Increased plasma levels of Gas6 and its soluble tyrosine kinase receptors Mer and Axl are associated with immunological activity and severity of lupus nephritis

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Abstract

Objective

Growth arrest-specific 6 (Gas6) and its receptors have been shown to play a crucial role in the homeostasis of the innate immune system by regulating apoptosis and inflammation. We aimed to verify whether an impairment of this system is associated with systemic lupus erythematosus (SLE) disease activity and with lupus nephritis (LN).

Methods

Plasma Gas6 and the soluble cleaved form of the receptors MerTK (sMer) and Axl (sAxl) concentrations were measured in n=59 SLE patients (n=44 with nephritis, 75%) and analysed in relationship to clinical and laboratory data.

Results

Patients with LN were characterised by higher Gas6 (19.0 ng/mL [16.8–24.5] vs. 16.5 ng/mL [13.89–18.91]; p=0.03) and sAxl plasma levels than those without LN (31.36 ng/mL [25.1–41.4] vs. 20.2 ng/mL [15.6–30.7]; p=0.03); conversely sMer plasma concentrations were similar between groups. All the three biomarkers studied were directly correlated to creatinine and daily proteinuria, being inversely related to creatinine clearance. 39 patients had a proteinuria level of <0.5 mg/day, 14 between 0.5 and 3.5 mg/day and 5 had ≥3.5 g/day; Gas6, sAxl and sMer plasma concentrations significantly increased for increasing degree of proteinuria (test for trend p=0.0002; p=0.02; p=0.009, respectively).These correlations were confirmed in multiple linear regression analysis models accounting for gender, age, disease duration and concomitant treatment.

Conclusion

Plasma Gas6, sAxl and sMer concentrations are associated with the severity of LN in patients affected by SLE. The excess cleavage of TAM receptors might contribute to LN pathogenesis.

Key words

systemic lupus erythematosus, lupus nephritis, Gas6, TAM receptors

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Introduction

Growth arrest specific 6 (Gas6) is a vitamin K-dependent protein, cloned in the early 90s, and identified as a ligand for TAM receptors (an acronym for three tyrosine-kinase receptors Tyro3, Axl and MerTK) (1). Gas6 is widely expressed and has pleiotropic effects, including haemostasis regulation (2), cell growth and survival, and cancer development (3). Furthermore, the Gas6/TAM system plays a crucial role in the homeostasis of the innate immune system by mediating the phagocytosis of apoptotic bodies (efferocytosis) and negatively regulating the inflammatory cascade (1). Specifically, it suppresses IL-1, IL-6 and TNF-α expression in Toll-like-receptors (TLR)-activated monocytes/macrophages (4) and downregulates inflammatory responses in activated dendritic cells through induction of Suppressor of cytokine signalling (SOCS) 1 and 3 (5).

The extracellular domains of both MerTK and Axl can be cleaved and released to form soluble (s) Mer and sAxl; the latter, particularly, behaves as a potent decoy-receptor for the TAM-ligand Gas6 (6, 7). The shedding mechanism can, therefore, negatively regulate MerTK/Axl signalling by reducing both the number of membrane-bound receptors and the availability of free ligand. Interestingly, sAxl has been proposed as a biomarker of endothelial damage in different human diseases (8-11). The silencing of this system in the triple TAM knockout mice promotes the hyper-activation of innate and adaptive immunity, as demonstrated by the occurrence of broad-spectrum autoimmune lupus-like clinical and serological manifestations (12). Previous reports have described disturbances of the Gas6/TAM system homeostasis in systemic lupus erythematosus (SLE) patients (13, 14), although with erratic conclusions. In the present study, we aimed to quantify Gas6, sMer and sAxl plasma concentrations in a cohort of Caucasian patients affected by SLE, and to assess their correlation with the disease activity and the presence and severity of lupus nephritis.

