Ameen *et al.*
Egyptian Rheumatology and Rehabilitation (2023) 50:30
https://doi.org/10.1186/s43166-023-00198-6

Egyptian Rheumatology and Rehabilitation

RESEARCH Open Access

A study of the association between Galectin-9 gene (LGALS9) polymorphisms and rheumatoid arthritis in Egyptian patients

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Abstract

Background Rheumatoid arthritis (RA) is an incessant synovial inflammation of an autoimmune origin, destroying articular cartilages and bones. Galectins are an evolutionarily conserved family of immune-modulatory animal lectins detected in a number of immune cells like T cells, fibroblasts and macrophages. Galectin 9 (Gal-9) has been the subject of many studies for being linked to regulation of both innate and adaptive immune reactions. The objective of the study was to evaluate the link between the Galectin-9 gene (LGALS9) polymorphisms and the susceptibility of RA in Egyptian patients, as well as, detection of the serum level of Gal-9 in RA and its association with LGALS9 polymorphisms, the activity of RA and radiological damage.

Methods A study of 85 participants; group (I): 60 RA cases and group (II): 25 apparently healthy subjects. RA Disease activity index (DAS-28) and Larsen index score were assessed. LGALS9 gene and serum Gal-9 were investigated.

Results rs4239242 TT genotype and T allele occurred more frequently in RA cases than controls with a significant difference ($P=0.006$; $P<0.001$ respectively). Gal-9 level was significantly higher among RA cases than control group ($P=0.017$). The Gal-9 level showed negative significant correlations with DAS-28 and Larsen score ($P<0.001$).

Conclusion RA is strongly linked to genetic alterations in the LGALS9 gene and the single nucleotide polymorphism (SNP) rs4239242 TT genotype in the Egyptian population. RA cases in remission or those with low disease activity had higher levels of serum Gal-9 in comparison to cases with moderate and high disease activity and this would be promising in the future of RA treatment.

Keywords Rheumatoid arthritis, Galectin-9, LGALS9 gene, Polymorphisms, DAS-28, Larsen score

Background

Rheumatoid arthritis (RA) is an incessant synovial inflammation of an autoimmune origin, destroying articular cartilages and bones with possible systemic manifestations. The significant characteristic of RA is the involvement of multiple cells and proteins which have been detected in abundance in the affected joint. The most commonly detected cells include T cells, neutrophils, B cells and macrophage. While among proteins and other mediators, the popular ones included tumor necrosis factor alpha

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(TNF-α), Interleukin-1 (IL-1), Interleukin-6 (IL-6), T helper-17 (Th17) cells, and anti-citrullinated peptide antibody (ACPA) which are autoantibodies used against citrullinated antigens. These infiltrations have been linked with joint inflammation, destruction, and angiogenesis [1, 2].

Galectins are an evolutionarily conserved family of immune-modulatory animal lectins detected in a number of immune cells like T cells, fibroblasts and macrophages. Only 11 of them have been described in humans involved in many biological processes [3, 4].

Galectin-9 (Gal-9) has been the subject of many studies for being linked to modulation of cellular polarity and adhesion, triggering of malignant cell apoptosis, and regulation of both innate and adaptive immune reactions [5–7]. Initially, Gal-9 was thought to be responsible for down regulation of the T cell immune reactions via triggering apoptosis in CD4+T helper 1 (Th1) and T helper 17 cells, but later was confirmed to stimulate the regulatory T cells [8–10].

Many mouse models of autoimmune diseases demonstrated an anti-inflammatory function of the high doses of Gal-9 [8, 9, 11–13]. While using lower concentrations of Gal-9 to treat resting mononuclear cells activated and expanded Interferon (IFN)- producing CD4+Th1 cells which suggest that the physiological concentrations Gal-9 could be involved in the immune pathology [14, 15].

The granulocytes (polymorph nuclear leukocytes) as well as the T cells are major regulators of the autoimmune response especially in RA [16–20]. When Gal-9 was introduced to human leukocytes in vitro, it activated the granulocytes which lead to an up regulated cytokine production, migration, and survival. Also, it up regulated the production of Peptidyl arginine deiminases 4 (PAD-4); causing intracellular citrullination of granulocyte proteins which have been significantly linked to the RA pathology. So, Gal-9 is suspected to be a stimulator of the immunopathology of RA [21].

The study in hands assesses the link between the Galectin-9 gene (LGALS9) polymorphisms and the susceptibility of RA in Egyptian patients. Also, evaluates the serum level of Galactin-9 in RA cases and its association with LGALS9 genotypes, the activity of disease and the radiological damage.

