UNIVERSITÀ DEL PIEMONTE ORIENTALE

SCHOOL OF MEDICINE

Department of Health Sciences

Master's degree in Medical Biotechnologies

Master Thesis

GAS6/TAM SYSTEM: POTENTIAL PROGNOSTIC BIOMARKERS FOR MULTIPLE SCLEROSIS.

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ABSTRACT

Introduction

The protein growth arrest specific 6 (Gas6) and its tyrosine kinase receptors Tyro-3, Axl, MerTK (TAMs) are ubiquitous proteins involved in regulation of inflammation, and apoptotic bodies clearance. Multiple sclerosis (MS) is the most common non-traumatic inflammatory demyelinating disease of the central nervous system (CNS) resulting in characteristic lesions, which lead to progressive and irreversible disability if not diagnosed and treated promptly. Gas6 and TAMs have been associated with neuronal remyelination and stimulation of oligodendrocyte survival.

Methods

In a retrospective cohort study, we enrolled 64 patients with a diagnosis of Clinical Isolated Syndrome (CIS), Radiological Isolated Syndrome (RIS) or Relapsing-Remitting (RR) MS according to McDonald 2017 at the end of the follow up. Before treatment, we collected serum, CSF and clinicals data (clinical course, presence of gadolinium-enhancing (Gad+) lesions and expanded disability status score (EDSS)). EDSS, MS severity score (MSSS) was assessed at the last clinical follow up visit. Gas6 and the levels of the circulating form of TAMs (sAxl, sMer and sTyro-3) were determined with ELISA kit (R&D Systems) and compared to neurofilaments (NFL) levels evaluated with SimplePlexTM fluorescence-based immunoassay. Data were analysed with Mann–Whitney, Kruskal-Wallis test and Spearman's rank correlation coefficient.

Results

All biomarkers were detectable in both serum and CSF with the exception of sMer which was not detected in the CSF. Moreover, high level of sAxl was associate with an EDSS ≤ 3 at diagnosis ($p = 0.037$) and in patients without EDSS progression ($p = 0.017$). Higher CSF Gas6 concentrations were found in patients with an EDSS ≤ 3 at diagnosis ($p = 0.04$), while serum Gas6 concentrations were inversely correlated to MSSS ($r^2 = -0.32$ and $p = 0.01$). Results were confirmed by multivariate analysis. Serum and CSF NFLs are associated with EDSS ($p = 0.005$ and $p = 0.002$) and directly correlate with MSSS ($r^2 = 0.27$ and $p = 0.03$; $r^2 = 0.39$ and $p = 0.001$).

Conclusions

Taken together our data suggest a protective role of Gas6 and its receptor in patients with MS making them potentially suitable severity disease biomarkers.

1. INTRODUCTION

1.1 Multiple sclerosis

Multiple sclerosis (MS) is an autoimmune disease that affects the central nervous system (CNS). The main features of this condition encompass inflammation, demyelination and progressive disability (Garg & Smith, 2015). In 2020 the estimated people affected by MS was 2.8 million with a global prevalence was 35.9 affected per 100,000 people and the annual incidence 2.1 every 100,000 people (Walton et al., 2020). The female-to-male ratio is between 2:1 and 4:1 depending on geographic area and population (Rangachari et al., 2022).

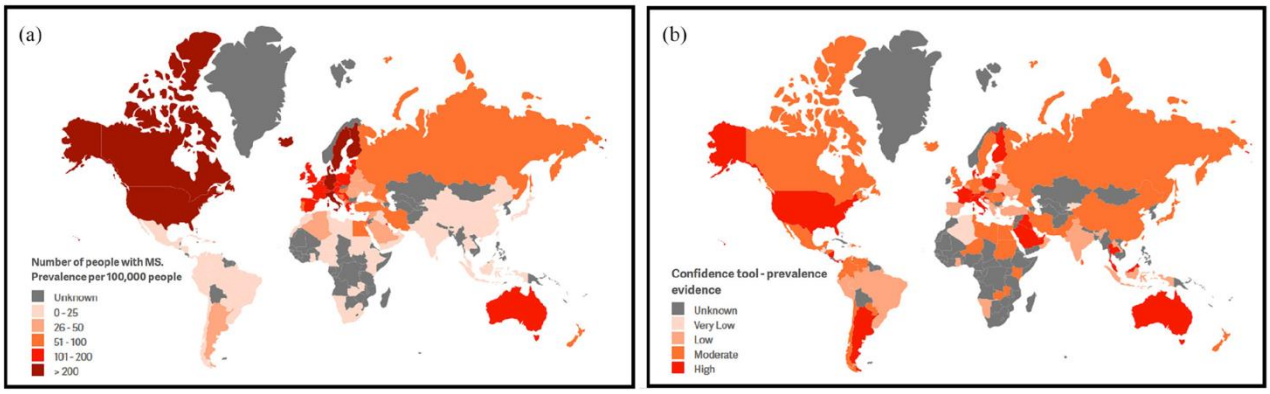


Figure 1. *Map showing the prevalence variation of MS for each country (Walton et al., 2020)*

The pathogenetic trigger of the disease is still unclear but what is known is the involvement of an inflammatory and neurodegenerative component. In particular, inflammation contributes to the activation of astrocytes that plays an important role in the phagocytic process by recruiting microglia into the site of demyelination and producing regenerative mediators (Sen et al., 2022). Moreover, inflammation leads to the destruction of oligodendrocytes (ODs), whose essential function is the myelination of neurons in the CNS (Trapp & Nave, 2008). The pathologic hallmark of MS is the development of demyelinating plaque, which are characterized by the presence of CD8+ and CD4+ T cells. Nevertheless, immunoglobulin-producing B cells also participate in the pathogenesis of the disease, as confirmed by the presence of oligoclonal bands (OCBs) (Deisenhammer et al., 2019).