Patients and methods

Fifty-nine SLE patients were consecutively enrolled at the Nephrology and Rheumatology outpatient clinic of the Ospedale Maggiore della Carità di Novara (Italy) over a 1-year period. After obtaining informed consent, 20-mL of whole blood were collected, and plasma and serum isolated and stored at -80°C. Patients' data were routinely recorded according to the local ethical guidelines. Procedures were performed in accordance with the code of conduct for responsible use of human tissue in medical research.

The following demographic, clinical and laboratory data were collected from patients records: age, gender, organ involvement, ongoing treatment and comorbidities, SLEDAI, SLICC, full blood cell count, serum creatinine, complement C3 and C4 fractions, 24-hour proteinuria, 24-hour proteinuria range (<0.5, 0.5–3.5, ≥3.5 g/dl), extended autoantibodies profile, erythrocyte sedimentation rate (ESR) and serum C-reactive protein (CRP). Clinical parameters and laboratory data were obtained on the same day as the sample collection. Thirty-four out of 59 patients (57.6%) were clinically required to undergo a renal biopsy, which was performed within one month from the recruitment date.

Plasma Gas6, sMer and sAxl were measured by an ELISA Kit DuoSet® (R&D Systems, Minneapolis, USA), according to the manufacturer's instructions.

Statistical analysis

The data were analysed using the biomedical statistical software package MedCalc v. 18.10.2 (MedCalc Software, Broekstraat 52, 9030, Mariakerke, Belgium). The Shapiro-Wilk test was performed to assess the normality of continuous variables. The measures of central tendency and dispersion used throughout the article were medians and interquartile ranges [IQR]. The Mann-Whitney test and the Kruskal-Wallis test were used to compare medians of continuous variables, as appropriate. Univariate (Spearman's correlation) and multiple linear regression analysis were used to test the associations between renal parameters and the plasma concentrations of the studied biomarkers. Jonckheere-Terpstra

Table I. Clinical features of the study population. Median values of continuous variables are presented along with interquartile range [IQR].

Demographics	
Age at sampling (years), median [IQR]	39.0 [31.5-53.7]
Female sex, n (%)	50 (84.7)
Disease duration (years), median [IQR]	6.0 [3.2-10.7]
Clinical manifestations	n. (% of total)
Lupus nephritis	47 (79.7)
Arthritis	36 (61.0)
Skin involvement	34 (57.6)
Haematological disorder	14 (23.7)
Serositis	11 (18.6)
Venous thrombosis	7 (11.9)
Arterial thrombosis	6 (10.2)
Neurological disorder	4 (6.8)
Autoantibodies	n. (% of total)
Antinuclear antibodies +	58 (98.3)
AntidsDNA +	45 (76.3)
Antiphospholipid antibodies +	13 (22.0)
Laboratory	median [IQR]
Creatinine (mg/dl)	0.8 [0.6-1.0]
Creatinine clearance (ml/min)	93.1 [63.0-112.8]
24h Proteinuria (g)	0.24 [0.07-0.60]
Haemoglobin (mg/dl)	12.4 [11.7-13.7]
Albumin (g/dl)	3.8 [3.6-4.2]
ESR (mm/h)	25.5 [12.0-36.0]
CRP (mg/dl)	0.15 [0.02-0.80]
C3 (mg/dl)	100.5 [83.0-118.0]
C4 (mg/dl)	19.5 [11.5-28.5]
SLICC damage index	1 [0-2]
SLEDAI	4 [2-6]
Treatment	n. (% of total)
Antimalarial	20 (33.9)
Corticosteroid	47 (79.7)
Immunomodulatory	36 (61.0)

ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; SLICC: Systemic Lupus International Collaborating Clinics.

test was used to compare medians of continuous variables for increasing degree of daily proteinuria. The level of statistical significance chosen was 0.05 (two-tailed).

Results

The main clinical features of the study population are described in Table I. As expected, the vast majority of SLE patients were females (n=50, 84.7%); the median age was 39.0 years [IQR 31.5–53.7].