Materials and methods

Materials

Sixty RA cases recruited from the outpatient's clinic and the inpatient's department of the Rheumatology, Rehabilitation and Physical Medicine from September 2020 to April 2021, diagnosed according to the American College of Rheumatology (ACR)/European League against Rheumatism (EULAR) 2010 RA classification criteria [22]. Other immunological disorders or renal diseases were excluded from the study. Twenty-five apparently healthy, sex and age matching represented the control group. The practical part of the study was done at the Clinical and Chemical Pathology department of the hospital.

Following the Helsinki Declaration, the Ethics Committee approved the study and each participant signed an informed written consent before joining the study. A full history was obtained from all participants who were then underwent a thorough clinical examination. The RA disease activity score (DAS-28 ESR) was used to determine the disease activity and classified as disease remission (score ≤ 2.6), low (score > 2.6–3.2), moderate (score > 3.2–5.1) and high disease activity (score > 5.1). Moreover, Plain x rays of both hands and feet were obtained and evaluated according to the Larsen Index Score [23].

Sample collection

Following a septic protocol, 7 ml of the peripheral venous blood were withdrawn from each subject and divided into 3 parts:

A) 1.6 ml were placed into Na citrate tube for ESR assessment.
B) 2 ml were placed into ethylene diamine tetra-acetate (EDTA) tube for CBC assessment and the remaining amount was preserved at − 80 °C for the real-time polymerase chain reaction (RT-PCR) investigations of Gal-9 rs4239242 polymorphism.
C) The rest of the blood sample was prepared to be used for clinical chemistry tests and ELISA via allowing it to clot in a serum separating tube at room temperature then centrifuging it at 3000 rpm for 10 min. CRP and kidney function test were also evaluated on the day of sampling.

Methods

Lab tests included

The automated cell counter Sysmex XS-800 I (Kobe, Japan) was used for the complete blood count (CBC), the Westergren method was used to detect the erythrocyte sedimentation rate (ESR), latex-enhanced nephelometry by a Behring Nephelometer was used for quantitative assessment of the CRP and the Biosystems A15 auto-analyzer (Barcelona, Spain) was used to investigate kidney functions. Rheumatoid factor (RF): detection by Nephelometry test, anti-Cyclic Citrullinated Peptide antibodies (Anti-CCP Abs): detection by ELISA and serum level of Gal-9 detection by Human (Gal-9) ELISA Kit Catalogue # 201–12-5670.

Molecular biology investigation

The quantitative real-time PCR (qRT-PCR) was used to evaluate LGALS9 gene single nucleotide polymorphism (SNP) genotype rs4239242.

The Gene JET Whole blood genomic DNA Purification mini kit was used to extract the genomic DNA from the EDTA blood. The kit offers a rapid and efficient purification of a high quality genomic DNA (Thermo Scientific, EU, Cat. # K0781).

Amplification by real-time PCR

Genotyping of LGALS9 gene SNP (rs4239242) was performed using forward primer 5′- ACACCCAGATCG ACAACTCCTG -3′ and reverse primer 5′- CAAACA GGTGCTGACCATCCAC -3′. The PCR amplification was done using Stepone Real Time PCR instrument (S/N 271003648) (Applied Biosystems, Singapore).

In a sterile microcentrifuge tube reagents were pipetted as follows:

Five µl TaqMan®Genotyping Master Mix, 0.25 µl TaqMan®SNP Genotyping Assay, 3.75 µl DNase/RNase free water and 1.0 µl DNA in a final volume/well 10.0 µl.

PCR was performed in thermal cycler according to the following thermal conditions:

Pre-PCR (1 cycle) 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min.

Statistical analysis

The Statistical package for Social Science (IBM Corp. Released 2017, IBM SPSS Statistics for Windows, and Version 25.0. Armonk, NY: IBM Corp.) was used to revise, code and tabulate the collected data. Deviations from Hardy–Weinberg equilibrium (HWE) expectations were determined using the chi-squared test. Polymorphisms and genotype frequencies were evaluated by gene counts. HWE indicates that the selected groups of study are reasonable for performing genetic analysis of this SNP. Mean SD (range) or median with interquartile range (IQR) were the formats used to present categorical data. Fisher's exact, chi square ($\chi 2$), Mann-Whitney, and Kruskal-Wallis tests were utilized for comparisons. Linear association between variables was presented by Spearman's correlation coefficients. Galectin-9's ability to differentiate between the RA and control groups was evaluated using receiver operating characteristics (ROC) curves. Both univariable and multivariable logistic regression analyses was constructed to find the factors that predict RA disease activity. A p value ≤ 0.05 was considered significant.