MS is characterized by different clinical presentations, which can vary from patient to patient. Symptoms are closely correlated with the affected areas of the brain (**Table 1**) (Filippi et al., 2018).

Table 1: *Typical and atypical clinical presentations of MS (Filippi et al., 2018)*

Presentation	Typical or atypical presentation	onset	Involvement	Signs or symptoms	Recovery
Optic neuritis	Typical	Sub- acute to chronic (hours to days)	Unilateral	Afferent pupillary defect Central visual blurring or scotoma Reduced visual acuity. Dyschromatopsia (colour blindness) Normal optic disc or optic disc swelling Mild unilateral orbital pain that is worsened by eye movements	Gradual recovery within 2–4 weeks after reaching peak severity
	Atypical	Acute (seconds to minutes)	Bilateral	Peripheral visual loss Altitudinal visual loss Retinal haemorrhages or exudates Severe optic disc swelling No light perception No or severe orbital pain. Photophobia	Progressive worsening or no recovery
Brainstem and/or cerebellar syndromes	Typical	Sub- acute and/or chronic (hours to days)	Unilateral and localized	Unilateral or bilateral internuclear ophthalmoplegia Multidirectional nystagmus Sixth cranial nerve palsy Ataxia or gait imbalance Vertigo Facial numbness or sensory loss Dysmetria and decomposition of complex movements	Gradual recovery starting within 2–4 weeks after reaching peak severity

				Dysarthria and slurred speech Dysphagia Hearing loss Nausea	
	Atypical	Acute (seconds to minutes)	Alternating syndromes	Vascular territory signs Isolated trigeminal neuralgia Fluctuating ocular or bulbar weakness Fever Meningism	Progressive worsening or no recovery
Myelitis	Typical	Sub-acute and/or chronic (hours to days)	Incomplete transverse myelitis Asymmetric involvement	Sensory involvement: paresthesias (numbness, tingling, pins- and-needles feeling, tightness, coldness and/or swelling of the limbs or trunk), Lhermitte sign, impairment of vibration and joint position sense, decreased pain and light touch perception and Uhthoff phenomenon. Motor deficits: pyramidal signs (Babinski sign, bright reflexes and clonus), spastic paresis and/or weakness (asymmetric) and spasticity Sphincter dysfunction: urinary urgency, hesitancy, urge incontinence, constipation and faecal incontinence.	Gradual recovery within 2–4 weeks after reaching peak severity

				Sexual dysfunction: erectile dysfunction and impotence	
	Atypical	Acute (seconds to minutes)	Complete transverse myelitis Complete Brown-Séquard syndrome Cauda equina syndrome Anterior spinal artery territory lesion Localized or radicular spinal pain	Progressive and symmetrical spastic paraparesis Progressive sensory ataxia (posterior column involvement) Sharp level to all sensory modalities Segmental loss of pain and temperature sensation Areflexia and/or spinal shock Acute urinary retention Severe pain	Progressive worsening or no recovery
Cerebral hemispheric syndromes	Typical	Sub- acute and/or chronic (hours to days)	Unilateral	Hemisyndrome (corticospinal tract involvement): hemiparesis and hemisensory deficits Campimetric deficits (optic radiation involvement)	Gradual recovery within 2–4 weeks after reaching peak severity
	Atypical	Acute (seconds to minutes)	Bilateral	Encephalopathy Epilepsy Cortical blindness Headache Intracranial hypertension	Progressive worsening or no recovery

1.1.2 Risk factors

Both environmental factors and genetic susceptibility are involved in the development of the disease. The only etiological factor ascertained is Ebstein Barr virus (EBV), even though the mechanism whereby the infection leads to the arise of MS is still unclear (Bjornevik et al., 2022). The most popular hypothesis is that through a mechanism of molecular mimicry EBV can lead to the production of autoreactive T cells and plasma cells (Wieland et al., 2022). Moreover, both passive and active cigarette smoke has been linked in a dose-dependent manner to the development of the disease (Manouchehrinia et al., 2022). Conversely, some studies have shown that snuff (oral tobacco) may have a protective role (Wu et al., 2023).

Recent evidence also correlates the level of exposure to sun ultraviolet radiation B (UV-B) with MS. Exposure to UV-B is the major determinant in the production of vitamin D in the human bodies. Low levels of vitamin D are a possible risk factor for the development of the disease while a normal status might play a protective role (Scazzone et al., 2021). Recently, other factor has been identified that could promote the onset of the disease such as: alcohol and coffee consumption, obesity at young age, organic solvent exposure and viral infection (Filippi et al., 2018).

Along with all environmental factors, genetic susceptibility may also contribute as a risk factor. Actually, 13% of the total cases of MS are familial, moreover, monozygotic twins are 35% more likely to develop the disease, while sibling is 3% more prone. Genome-wide association identified roughly 200 polymorphisms, the majority are related to HLA class I and II, these interacting with particular environmental factors confer a higher risk of developing the disease (Marrosu et al., 2006).