The median Gas6 plasma concentration was 18.1 [16.2–23.2] ng/mL, being similar in patients with and without skin, joint or haematological involvement (see Table II). The overall sAxl median plasma concentration was 30.1 [20.1–40.5] ng/mL, while median sMer plasma level was 23.5 [19.2–27.7] ng/mL. As observed for Gas6, we could not detect significant differences in sAxl/sMer plasma levels depending on the presence/absence of skin, joint and haematological manifestations. Moreover, plasma levels of Gas6/sAxl/sMer were comparable between females and males (Table II).

As reported in Table I, 47/59 patients were diagnosed with lupus nephritis (LN). These patients were characterised by significantly higher Gas6 (19.0 ng/mL [16.8–24.5] *vs*. 16.5 ng/mL [13.9–18.9]; p=0.03) and sAxl (31.4 ng/mL [25.1-41.4] *vs*. 20.22 ng/mL [15.6–30.7]; p=0.03) plasma levels in comparison with those without renal involvement. Conversely, sMer plasma concentration was comparable between groups (23.5 ng/ml, [19.2–31.0] *vs*. 22.7 ng/ml [18.9–25.4], respectively; p=0.42) (Fig. 1).

To evaluate whether the levels of circulating TAM receptors and ligand would be associated with specific clinical variables, we correlated the plasma concentration of Gas6, sAxl and sMer with age (years), SLEDAI and SLICC scores, 24-hour proteinuria (mg), Hb (g/dl), ESR (mm/h), CRP (mg/dl), IgG (mg/dl), C3 (mg/dl), C4 (mg/dl), creatinine (mg/dl), creatinine clearance (ml/min), anti-dsDNA (IU/ml), and CKD class.

Concerning general disease activity markers, plasma concentration of Gas6 and sMer were both significantly positively correlated with SLEDAI (ϱ=0.27, p=0.04; ϱ=0.45, p=0.0005). A positive significant correlation was also

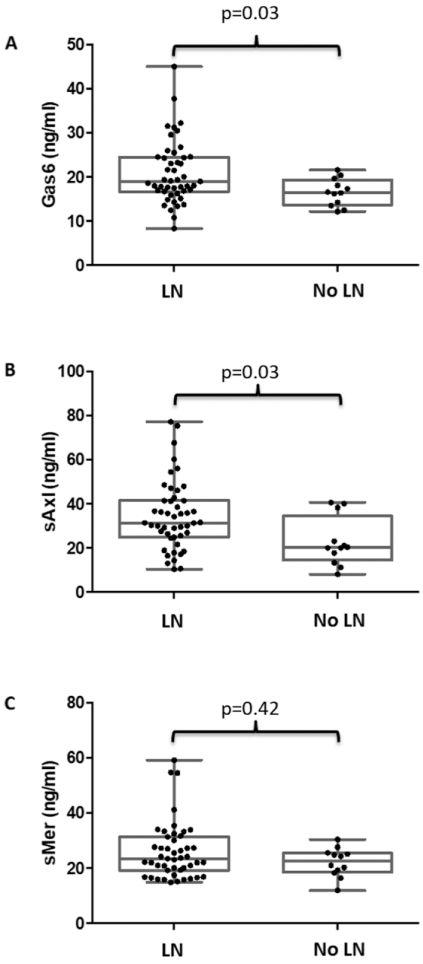

Fig. 1. Comparison of Gas6 (**A**), sAxl (**B**) and sMer (**C**) between patients with lupus nephritis (LN) and systemic lupus erythematosus without renal involvement (No LN).

Table II. Comparison between Gas6, sMer and sAxl plasma concentrations according to sex and organ involvement.

