)	
	Control (N=50)	Patients (N=50)
Gal-9		
Median	362.43	332.80
Minimum	51.27	231.84
Maximum	2541.07	1165.93
"p-value"	0.33^{ns}	
TIM-3		
Median	623.03	511.45
Minimum	0.00	87.86
Maximum	4616.06	1703.38
"p-value"	0.026*	

*="p-value"<0.05, ns=not significant, Mann-Whitney U test: for non-parametric comparison of two independent groups. Data presented as median values.
TIM-3=T-cellimmunoglobulin and mucin-domain containing-3, RRMS=relapsing-remitting multiple sclerosis

Table 1 Demographic characteristics between study groups with age distribution comparison

Variables			Study groups (N=100)	
			Control(N=50)	Patients (N=50)
Age (years)		Mean	33.2±2.9	33.3±2.98.2
		Range	27-38	20-50
		"p-value"	0.6221^{ns}	
Sex	Frequency	Male	10 (20.0%)	15 (30.0%)
		Female	40 (80.0%)	35 (70.0%)
		"p-value"	0.500^{ns}	
Age Group	Patients	Control	Total	"p-value"
20-30	22 (44.0%)	11 (22.0%)	33	
31-40	17 (34.0%)	39 (78.0%)	56	0.001**
41-50	11 (22.0%)	0 (0.0%)	11	
Total	50 (100.0%)	50 (100.0%)	100	

Chi-square test, ns=non-significant, S.D.=standard deviation, **significant at 0.001 level

Biomarker levels by EDSS severity

Patients were stratified into two groups based on EDSS scores: mild disability (EDSS=1, n=30) and moderate-to-severe disability (EDSS≥4.5, n=20) (Table 3). TIM-3 levels between the two groups were mild: 623.42 vs. moderate-severe: 604.29 pg⁄ml, "p-value"=0.868, with no significant differences. Similarly, Gallevels were nearly indistinguishable between severity groups (mild: 362.54 pg⁄ml vs. moderate-severe: 357.28 pg⁄ml, "p-value"=0.959). Greater variability was observed in the moderate-severe group despite similar medians.

Analysis of biomarker levels by disease duration:

Table 4 shows that the TIM-3 level increased gradually throughout the disease duration (from 594.87 to 667.57), indicating a 12% rise from early to late stages, with "p-value"=0.637. Gal-9 level showed a non-linear pattern, with an initial elevation at the intermediate period (12.8% elevation) and a minor decrease in the last stage ("p-value"=0.712).

Spearman correlation matrix among patient groups

Among patient groups, there was a greater positive correlation (r=0.582, "p-value"=0.001) between the two markers (TIM-3 and Gal-9). Strong inter-correlations were observed among clinical parameters: EDSS correlated positively with disease duration (r=0.435, "p-value"=0.002), and Relapse Frequency (r=0.757, "p-value"<0.0001), also disease duration correlated with Relapse Frequency (r=0.416, "p-value"=0.003). In contrast, neither TIM-3 nor Gal-9 showed significant correlations with any clinical parameter. TIM-3 did not correlate with EDSS (r=0.032, "p-value"=0.824), disease duration (r=0.079, "p-value"=0.585), and relapse frequency (r=0.111, "p-value"=0.444). Similarly, Gal-9 showed no correlation with EDSS (r =-0.010, "p-value"=0.945), disease duration (r=0.073, "p-value"=0.614), or relapse frequency (r=-0.022, "p-value"=0.877), as shown in Table 5.