1.1.3 Clinical course

MS do not have a single and established clinical course (**Figure 2**) (Klineova & Lublin, 2018a). Even though it is not included in the possible presentation of MS, radiologically isolated syndrome (RIS) is a possible first manifestation of the disease. In this stage there is no real diagnosis of MS, but only abnormalities in the Magnetic Resonance Imaging (MRI) examination that may suggest a possible ongoing demyelization phenomenon (Lebrun-Frénay et al., 2023.). Subsequently, RIS can evolve in Clinically Isolated Syndrome (CIS) whit a conversion rate of 30-35%. CIS is known to be the first presentation of the disease in approximately 80% of MS patients. CIS is an unexpected neurological event involving one or more areas of CNS, which may have an acute or subacute clinical course (Rossi et al., 2015). Unlike RIS, CIS requires the demonstration of the presence of neurological impairment (Miller et al., 2012). In presence of normal MRI scan, the likelihood of developing clinically definitive MS (CDMS) is around 20%, while in case of abnormal MRI or CSF T2 lesions or OCBs the conversion rate increases up to 80% (Uher et al., 2014).

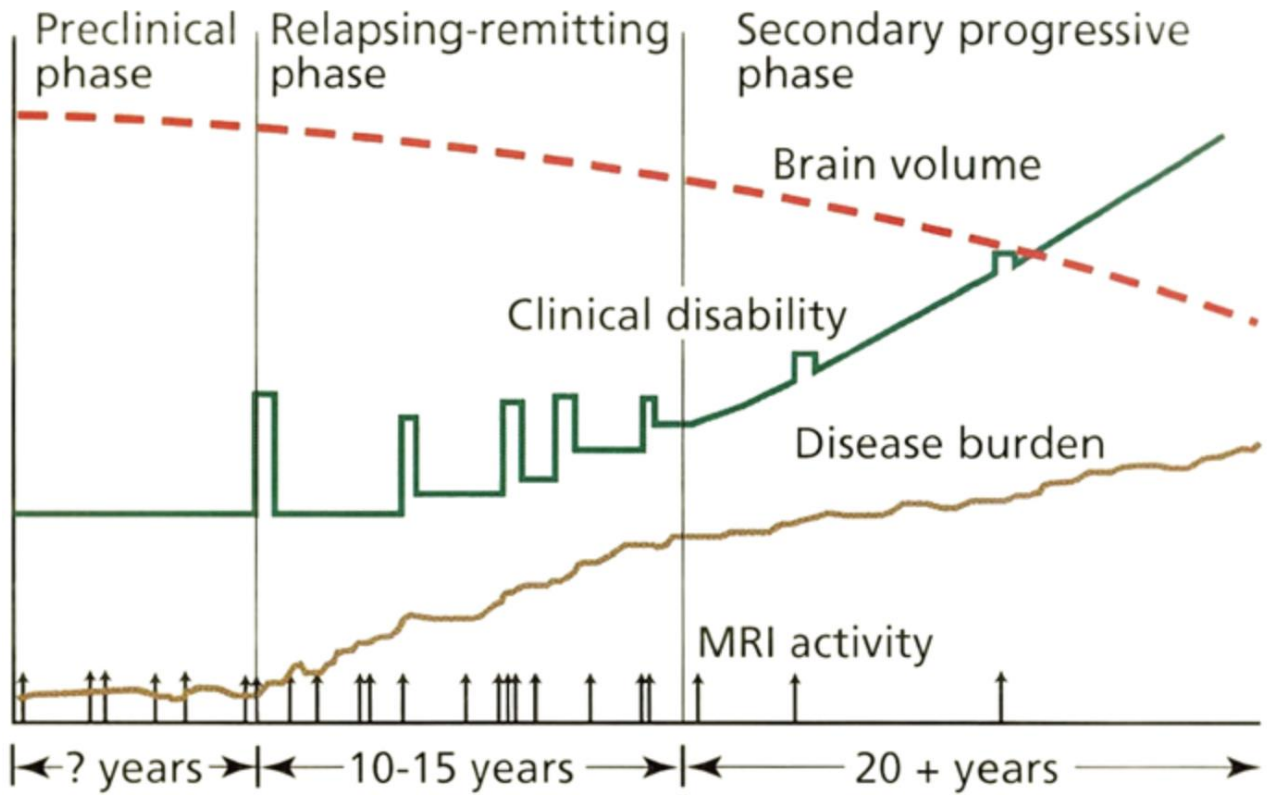


Figure 2. *Possible clinical courses of MS. The black arrows indicate disease progression identifiable with an MRI, the green line shows the progressive accumulation of disability due to attacks, while the brown line emphasises the burden of disease (Https://Www.Clevelandclinicmeded.Com/Medicalpubs/Diseasemanagement/Neurology/Multiple_sclerosis/, n.d.).*

CDMS may have different clinical courses: relapse-remitting MS (RRMS), primary progressive MS (PPMS) and secondary progressive MS (SPMS); among them, RRMS is the most common (85% of patients). This pattern is characterized by alternating relapses and periods of stability (as shown in **Figure 3**) in which there is no disease progression but instead a recovery of symptoms acquired during relapse (Ruiz et al., 2019). The time to relapse is variable but usually do not exceed 1.5 event per year. The neurological symptoms during relapses have a wide spectrum (already reported in **Table 1**). Recovery from relapse can be both complete and incomplete, around 30% of patients experiencing residual effects (Baecher-Allan et al., 2018). The presence of residual impairment may be an indicator of irreversible axonal lost or incomplete remyelination, while a full recovery from relapse suggests that the process of remyelination is ongoing. Nonetheless, inflammation and relapse frequency decreases with disease progression and age.