	Gas6 (ng/ml)			sAxl (ng/ml)			sMer (ng/ml)		
	Median [IQR]	Median [IQR]	*p*-value	Median [IQR]	Median [IQR]	*p*-value	Median [IQR]	Median [IQR]	*p*-value
Gender (F/M)	18.0 [16.3-23.3]	18.1 [15.3-23.4]	0.65	29.9 [20.0-40.6]	30.1 [28.2-39.5]	0.64	22.7 [19.2-27.6]	24.2 [19.9-30.4]	0.75
Skin involvement (n/y)	17.7 [14.6-23.1]	19.1 [16.6-24.3]	0.23	29.3 [19.5-36.6]	31.5 [20.4-41.4]	0.28	23.8 [18.8-28.0]	23.3 [19.2-27.7]	0.62
Haematological involvement (n/y)	18.1 [16.1-23.5]	18.4 [17.4-23.0]	0.69	29.6 [20.0-40.8]	34.8 [21.6-40.1]	0.65	23.1 [19.3-27.4]	24.7 [16.5-32.6]	0.68
Joint involvement (n/y)	17.9 [15.4-23.8]	19.1 [17.3-23.0]	0.33	29.7 [18.8-39.4]	31.4 [28.0-44.8]	0.15	24.0 [19.4-28.9]	23.1 [17.3-26.7]	0.59

IQR: interquartile range; F: females; M: males; n: no; y: yes. Medians were compared between groups by Mann-Whitney test.