Results

The current study included 60 RA cases; their mean age was 42.7 ± 10.9 years. They were 10 males (16.7%) and 50 females (83.3%). Control group was selected to be matched in age and gender ($P > 0.05$ for each).

Among the examined RA cases, the mean disease duration was 9.3 ± 7.1 years, mean number of tender joints was 6.6 ± 4.8, mean number of swollen joints was 3 ± 3.1, mean DAS-28 was 5.1 ± 1.3, mean Larsen score was 14.4 ± 6.9, mean Hb was 11.4 ± 1.4 g/dL, mean WBCs was $7.6 \times 10^9 \pm 2.4/mm^3$, mean ESR was 48.9 ± 30.7 mm/h, mean platelet was $290 \times 10^9 \pm 101.6/mm^3$, mean urea was 30.3 ± 3.7 mg/dL, mean serum creatinine was 0.9 ± 0.2 mg/dL, 65% had positive CRP, mean CRP titer was 18.8 ± 21.2, 80% had positive RF, mean titer was 71.2 ± 50.7, 88.3% had positive ACPA, mean level was 66.3 ± 52.7. Among the RA cases, 3.3% achieved remission, 18.3% had low disease activity, 14.8% had moderate and 62.3% had high disease activity (Table 1).

RA cases were significantly linked to higher frequency of TT genotype, T allele ($p = 0.006, < 0.001$ respectively), with risk to develop RA (OR = 3.794, 2.185 respectively) (Table 2).

Compared to the control group, cases of RA had significantly higher serum levels of Gal-9 ($P = 0.017$) (Fig. 1).

Gal-9 level was assessed in patients and control groups carrying different genotypes, no significant differences were found regarding Gal-9 serum level between different genotypes ($p = 0.443$, $p = 0.202$ respectively) (Fig. 2).

A receiver operating characteristic curve of Gal-9 levels was conducted for discrimination between RA cases and control groups. At best cut of value of Gal-9 was 142.7 pg/ml, sensitivity was 53.3%, specificity was 88%, positive predictive value (PPV) was 53.3%, negative predictive value (NPV) was 88%, and accuracy was 63.5% (Fig. 3).

Gal-9 level showed highly statistically significant negative correlations with DAS-28 and Larsen score ($p < 0.001$) (Fig. 4). However, Galectin-9 serum level showed non-significant negative correlation with Anti-CCP-Abs titer ($p = 0.435$).

Patients who were in remission and had low disease activity had higher serum levels of Gal-9 than those with moderate or high disease activity ($P < 0.001$) (Table 3).

No statistically significant associations were found regarding RA disease duration, number of tender joints, number of swollen joints, DAS-28, and Larsen score with *LGALS9* genotypes in RA group ($P = 0.330$, $P = 0.401$, $P = 0.241$, $P = 0.126$ and $P = 0.357$) respectively.

Table 1 Characteristics of RA group

			RA	
			N = 60	
			Mean ± SD (range)	
Disease duration (years)			9.3 ± 7.1 (1–30)	
Number of tender joints			6.6 ± 4.8 (2–28)	
Number of swollen joints			3.1 ± 3.1 (0–16)	
Disease activity score (DAS-28)			5.1 ± 1.3 (3–8)	
Larsen score			14.4 ± 6.9 (5–40)	
			RA	
			***N* = 60**	
HB (g/dl)		Mean ± SD	11.4	± 1.4
WBCs ($\times 10^3$ /mm^3)		Mean ± SD	7.6	**±** 2.4
ESR (mm/1st h)		Mean ± SD	48.9	**±** 30.7
Platelets($\times 10^3$ /mm^3)		Mean ± SD	290	**±** 101.6
Urea (mg/dl)		Mean ± SD	30.3	± 3.7
Creatinine (mg/dl)		Mean ± SD	0.9	± 0.2
CRP	Negative	*N*, %	21	35.0%
	Positive	*N*, %	39	65.0%
CRP titre (mg/l)		Mean ± SD	18.8	**±** 21.2
RF	Negative	*N*, %	12	20.0%
	Positive	*N*, %	48	80.0%
RF titre (IU/ml)		Mean ± SD	71.2	**±** 50.7
Anti-CCP-Abs	Negative	*N*, %	7	11.7%
	Positive	*N*, %	53	88.3%
Anti-CCP-Abs titre (U/ml)		Mean ± SD	66.3	**±** 52.7