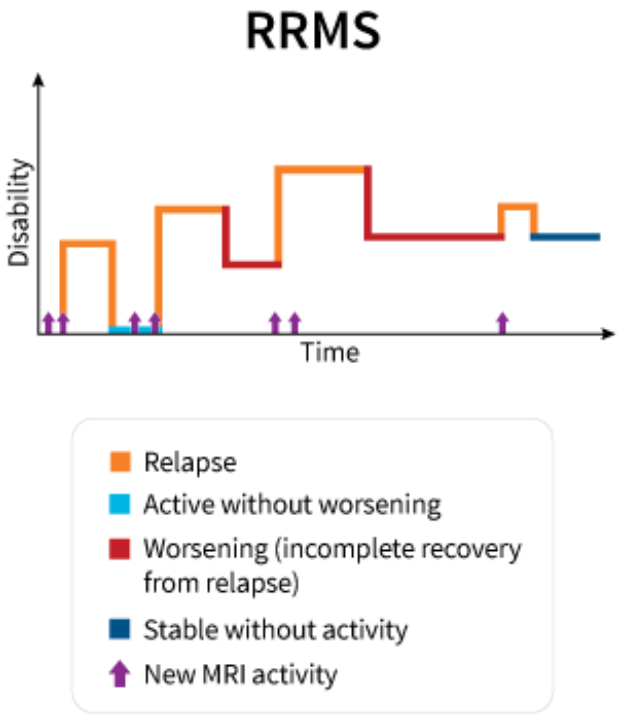


Figure 3. *Disease course of relapsing remitting multiple sclerosis (Klineova & Lublin, 2018b)*

RRMS could evolve in SPMS over time (**Figure 4A**) with an average time of 19 years after the onset of RRMS (Inojosa et al., 2021). This stage of the disease in characterized by a progression of symptoms, although there is no new evidence in the MRI. A progressive phenotype, without the initial RR phase, is found in about 10-20% of the patients and it is called PPMS (**Figure 4B**) (A. Thompson, 2004).

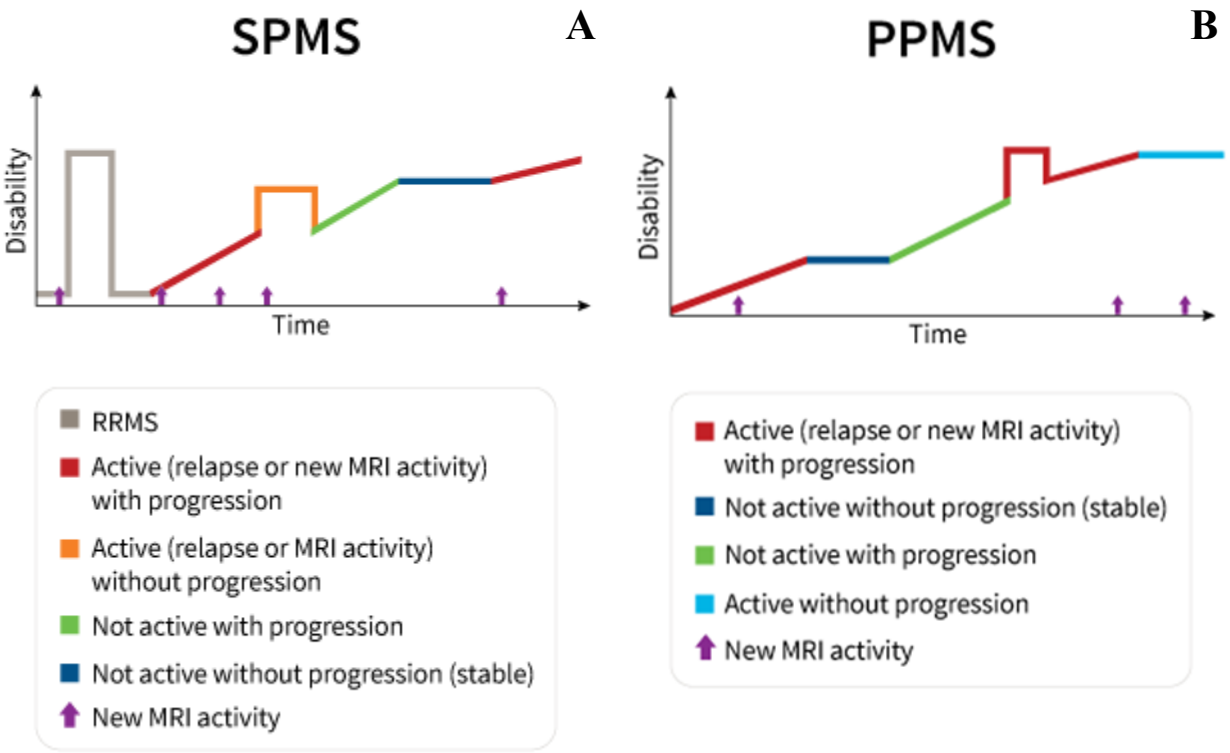


***Figure 4**. Disease course of secondary progressive multiple sclerosis (**A**) and primary progressive multiple sclerosis (**B**) (Klineova & Lublin, 2018b).*

1.1.4 Diagnosis

Diagnosing MS can be complex. Beyond medical history and physical examination, MRI is an extremely valuable tool in MS diagnosis for detecting macroscopic abnormalities in the brain and spinal cord (**Figure 5**) (Tomassini et al., 2020). It is performed using *Gadolinium* as contrast agent, a chemical compound with high molecular weight capable of diffusing into the CSF in the presence of BBB damage. A lumbar puncture can be useful in order to obtain supplementary diagnostic markers, such as increased IgG index and presence of OCB (Filippi et al., 2022).