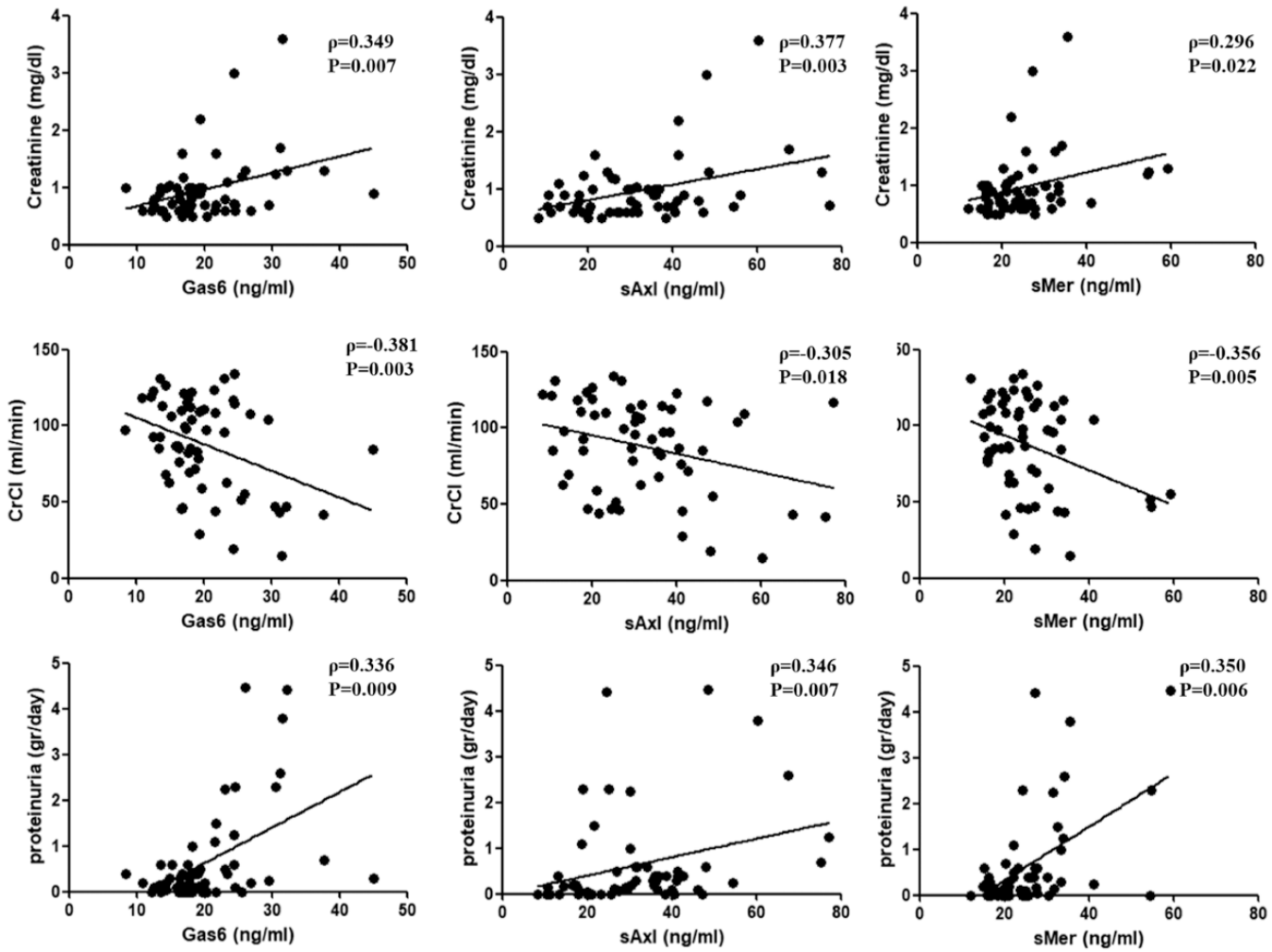

Fig. 2. Correlation between Gas6, sAxl and sMer plasma concentrations and markers of renal damage. CrCl: creatinine clearance.

observed between sAxl plasma concentration and SLICC (ϱ=0.37; p=0.005), C4 (ϱ=0.31; p=0.02) and ESR (ϱ=0.27; p=0.04). Conversely, sMer was negatively correlated to C3 (ϱ=-0.40; p=0.0002) (data not shown).

Notably, with specific regards to renal parameters, all the three biomarkers included in this study were positively correlated with plasma creatinine concentration and 24-hour proteinuria, and, consistently, negatively associated with the creatinine clearance, an index of renal functionality (Fig. 2). Thiry-nine patients had a proteinuria level of <0.5 g/day, 14 between 0.5 and 3.5 g/day and 5 had ≥3.5 g/day. As shown in Figure 3, increasingly high plasma levels of each of the TAM-family biomarkers (Gas6, sAxl and sMer) were associated with more severe proteinuria (test for trend p=0.0002; p=0.02; p=0.009, respectively). Conversely, Gas6, sAxl and sMer plasma concentrations were not dissimilar according to the different histological classes of LN (data not shown).

Multiple linear regression analysis

The above-described correlations between plasma concentration of Gas6, sAxl, sMer and renal parameters were further confirmed in a multiple linear regression analysis model including gender, age, disease duration and concomitant treatment. More specifically, Gas6 was an independent predictor of both 24-hour proteinuria (p=0.003) [along with male gender (p=0.01)], and creatinine clearance (p=0.004). Similarly, sMer predicted 24-hour proteinuria (p=0.004), along with male gender (p=0.04), and creatinine clearance (p=0.002), along with age (p=0.04). Finally, sAxl predicted 24-hour proteinuria (p=0.008), along with male gender (p=0.04), and creatinine clearance (p=0.02).

Discussion

The highly pleiotropic vitamin K-dependent protein Gas6 and its receptors are known to play a major role in fibrosis development and the regulation of apoptotic body clearance and inflammation, therefore representing not only a potential biomarker in different human diseases but also a promising therapeutic target (15-23). This is particularly true in SLE, where defective clearance of apoptotic debris, complement deficiency and persistent inflammation are critical pathogenetic mechanisms (1, 24). Furthermore, TAM knock-out mice are characterised by the development of multiple autoimmune manifestations, with a typical "lupus-like" phenotype (12, 25). In this study, we investigated a cohort of patients with a high prevalence of LN to better define the relationship between Gas6/TAM receptors and kidney involvement in SLE. Our results