HB hemoglobin, *WBCs* white blood cells, *ESR* erythrocyte sedimentation rate, *CRP* C–reactive protein, *RF* rheumatoid factor; *Anti-CCp Abs* anti-cyclic citrullinated peptide antibodies

Table 2 Distribution of the LGALS9 (genotypes and alleles) in patients with RA and healthy individuals

		Control		**RA**		***P***	**OR**	**95% CI**	
		***N* = 25**		***N* = 60**					
		N	**%**	***N***	**%**				
LGALS9	CC	5	20	4	6.7	–	1	(Reference)	
	CT	15	60	18	30	0.591 (NS)	1.289	0.510	3.257
	TT	5	20	38	63.3	**0.006 (S)**	3.794	1.458	9.876
	CT + TT	20	80	56	93.3	0.084 (NS)	2.167	0.903	5.203
	C	25	50	26	21.7	–	1	(Reference)	
	T	25	50	94	78.3	**< 0.001 (HS)**	2.185	1.425	3.351

OR odds ratio, *CI* confidence interval, *P* probability, *NS* non-significant *P* (> 0.05), *S* significant *P* (≤ 0.05), *HS* highly significant *P* (< 0.001), *LGALS9* Galectin-9 gene

Ordinal regression analysis was used for predicton of higher RA activity using age, gender, family history, duration, NTJ, NSJ, ESR, CRP, anti-CCP-Abs, RF, Gal-9 level and genotypes as covariates. Higher ESR, number of tender joints (NTJ), number of swollen joints (NSJ) and lower Gal-9 serum level were associated with higher activity in univariable analysis. However, on conducting multivariable analysis using significant covariates in univariable analysis revealed that only higher NTJ, NSJ, and lower level of Gal-9 was considered as independant predictor of higher RA activity (Table 4).

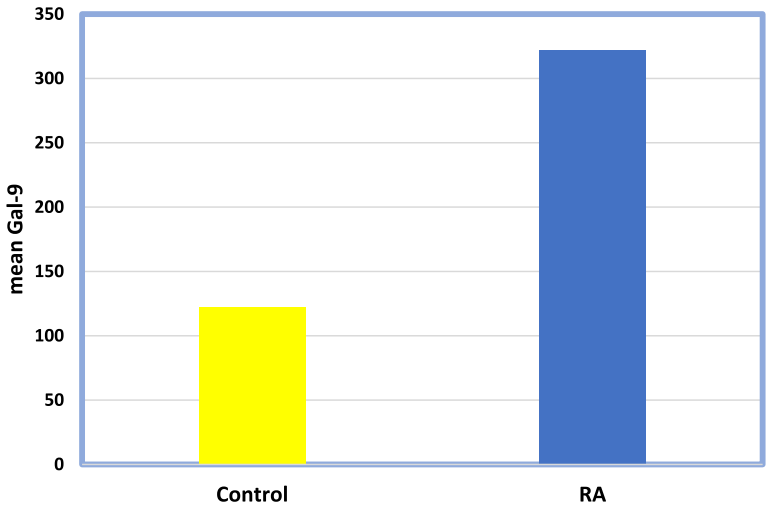


Fig. 1 Gal-9 levels between RA and control groups. When compared to the control group, cases of rheumatoid arthritis had significantly higher serum levels of Gal-9 ($p=0.017$)

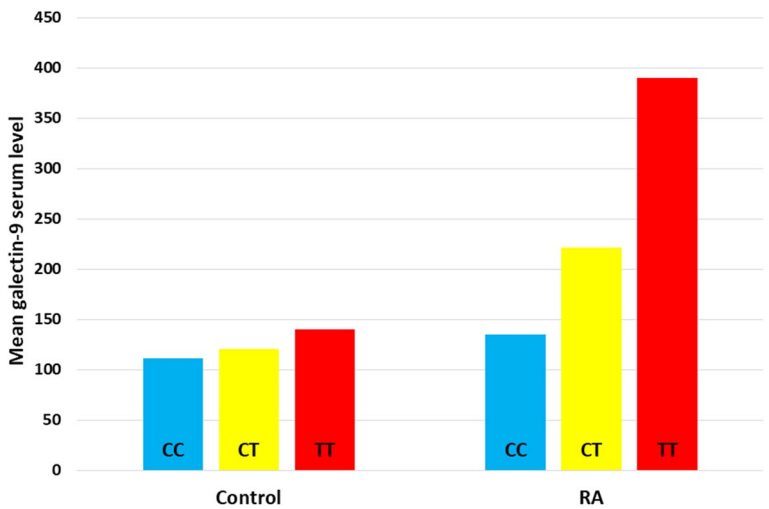


Fig. 2 Association between LGALS9 genotypes and serum levels of Gal-9 in RA and control groups. Gal-9 level was assessed in patients and control groups carrying different genotypes, no significant differences were found regarding Gal-9 serum level between different genotypes ($p=0.443$, $p=0.202$ respectively)