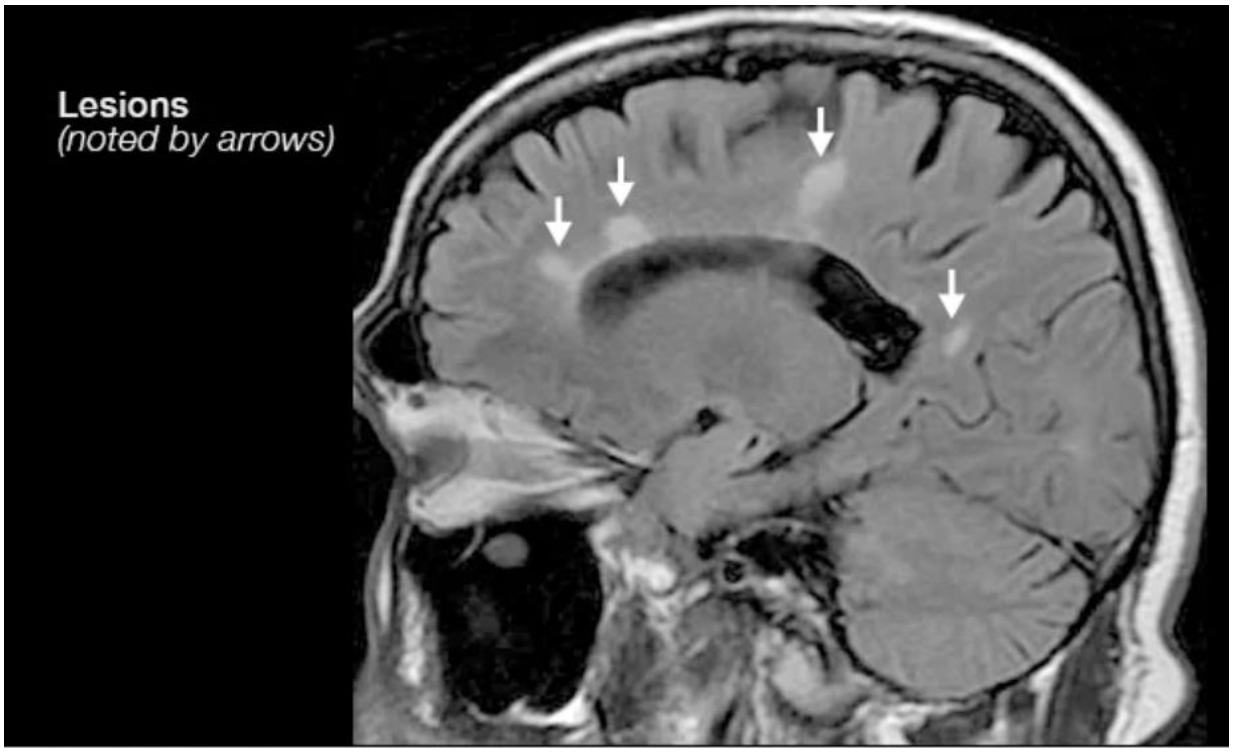


Figure 5. *This image shows an MRI scan of a patient with MS. The lighter areas indicated by the white arrows indicate injured areas with loss of myelin.* (*Https://Www.Mayoclinic.Org/Diseases-Conditions/Multiple-Sclerosis/Multimedia/Multiple-Sclerosis-Mri-Scan/Img-20135010*, n.d.)

1.1.5 McDonald criteria

The diagnostic criteria currently used are the 2017 version of the McDonald criteria (A. J. Thompson et al., 2018). To make a diagnosis, the presence of lesions scattered over time and space is required, which can be identified based on the following criteria (**Table 2**):

1. Dissemination in time and space (DIS and DIT) is demonstrated through medical history and clinical examination. This involves experiencing two or more attacks separated by at least 1 month, each lasting more than 24 hours, and showing two or more clinical signs indicating involvement of different anatomical sites, without the need for additional criteria.
2. If there have been two or more previous attacks (DIT), evidence during neurological examination indicates involvement of a single area, but there is evidence of DIS shown by MRI or subsequent attack.
3. A single attack is considered if evidence during neurological examination indicates involvement of at least two systems (DIS) and there is evidence of DIT demonstrated by MRI (lesions that enhance with contrast and black holes) or positive oligoclonal bands (OCB), or if a second clinical attack occurs.
4. In cases where there is a clinical attack with evidence during neurological examination indicating involvement of a single area (DIS and DIT not supported by medical history and clinical examination), it suggests clinically isolated syndrome. In such cases, to demonstrate DIS and DIT, an additional attack in another area with different signs during neurological examination and additional lesions on MRI are necessary. Alternatively, to demonstrate DIT, a positive cerebrospinal fluid examination is required.
5. If there is no history of attacks or presence of lesions, but there is a positive result for two or three criteria including cerebrospinal fluid examination, brain MRI, or spinal cord MRI, it is defined as radiologically isolated syndrome (RIS).

Table 2. *The 2017 McDonald criteria for diagnosis of multiple sclerosis in patients with an attack at onset*

	Number of lesions with objective clinical evidence	**Additional data needed for a diagnosis of multiple sclerosis**
≥2 clinical attacks	≥2	None*
≥2 clinical attacks	1 (as well as clear-cut historical evidence of a previous attack involving a lesion in a distinct anatomical location)	None*
≥2 clinical attacks	1	Dissemination in space demonstrated by an additional clinical attack. implicating a different CNS site or by MRI
1 clinical attack	≥2	Dissemination in time demonstrated by an additional clinical attack or by MRI OR demonstration of CSF-specific oligoclonal bands
1 clinical attack	1	Dissemination in space demonstrated by an additional clinical attack implicating a different CNS site or by MRI. Dissemination in time demonstrated by an additional clinical attack or by MRI OR demonstration of CSF-specific oligoclonal bands