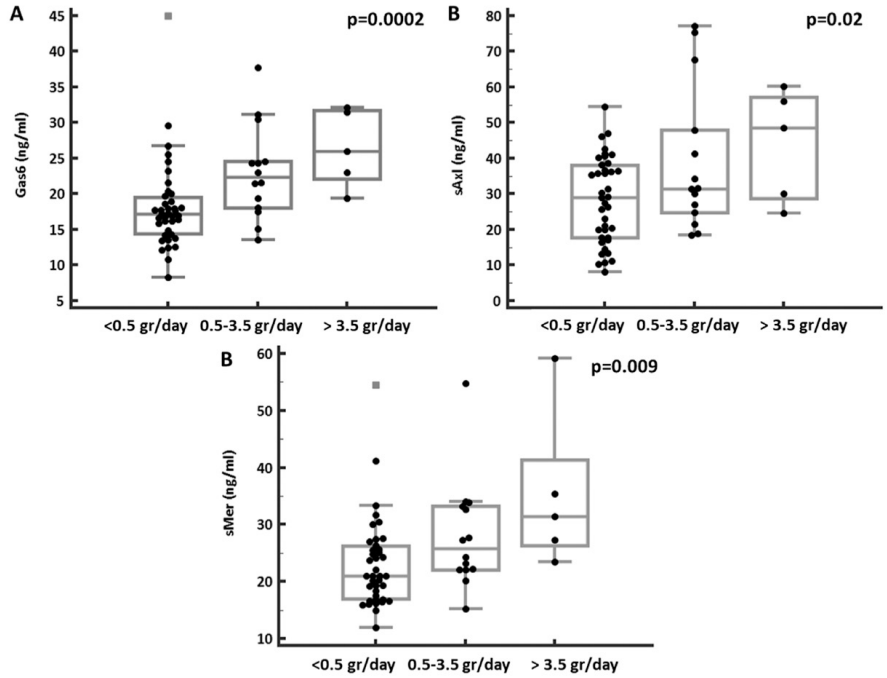

Fig. 3. Gas6, sAxl and sMer plasma levels according to the degree of daily proteinuria.

need to be interpreted at the light of the existing literature.

SLE is a heterogeneous chronic autoimmune condition characterised by a complex pathogenesis, only partially known. As several different biological systems are involved, a better understanding of the mechanisms leading to lupus development would allow the identification of disease biomarkers and the advancement of novel therapeutic strategies (26). Available data in the literature suggest that alterations of plasma Gas6, sAxl and sMer levels occur during SLE course (14, 27, 28); moreover, Gas6 and sAxl have been proposed as biomarkers for kidney involvement in this context, since plasma levels of Gas6 and sAxl are higher in SLE patients with LN than in non-renal lupus (29, 30). Our data confirm this hypothesis and, even more strikingly, suggest that Gas6, sAxl and sMer plasma levels significantly correlate with disease activity and parameters of renal involvement, including the degree of daily proteinuria and functional renal decay, measured either by creatinine or by creatinine clearance. Interestingly, these significant correlations were confirmed in multivariate models accounting for other potential confounders. Conversely, no associations were observed with the different histopathological aspects of LN, although the relatively small sample size might have affected this observation. The independent relationship between proteinuria and Gas6, sAxl and sMer levels also hints that these molecules may be highly expressed in patients with LN and CKD because of their pathogenetic role in the mechanisms leading to glomerulonephritis and not merely as a result of a retentive effect due to impaired renal function.