Discussion

A distinct Pathophysiology; related to RA, mainly mediated by T cells which infiltrates the affected joint along with many other immune cells like neutrophils, macrophages, B cells, and dendritic cells [24].

Gal-9 has multiple cell surface receptors with the most important are the T cell immunoglobulin domain and the mucin-domain-containing molecule-3 (Tim-3), with an expression on CD4 + Th1 cells, CD8 + cytotoxic T cells, and CD11b + dendritic cells (DC), but not on Th2 cells or macrophages [25].

Gal-9 and its receptors trigger T cells apoptosis via regulating the immune response induced by Th1 and Th17 cells [26]. Gal-9 triggers apoptosis of fibroblast like synoviocytes (FLS) which could protect the joint from synoviocyte hyperproliferation. So, upregulation of Gal-9 and Gal-9-Tim-3 pathway could be promising in the future of RA therapy [27].

On the contrary, some studies reported Gal-9 as a participant in the immunomodulatory of RA exerting its effect on granulocytes which triggers spontaneous apoptosis, migration and release of the pro-inflammatory

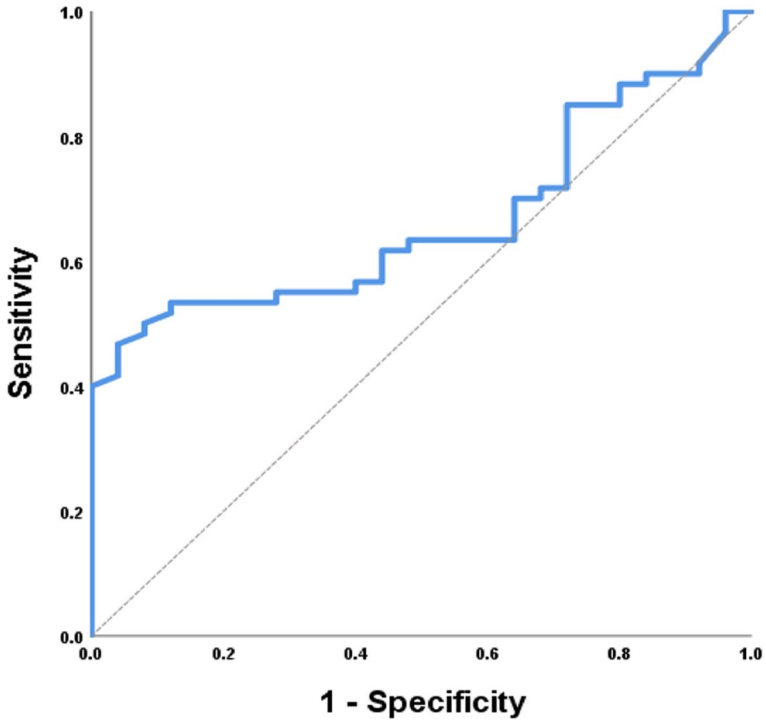


Fig. 3 ROC curve of Gal-9 level for discrimination between RA cases and control groups. A receiver operating characteristic curve of Gal-9 levels was conducted for discrimination between RA cases and control groups. At best cut of value of Gal-9 was 142.7 pg/ml, sensitivity was 53.3%, specificity was 88%, PPV was 53.3%, NPV was 88%, and accuracy was 63.5%

mediators IL-8 and TNF. It also markedly increases the intracellular level of the enzyme PAD-4 which is responsible for catalyzing the citrullination of proteins which are key predictors of RA [21].

The study in hands investigated the link between the Gal-9 gene (LGALS9) polymorphisms and the susceptibility of RA in Egyptian patients. Also, the study evaluated the level of Gal-9 in the serum of RA patients and its relationship with LGALS9 polymorphism, disease activity and the radiological damage.

Our results showed that RA cases demonstrated a significantly higher frequency of SNP rs 4239242 TT genotype and T allele, with risk to develop RA. The control group had more heterozygous TC (60.0%) compared to RA patients (30.0%). These results coincided with the study of Vilar et al. [28] who found that the SNP rs 4239242 TT genotype was positively associated with RA and heterozygous TC were more prevalent in controls than RA cases.