1.1.6 Disability scale

The Expanded Disability Status Scale (EDSS) was developed in 1983; it is a scoring system based on neurological examination. EDSS ranges from 0 to 10 subdivided in 20 steps, each of them represents an increase of 0.5 in the scale (**Figure 6**). The EDSS score is calculated by a neurologist and assesses ambulation and seven functional system (FS) namely: visual, brainstem, pyramidal, cerebellar, sensory, bowel and bladder and cerebral function (Meyer-Moock et al., 2014a). The influence of these parameters in the final score varies from 0 to 4/5/6, nonetheless, ambulation has a predominant role on the formulation of the final score (Şen, 2018a). Moreover, the EDSS scale can be segmented in three portions: from the score 0 to 4 suggests a mild to moderate disability, from 4.5 to 7.5 it reflects a limitation of ambulation, above 7.5 indicates the absence of ambulation in patients, a score of 10 corresponds to the patient's death (Lublin et al., 2022). However, EDSS has some limitations such as the fact that it is mainly focused on walking ability and the final score is operator-dependent and the result can vary depending on the examiner.

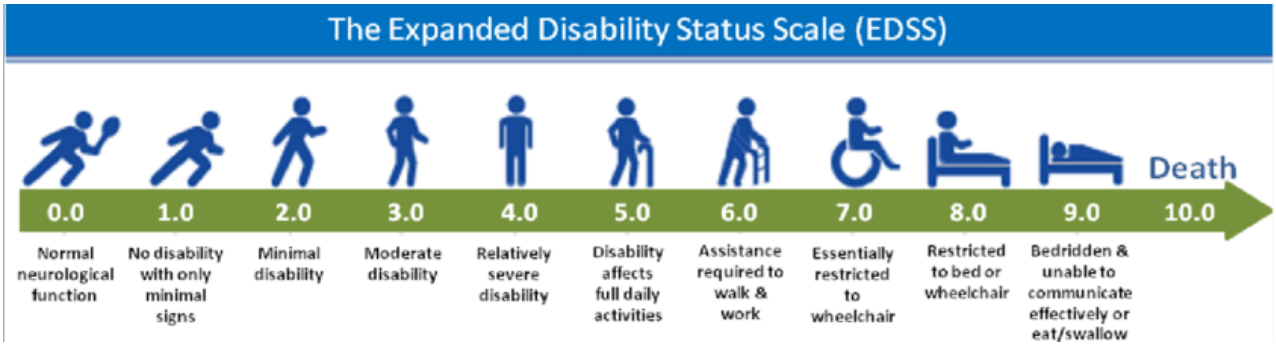


Figure 6. *EDSS disability scale, main disability characteristics are specified for each value (Spiteri & Allensbach, n.d.)*

Multiple Sclerosis Severity Score (MSSS) is defined as *"the decile of the EDSS within the range of patients who have had the disease for the same disease duration"* (**Figure 7**). This disability measurement scale relates EDSS to years since the onset of disease. MSSS is used for the classification proposed by Herbert in 2006 (**Figure 8**). In this classification MS patients are divided into six equi-populated tiers of disability depending on their MSSS (Charlson et al., 2016a).