The association between high Gas6, sAxl and sMer concentration and the severity of kidney involvement in SLE patients has been reported before (13, 30, 31). Although the increased levels of Gas6 and the soluble forms of its receptors in SLE, correlating with disease activity and nephritis severity, have been increasingly recognised, the reason for this occurrence remains unclear. One plausible explanation for this finding is related to a deranged cleavage of the membrane receptor. The extracellular domains of TAM receptors can be proteolytically cleaved by metalloproteases to yield soluble forms of the receptor (sTAM). A disintegrin and metalloproteinase 10 (ADAM10) and 17 are the two enzymes primarily responsible for the generation of sTAM. The real biological function of these soluble forms is yet to be clarified, however, they probably act as decoy receptors and down-regulate Gas6/TAM signalling (32). Therefore, it is reasonable to postulate that the increase in sMer and sAxl reflects an increased proteolytic cleavage, which in turns drives a defective biological activity of the system. It has been previously shown that rat glomeruli express MerTK and that this receptor plays a pivotal role in the protection from serum-induced nephritis (33). Thus, cleavage of the renal MerTK might contribute to the development of kidney inflammation and glomerular damage, leading to proteinuria. It is also plausible that an increase in sMer is associated with defective clearance of apoptotic bodies, favouring the development of autoreactive B cells and the production of pathogenetic autoantibodies and immune complexes acting on the kidney. Consistently, shedding of Axl ectodomain and the subsequent raise in sAxl render mice prone to lupus development (34). Taken together, these findings suggest that a generalised dysregulation of the Gas6/TAM axis is a hallmark of this condition. TAM receptors cleavage might impair the Gas6/TAM system because of both the reduced number of available membrane receptors and the decreased amount of active ligands (Gas6 and Protein S) accessible to activate the membrane-bound receptors.

Sera rich in sAxl, sMer, and sTyro3 obtained from SLE patients are able to inhibit phagocytosis in healthy macrophages, further implying that this impairment is, at least partially, due to the "decoy" activities of sTAM receptors (14).

Whereas there is a wide acceptance of the association between sTAM plasma levels and SLE activity, the evidence about the role of Gas6 levels is more heterogeneous. Our data are concordant with those of other authors reporting higher Gas6 plasma levels in SLE patients (27), particularly in the presence of renal involvement (31), but other studies offered different conclusions; for instance, Suh *et al*. found no significant overall differences between the levels of Gas6 in SLE patients and healthy controls (35). Since SLE is a highly

heterogeneous condition with multiple factors, including ethnicity and genetic determinants, profoundly influencing the disease outcome, a comparison of different cohorts might be particularly challenging. Moreover, Gas6 quantification by itself is unlikely to be the ideal method to assess the activity of the Gas6/TAM axis, which is complex and highly integrated. Hence, since the contribution of the soluble forms of TAM receptors is not negligible, this must be taken into account to comprehensively investigate the impact of this biological system in specific diseases

The main limitation of our study is linked to the retrospective design and lack of a prospective follow-up that could limit the interpretation of data. Moreover, the majority of patients enrolled in this study were already on treatment and at different stages of the disease. Additionally, the prevalence of LN was high (74.6%) due to the recruitment of most patients from a Nephrology clinic.

In conclusion, Gas6/TAM system is deranged in SLE patients, but whether this dysregulation is pathogenetically relevant or a simple epiphenomenon of the disease remains controversial. SLE patients, especially with renal involvement, seem to be more prone to heightened TAM receptors cleavage, which might reduce Gas6/TAM axis activation and cause impaired deactivation of inflammatory responses and defective clearance of apoptotic bodies. Thus, it is reasonable to postulate that Gas6 and its receptors may be utilised as potential diagnostic and/or prognostic biomarkers of renal disease in lupus, and the Gas6/TAM system could represent a future therapeutic target for the management of SLE.

Results coming from pre-clinical studies suggest that targeting the Gas6/Axl pathway is a promising strategy for treating lupus nephritis (36); for instance, a selective inhibitor of Axl (R428) has been reported to be effective in a murine model of human lupus nephritis (37). Blocking the proteolytic cleavage of the membrane receptors represents an alternative tentative approach, and the restoration of TAM function by targeting sTAM proteases has been already proposed as a therapeutic strategy in SLE (38).

Therefore, further studies are needed in this field, to better comprehend the real relevance of Gas6/TAM system in the pathogenesis of SLE since this might contribute to the development of novel target therapies.

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