Table 3 Biomarker levels by EDSS severity

Biomarker	Mild (EDSS=1, n=30)	Moderate sever (EDSS ≥4.5, n=20)	"p-value"
TIM-3 (pg⁄ml)	623.42 (0.00-1164.96)	604.29 (144.89-4616.07)	0.868 ns
Gal-9 (pg⁄ml)	362.54 (51.27-826.24)	357.28 (203.76-2541.07)	0.959 ns

Mann-Whitney test. Data presented as median (range). ns=not significant, TIM-3=T-cellimmunoglobulin and mucin-domain containing-3, EDSS=expanded disability status scale

Table 4 Biomarker levels by disease duration

Biomarker	Early (≤6m, n=22)	Intermediated (7-12m, n=14)	Late (>1y, n=14)	"p-value"
TIM-3 (pg⁄ml)	594.87	654.42	667.57	0.637 ns
Gal-9 (pg⁄ml)	334.47	377.43	368.56	0.712 ns

Kruskal – Wallis test, data presented as median, ns=non-significant, TIM-3=T-cellimmunoglobulin and mucin-domain containing-3

Spearman correlation matrix among control groups

Interestingly, the control group showed an even stronger significant correlation between TIM-3 and Gal-9 (r=0.659, "p-value"=0.0001) compared to patients. Neither marker correlated significantly with demographic variables in controls (Table 6).

ROC curve analysis assessed the diagnostic performance of Gal-9 and TIM-3

The Gal-9 biomarker demonstrated poor diagnostic value (AUC=0.556, "p-value"=0.338). The TIM-3 biomarker outperformed Gal-9 with an AUC of 0.628, reaching statistical significance ("p-value"=0.027) (Table 7).

This indicates that TIM-3 has an AUC of 0.628, fair but not strong discriminative ability, and neither TIM-3 nor Gal-9 demonstrated discriminatory ability for EDSS, relapse frequency, or disease duration (Table 8).

Table 5 Combined spearman correlation matrix for biomarkers and clinical parameters among patient groups

Parameters	Disease duration		EDSS		Relapse frequency		TIM-3		Galectin-9	
	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
Disease duration	1	–	0.435**	0.002	0.416**	0.003	0.079ns	0.585	0.073ns	0.614
EDSS			1	–	0.757**	0.0001	0.032ns	0.824	– 0.010ns	0.945
Relapse frequency					1	–	0.111ns	0.444	–0.02ns	0.877
TIM-3							1	–	0.582**	0.0001

Spearman correlation test, **Correlation is significant at the 0.001 level, ns=not significant, TIM-3=T-cellimmunoglobulin and mucin-domain containing-3, EDSS=expanded disability status scale

Table 6 Spearman correlation matrix among control groups

Variables	TIM-3		Gal-9		Age	
	r	"p-value"	r	"p-value"	r	"p-value"
Gal-9	0.659*	0.0001	–	–	–0.52ns	0.09
TIM-3	–	–	0.659*	0.0001	–0.61ns	0.07
Age	–0.61ns	0.07	–0.52ns	0.09	–	–
Sex	0.24ns	0.08	0.163ns	0.25	0.006ns	0.96

**Correlation is positive, significant at the 0.01 level, ns=non-statistically significant, TIM-3=T-cellimmunoglobulin and mucin-domain containing-3

Table 7 ROC analysis for Gal-9 and Tim-3 markers

Variables	AUC	Std. Error	"p-value"	95% Confidence Interval	
Gal-9	0.556	0.058	0.338	0.442	0.670
TIM-3	0.628	0.056	0.027*	0.518	0.738

*significant at level 0, 01, AUC=The Area under the Curve, TIM-3=T-cellimmunoglobulin and mucin-domain containing-3, ROC=receiver operating characteristic

Table 8 ROC analysis for clinical stratification

Clinical Parameters	Biomarker	AUC	95% CI	"p-value"
EDSS Severity (Mild vs Moderate Severe)	TIM-3	0.497	0.333 – 0.661	0.967 ns
	Gal-9	0.508	0.344 – 0.672	0.924 ns
Relapse Frequency (Single vs Multiple)	TIM-3	0.524	0.356 – 0.692	0.767 ns
	Gal-9	0.591	0.423 – 0.759	0.279 ns
Disease Duration (Early vs Late)	TIM-3	0.578	0.396–0.760	0.419 ns
	Gal-9	0.539	0.357–0.721	0.689 ns