In addition, Xu et al. [29] reported a positive association between TT genotype (rs4239242) of LGALS9 gene and the incidence of RA; they also detected the prevalence of TC genotype among controls in comparison to RA patients.

Our study didn't detect any link between the selected polymorphisms and the level of Gal-9 in the serum which is similar to the data demonstrated by Vilar et al. [28].

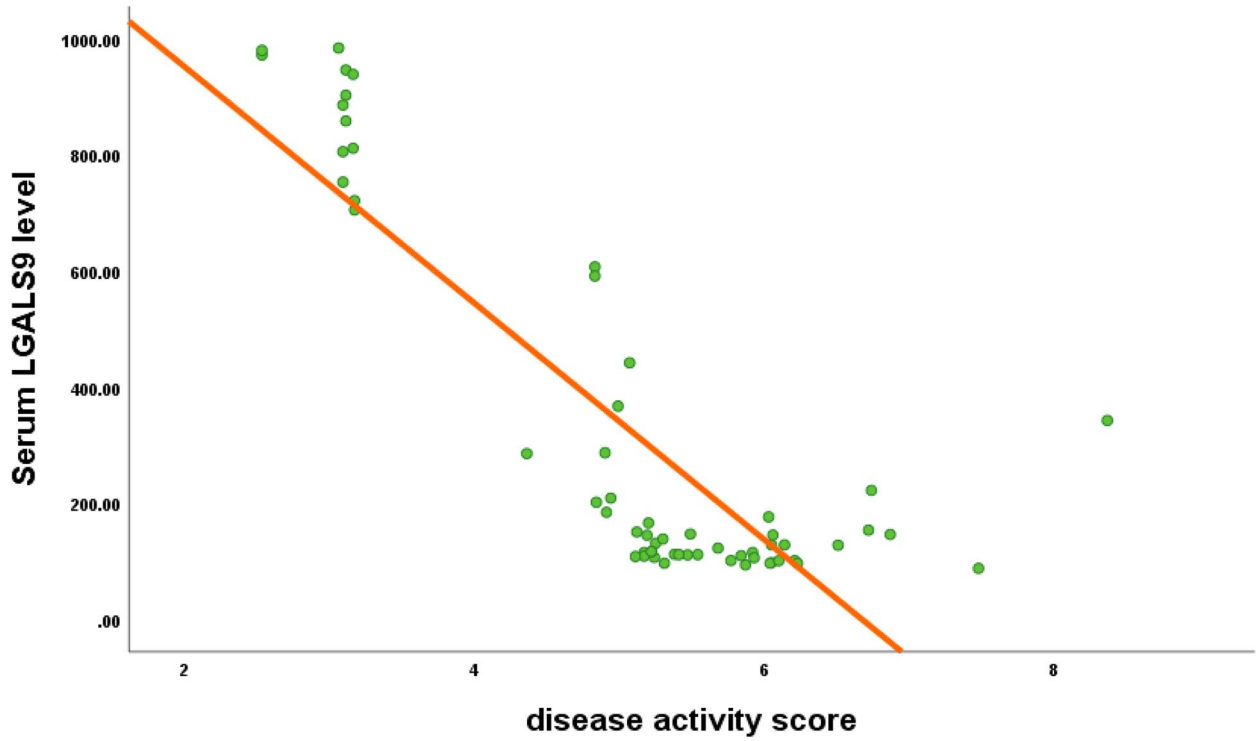
We also didn't detect any link between the selected polymorphisms and duration of RA, NTJ and NSJ, DAS-28 nor the Larsen score.

The present study showed that RA cases had significantly higher serum Gal-9 when compared to control group. In agreement with our results, Vilar et al. [28], Wang et al. [26], Wiersma et al. [21], and Fujita et al. [30] reported a significantly elevated Gal-9 serum levels in RA cases in comparison to controls.

Also, Lee et al. [31] reported a higher mRNA level of Gal-9 in peripheral blood mononuclear cells (PBMCs) of RA cases than healthy controls.

In the current work, we noticed that RA cases in remission or those with low disease activity had higher concentrations of serum Gal-9 in comparison to cases with moderate and high disease activity. Similarly, Vilar et al. [28] reported higher serum Gal-9 in RA cases in remission than those with moderately active RA.