	0	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9	9.5	EDSS
1	0.67	2.44	4.30	5.87	7.08	7.93	8.64	9.09	9.35	9.50	9.63	9.74	9.84	9.90	9.94	9.97	9.98	9.98	9.99	
2	0.53	2.01	3.69	5.24	6.46	7.27	7.98	8.58	8.95	9.18	9.38	9.59	9.79	9.88	9.93	9.97	9.99	9.99	9.99	
3	0.45	1.77	3.34	4.82	6.00	6.81	7.54	8.14	8.55	8.83	9.07	9.35	9.63	9.77	9.86	9.92	9.97	9.98	9.99	
4	0.35	1.45	2.87	4.27	5.41	6.24	6.98	7.65	8.12	8.42	8.70	9.08	9.47	9.68	9.80	9.88	9.95	9.98	9.99	
5	0.30	1.28	2.60	3.90	4.95	5.79	6.58	7.26	7.75	8.08	8.38	8.83	9.32	9.60	9.76	9.86	9.95	9.98	9.99	
6	0.25	1.13	2.33	3.54	4.55	5.38	6.14	6.81	7.33	7.66	7.98	8.50	9.08	9.45	9.68	9.81	9.93	9.97	9.99	
7	0.24	1.04	2.10	3.17	4.13	4.96	5.75	6.46	6.98	7.32	7.65	8.24	8.91	9.33	9.59	9.76	9.90	9.95	9.99	
8	0.21	0.94	1.92	2.93	3.81	4.57	5.36	6.10	6.61	6.95	7.32	7.97	8.71	9.21	9.55	9.74	9.89	9.96	9.99	
9	0.21	0.88	1.76	2.65	3.45	4.17	4.93	5.64	6.14	6.50	6.90	7.65	8.53	9.09	9.47	9.70	9.87	9.95	9.99	
10	0.19	0.78	1.53	2.34	3.10	3.79	4.55	5.28	5.77	6.14	6.58	7.39	8.31	8.92	9.34	9.61	9.83	9.94	9.99	
11	0.17	0.71	1.40	2.13	2.82	3.46	4.21	4.94	5.42	5.82	6.30	7.18	8.15	8.79	9.24	9.52	9.78	9.92	9.98	
12	0.16	0.64	1.28	1.98	2.64	3.25	3.94	4.63	5.13	5.54	6.03	6.92	7.93	8.63	9.13	9.43	9.71	9.88	9.97	
13	0.13	0.57	1.14	1.80	2.44	3.05	3.70	4.38	4.91	5.32	5.80	6.74	7.83	8.55	9.03	9.34	9.65	9.85	9.96	
14	0.11	0.49	1.03	1.70	2.33	2.91	3.55	4.26	4.82	5.23	5.70	6.56	7.59	8.34	8.86	9.20	9.57	9.82	9.95	
15	0.10	0.45	0.99	1.64	2.26	2.82	3.44	4.14	4.68	5.09	5.51	6.33	7.41	8.17	8.70	9.11	9.51	9.78	9.94	
16	0.09	0.38	0.85	1.42	1.99	2.56	3.17	3.86	4.41	4.81	5.18	6.00	7.14	7.97	8.54	9.04	9.49	9.75	9.94	
17	0.05	0.32	0.76	1.28	1.77	2.30	2.95	3.65	4.17	4.55	4.94	5.74	6.89	7.77	8.38	8.99	9.52	9.79	9.96	
18	0.04	0.26	0.66	1.12	1.57	2.09	2.70	3.37	3.89	4.27	4.62	5.43	6.62	7.54	8.23	8.94	9.51	9.78	9.96	
19	0.05	0.28	0.63	1.00	1.39	1.89	2.50	3.19	3.72	4.12	4.49	5.35	6.59	7.51	8.22	8.98	9.57	9.81	9.96	
20	0.05	0.26	0.59	0.94	1.29	1.71	2.29	2.99	3.51	3.93	4.30	5.15	6.43	7.45	8.23	8.98	9.58	9.80	9.95	
21	0.05	0.30	0.66	1.02	1.39	1.77	2.34	2.97	3.43	3.83	4.21	5.09	6.35	7.33	8.08	8.87	9.49	9.77	9.96	
22	0.04	0.23	0.54	0.90	1.28	1.66	2.20	2.82	3.29	3.69	4.09	5.04	6.35	7.35	8.10	8.84	9.42	9.73	9.95	
23	0.05	0.27	0.58	0.91	1.26	1.64	2.19	2.78	3.21	3.69	4.19	5.16	6.47	7.46	8.20	8.87	9.43	9.75	9.95	
24	0.05	0.24	0.52	0.86	1.25	1.63	2.15	2.71	3.09	3.52	4.01	5.03	6.36	7.38	8.15	8.81	9.39	9.74	9.96	
25	0.05	0.23	0.47	0.77	1.15	1.56	2.05	2.53	2.84	3.21	3.74	4.88	6.26	7.24	8.00	8.73	9.35	9.75	9.98	
26	0.05	0.20	0.45	0.78	1.17	1.58	2.08	2.63	2.99	3.40	3.95	5.02	6.39	7.44	8.21	8.89	9.48	9.80	9.96	
27	0.05	0.22	0.48	0.78	1.15	1.56	2.03	2.56	2.91	3.29	3.86	4.93	6.33	7.38	8.14	8.91	9.56	9.85	9.98	
28	0.04	0.17	0.40	0.74	1.16	1.52	1.88	2.39	2.76	3.04	3.46	4.54	5.99	7.07	7.90	8.75	9.45	9.80	9.98	
29	0.03	0.18	0.47	0.80	1.19	1.51	1.79	2.27	2.68	3.01	3.41	4.35	5.68	6.76	7.66	8.62	9.38	9.75	9.96	
30	0.01	0.13	0.45	0.82	1.19	1.45	1.69	2.23	2.75	3.13	3.50	4.35	5.61	6.66	7.54	8.47	9.27	9.67	9.91	
Years																				

=1st Decile
=2nd Decile
=3rd Decile
= 4th Decile
= 5th Decile
= 6th Decile
= 7th Decile
= 8th Decile
= 9th Decile
= 10th Decile

Figure 7. *MSSS disability scale (Roxburgh et al., 2005)*

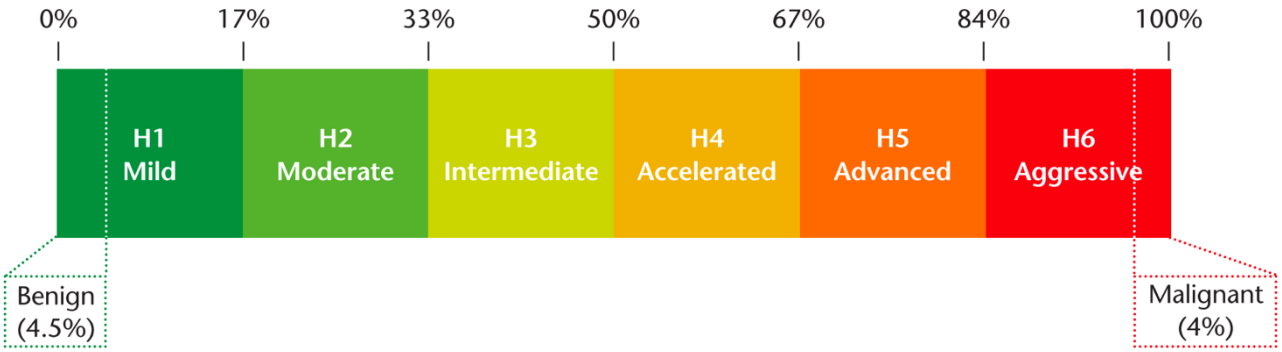


Figure 8. *Herbert's severity grading divides patients with multiple sclerosis into six approximately equipopulated tiers of disability based on their Multiple Sclerosis Severity Score (Charlson et al., 2016b)*

Another way to evaluate the disease progression is the Age-Related Multiple Sclerosis Severity (ARMSS). It is and adjustment of the MSSS algorithm taking into account the age of the patients. This kind of severity score offers several advantages compared to MSSS; the patient's age is easier to obtain than the date of onset of symptoms (Manouchehrinia et al., 2017). Date of symptoms onset is influenced by the patient's ability to recall past events, and moreover the skill of the physiologist in interpreting them must be taken into account. ARMSS is also useful to compare patients from different courts and returns a more comprehensive picture of the patient's disability score (Kirk et al., 2020).