ROC=receiver operating characteristic, AUC=area under the curve, CI=confidence interval, ns=not significant ("p-value">0.05), TIM-3=T-cellimmunoglobulin and mucin-domain containing-3, EDSS=expanded disability status scale

Subgroup analysis by treatment status

Patients were divided into groups based on their disease-modifying therapy (DMT) status. Among the 50 RRMS patients, 27 (54.0%) were on interferon-based DMTs, while 23 patients (46.0%) were not on any DMT. TIM-3 levels were not significantly different between treated and untreated individuals (median 696.48 vs. 594.77, "p-value"=0.529). Similarly, there was no significant difference in Gal-9 levels by treatment status (median 357.28 vs 372.40, "p-value"=0.892).

Discussion

This study provides unique insights into the role of immune checkpoint molecules in RRMS, with a specific focus on Gal-9 and TIM-3. By incorporating immunological, demographic, and investigative analyses, we establish that TIM-3 is significantly increased in patient groups, while Gal-9 biomarker reflects a substantial heterogeneity without diagnostic importance.

Demographic considerations

Both study groups revealed comparable distributions in terms of age and gender, minimizing the likelihood that demographic factors influenced the observed differences in biomarkers. The slightly higher age range among patients, especially within the 41–50 years group, may be attributed to the progressive nature of MS, where immune dysregulation and cumulative environmental exposure trigger disease onset later in life[16].

Gal-9: immunoregulatory checkpoint with limited diagnostic utility

Although the median Gal-9 levels in patients were slightly higher than those in controls, the difference remained statistically nonsignificant. Notably, the substantial variation in Gal-9 concentrations among patients suggests that its immunological role varies across patients. Gal-9 interacts with TIM-3, promoting the apoptosis of the Th1 and Th17 subsets while enhancing the stability and expansion of regulatory T cells (Tregs)[17]. The variability noted in Gal-9 levels among patients could indicate different underlying immune states. Elevated Gal-9 concentrations in some patients could represent a compensatory mechanism aimed at suppressing inflammation, while reduced levels in others may reflect deficient tolerance mechanisms[18], potentially contributing to sustained immune activation. The limited discriminative ability noted in the ROC analysis (AUC=0.556) further reflects that Gal-9 alone is insufficient to reliably differentiate patients from healthy controls. However, its interaction with TIM-3 underscores its biological relevance within the disease pathways.

TIM-3: marker of immune exhaustion and dysregulation

Serum TIM-3 levels, in contrast, varied significantly among patients, ranging from almost undetectable quantities to over 4600 units. The pronounced elevation in median TIM-3 levels, combined with its moderate diagnostic performance, indicates that TIM-3 could serve as a biomarker promoting immune checkpoint imbalance[19]. Furthermore, the pronounced variability suggests that TIM-3 may reflect not only the presence of disease but also its stage, severity, and clinical phenotype. The detected increase further supports the concept that the immune checkpoint dysfunction is an integral feature of MS disease pathophysiology.

Correlation patterns: divergent regulation in health and disease

The results demonstrate more significant positive correlations between the two markers in controls than in patients. This is attributed to the fundamental biological coupling between these markers regardless of disease state, further suggesting homeostatic regulation. Specifically, TIM-3 expression on different immune cells aligns with proportional Gal-9 ligand availability, thereby preserving tolerance pathways[20]. This strong coupling provides immune checkpoint pathways that operate within physiological boundaries, preventing excessive immune activation.

In RRMS patients, the slightly weaker, though still significant, correlation may reflect partial uncoupling of this regulatory axis. Different mechanisms could illustrate this: dysregulation of TIM-3 expression on pathogenic T cells that have escaped normal checkpoint control, Gal-9 production affected by inflammatory cytokines, and disease heterogeneity, where some patients exhibit more dysfunctional immune checkpoints than others[21,22]. The persistent correlation, despite illness, suggests that this pathway, while disrupted, remains partially functional and may reflect a therapeutic target for restoring immune tolerance.