Mansour et al. [32] evaluated the effect of Gal-9 administration on gouty arthritis and reported a reduction in both the inflammatory cell infiltrates and the



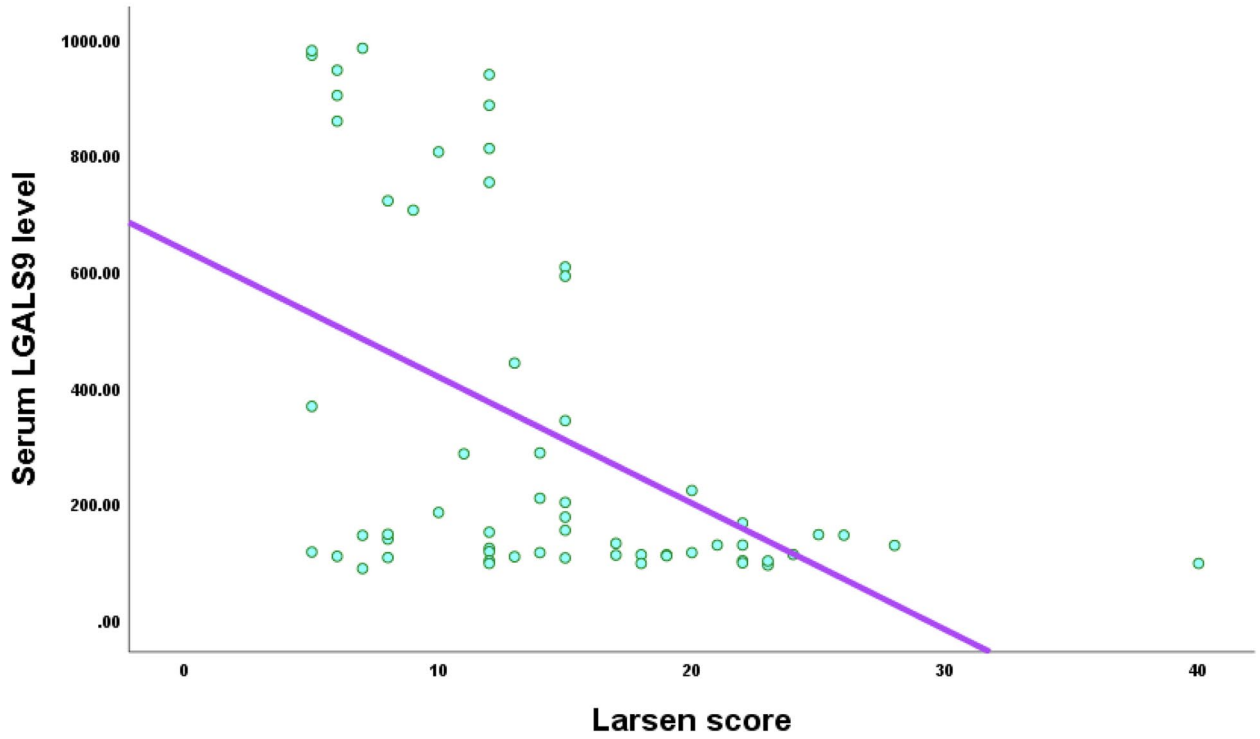


Fig. 4 Correlations of Gal-9 serum level with DAS-28 & Larsen score in RA group. Gal-9 level showed highly statistically significant negative correlations with RA disease activity (DAS-28) and Larsen score ($p < 0.001$)

Table 3 Association of RA disease activity (DAS-28) with Gal-9 serum level

		Galectin-9 level (pg/ml)		*p*
		Mean	SD	
Activity	Remission	975.6	5.6	**< 0.001 (HS)**
	Low	846.3	94.6	
	Moderate	352.7	162.1	
	High	128.8	44.6	

Kruskal–Wallis test was used for comparison. *P* probability, *HS* highly significant *P* (< 0.001)

pro-inflammatory cytokines released by neutrophils/inflammatory monocytes (e.g., IL-1a/b, TNF-a, junctional epithelium (JE), keratinocyte chemoattractant (KC) and Th17/Treg cell subtypes (e.g., IL-10, IL-17, GCSF) at the affected joint, suggesting the anti-inflammatory effect of Gal-9.

Contrarily, Fujita et al. [30] reported an elevated serum level of Gal-9 in RA cases which correlated positively with the disease activity without high titers of anti-cyclic citrullinated peptide (ACCP), also, Sun et al. [33] showed a positive association between the RA activity and both the Gal-9 level in the serum and PBMCs in 77% of RA cases.