Importantly, this pattern—maintained but weakened correlation in disease—has been observed with other immune regulatory molecules in autoimmune conditions, supporting the concept that MS involves partial rather than the complete breakdown of tolerance mechanisms[23].

Lack of correlation with clinical parameters

Biomarker concentrations and clinical indices of disease, including EDSS scores, relapse frequency, and disease duration, did not significantly correlate in this investigation, for the following reasons. First Non-linear Dynamics: Biomarker expression variations might have non-linear paths, necessitating longitudinal monitoring or alternate temporal stratification in order to record significant time points. Second, the absence of association with EDSS score may highlight a key limitation of this scale, as it primarily estimates accumulated neurological disability rather than ongoing immune-mediated activity. As markers of T-cell exhaustion and immune checkpoint activation, TIM-3 and Gal-9 may better capture underlying immunological processes rather than the downstream neurological consequences of inflammation. Similar discrepancies between inflammatory mediators and clinical impairment have been found in previous MS studies, underscoring the complexity of interpreting immune biomarkers in this context[24]. Third, the absence of correlation with disease duration, ranging from 1 to 36 months, reveals that immune checkpoint dysregulation emerges early in the illness and remains relatively stable over time, rather than intensifying. However, the nature study and limited temporal window restrict the ability to estimate very early (preclinical) or extended (>3 years) disease stage, where such a relationship may differ. Fourth, no present correlation with recent relapse frequency reflects that these biomarkers are not indicators of acute disease activity but rather reflect an

underlying state of immune dysregulation that remains even through clinical remission[25,26].

These findings should be interpreted in light of certain limitations, especially the study design, which captures biomarker levels at a single time point and thus cannot estimate dynamic variables in relation to relapse or treatment response.

Limitations and future direction

First, MRI data were unavailable due to patient privacy rules and limited access in the outpatient context. Second, while treatment status was documented and analyzed, comprehensive data on treatment duration, adherence, previous therapies, and reasons for treatment decisions were unavailable. The lack of pre-treatment baseline measurements limits the interpretation of treatment effects, and we cannot exclude the possibility that the treatment and non-treatment groups differed in disease characteristics, influencing therapeutic decisions. Third, while clinical data (EDSS, disease duration, relapse frequency) were documented, predominantly mild-to-moderate disease severity (EDSS 1-5) may have limited power to detect correlations with clinical parameters. The absence of very early disease (<1 month) or advanced disease (EDSS >5) patients may have constrained the observable range of biomarker-clinical relationships. Although the mean age did not differ significantly between groups, the age distribution was broader in the patient group, which may represent a potential source of residual confounding. This imbalance could have influenced biomarker variability and should be considered when interpreting the results. Future studies with tighter age matching or age-adjusted models are warranted.

Conclusion

This study demonstrates that serum TIM-3, but not Gal-9, is considerably higher in RRMS with marked heterogeneity, indicating underlying immunological dysregulation. Although Gal-9 has little diagnostic efficacy, its strong correlation with TIM-3 confirms its immunological significance.

TIM-3 can differentiate RRMS patients from healthy controls, but without detecting disease severity. These findings are hypothesis-generating. The lack of correlation may indicate the study design's limitations, or that the biomarkers reflect continuous immunological dysregulation instead of acute disease activity.

Future research requires prospective longitudinal designs with serial sampling, a wider disease severity spectrum, and MRI correlation. Despite these obstacles, this study provides the first evidence of TIM-3 dysregulation in Iraqi RRMS patients, laying the groundwork for further study into immune checkpoint pathways in MS pathogenesis.

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Conflict of interest

All authors declare they have no conflict of interest.

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