The present study revealed that Gal-9 serum level showed significant negative correlations with the number of swollen joints, the number of tender joints, ESR titer, CRP titer, DAS-28 and Larsen score. In agreement with our study, Liu et al. [34] reported a negative correlation between the RA activity and the percentage of Gal 9 + cells in synovium and the level of Gal-9 in the synovial fluid. But, Fujita et al. [30] detected an elevated Gal-9 level in the plasma of RA cases with progressive joint damage.

While Wiersma et al. [21] and Fujita et al. [30] found that the RA activity was significantly correlated with the level of Gal-9 and Sun et al. [33] found a moderate positive correlation between Gal-9 levels and CRP, Simple Disease Activity Index (SDAI), and DAS28 in RA cases at baseline.

Per our results, the serum level of Gal-9 was not linked to the anti-CCP-Abs titer which agrees with the data reported by Fujita et al. [30] and Sun et al. [33].

The study in hands excluded all cases suffering from other autoimmune disease which could have an impact

Table 4 Regression analysis for prediction of RA disease activity

	Univariable				Multivariable			
	p	OR	95% CI		*p*	OR	95% CI	
Age	0.157 (NS)	0.977	0.947	1.009				
Gender	0.508 (NS)	1.361	0.546	3.393				
Family history	0.894 (NS)	0.942	0.388	2.285				
Duration	0.335 (NS)	1.027	0.973	1.084				
NTJ	**< 0.001 (HS)**	2.195	1.638	2.941	**< 0.001 (HS)**	2.458	1.560	3.872
NSJ	**< 0.001 (HS)**	2.145	1.587	2.900	**0.006 (S)**	1.882	1.201	2.948
ESR	**< 0.001 (HS)**	1.039	1.019	1.059	0.185	2.959	.465	5.841
CRP titer	0.129 (NS)	1.013	0.996	1.030				
Anti-CCP-Abs titer	0.101 (NS)	1.010	0.998	1.023				
RF titer	0.514 (NS)	1.003	0.995	1.011				
LGALS9 (CT + TT)	0.283 (NS)	0.581	0.215	1.567				
***Galectin-9 serum* level**	**< 0.001 (HS)**	0.983	0.974	0.992	**0.004 (S)**	0.943	0.914	0.974

OR odds ratio, *CI* confidence interval, *P* probability, *NS* non-significant *P* (> 0.05), *S* significant *P* (≤ 0.05), *HS* highly significant *P* (< 0.001), *NTJ* number of tender joints, *NSJ* number of swollen joints, *ESR* erythrocyte sedimentation rate, *CRP* C–reactive protein, *RF* rheumatoid factor, *Anti-CCP Abs* anti-cyclic citrullinated peptide antibodies, *LGALS9* Galectin-9 gene

of the targeted gene level. Yet, the results could be limited by the relatively small population so future studies on larger populations are needed to confirm the findings.

Conclusion

Rheumatoid arthritis is strongly linked to genetic alterations in the LGALS9 gene and the SNP rs4239242 TT genotype in the Egyptians. The detected polymorphisms in LGALS9 could be promising in the future of RA research. RA cases in remission or those with low disease activity had higher concentrations of serum Gal-9 in comparison to cases with moderate and high disease activity and this could be promising in the future of RA treatment.

Abbreviations

RA	Rheumatoid arthritis
Gal-9	Galectin-9
CRDs	Carbohydrate recognition domain
SNP	Single nucleotide polymorphism
NTJ	No of tender joints
NSJ	No of swollen joints
DC	Dendritic cells
Th1	T helper type 1
Th17	T helper type 17
PBMCs	Peripheral blood mononuclear cells

Acknowledgements

Not applicable.

Authors' contributions

All authors have read and approved the manuscript. Idea suggestion, study design: Magda Abd el-Aziz Zidan, Seham Gouda Ameen, Walid Abd Ellatif Abd Elhalim and Arwa S. Amer. Data collection and analysis: Nessma Fathy Elshahat, Arwa S. Amer and Seham Gouda Ameen. Manuscript writing and final revision: Arwa S. Amer, Walid Abd Ellatif Abd Elhalim, Seham Gouda Ameen and Magda Abd el-Aziz Zidan.

Funding

This research did not receive any specific grant from funding agencies whether public, commercial, or not-for-profit sectors.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

An informed written consent was taken from all patients and subjects' participating in this study and the protocol was approved by the ethical committee of Benha Faculty of Medicine no MS 17-8-2020.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 8 May 2023 Accepted: 22 June 2023
Published online: 10 July 2023

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