1.2 Neurofilaments

Neurofilaments (NFs) are the major components of the mature neurons; they are part of the intermediate filament family and together with microtubules and microfilaments form the neuronal cytoskeleton (Gordon, 2020). NFs are divided in three categories according to the molecular weight: 68 kD for the light neurofilaments (NFLs), 150 kD for the medium neurofilament (NFMs) and between 190 and 210 for the heavy (NFHs). The final protein is a heterotrimer, every monomer is composed of NF-L as core of the peptides and then dimerize whit NF-M or NF-H (Petzold, 2022). Neurofilaments play a crucial role in the structure and function of neuronal axons, the main role is to contribute to the structural support, while their differential gene expression and phosphorylation influence the diameter and growth of the axons, the conductibility and the level of myelination. Neurofilaments are one of the only few possible biomarkers, not only for MS, but also for other pathology in which damage to neurons is implicated (Williams et al., 2021). After the damage to the neurons, NFL are released in the interstitial fluid in the CNS and then in the CSF, if the BBB is damaged, they could be scattered in the bloodstream (Bomont, 2021).

1.3 Gas6 (growth arrest specific gene 6)

Gas6 gene was isolated for the first time in 1988; five years later the gene was cloned in humans by the same scientific group. The protein produced by the Gas6 gene was identified as a member of the vitamin K-dependent (VKDPs) family and shares the 46% of its sequence with another important protein of this family is protein S (PROS-1), which plays a crucial role in the complement activation and coagulation cascade. The length of Gas6 gene is 2556 nucleotides and encode for a 70kD protein composed by 678 ammino acids (**Figure 9**) (Mark et al., 1996); it is a multimodular protein that contain three domains:

- N-terminal domain also called Gla module and it the domain of γ-carboxyglutammine acid residues.
- Four epidermal grow factor (EGF) modules rearranged in tandem constitute the central domain of the protein.
- C-terminal steroid hormone-binding globulin (SHBG)-like domain that contains two laminin-globular (LG) modules.
- Even though Gas6 and PROS-1 incorporate a disulfide-bridged thumb loop, Gas6 does not seem to be cleaved by serine proteases. (Bellido-Martín & de Frutos, 2008)

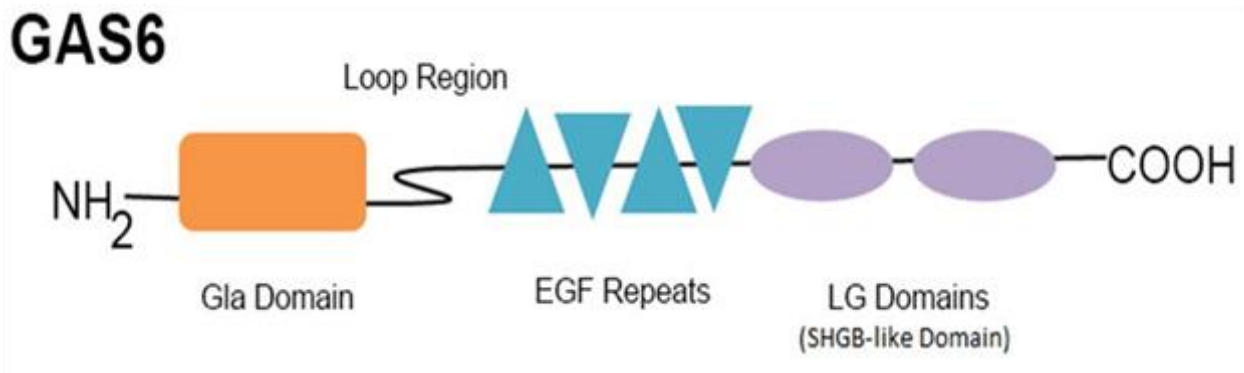


Figure 9. *Gas6 structure. Abbreviation: Gla domain = γ-carboxyglutammine; EGF repeats = epidermal grow factor; LG Domains = laminin-globular; SHGB-like Domain = steroid hormone-binding globulin* (Law et al., 2018a).

Gas6 and the other protein belonging to the VKDPs family require carboxylation to become biologically active. In presence of reduced vitamin K the enzyme γ-glutamyl carboxylase (GGC) facilitates the carboxylation of glutamic acid residue within the GLA domain (Danziger, 2008). Gas6 is quite unique among the other proteins of the vitamin-K dependent family because its synthesis does

not take place in the liver; indeed, the liver level of Gas6 is lower than PROS1, while the level in other tissue is higher compared to the other protein of the same family. This is confirmed by the evaluation of the mRNA and protein in murine model, that ascertains their presence in various murine tissues such as the central nervous system (SNC), heart, lung, Sertoli cells, Leydig cells, gastric epithelium, endothelial cells, vascular smooth muscle cells (VSMCs) (Yin et al., n.d.).

Gas6, together with its receptor, is involved in a wide range of biological processes (**Figure 10**) and, despite its name, the main roles are cell homeostasis, differentiation and proliferation and rescue from apoptosis (Torii & Yamauchi, 2016). According to the current literature, Gas6 plays a key role in homeostatic processes such as microglial efferocytosis and modulation of the function of BBB and in some pathological conditions of the CNS, such as Alzheimer's disease (Goudarzi et al., 2020).

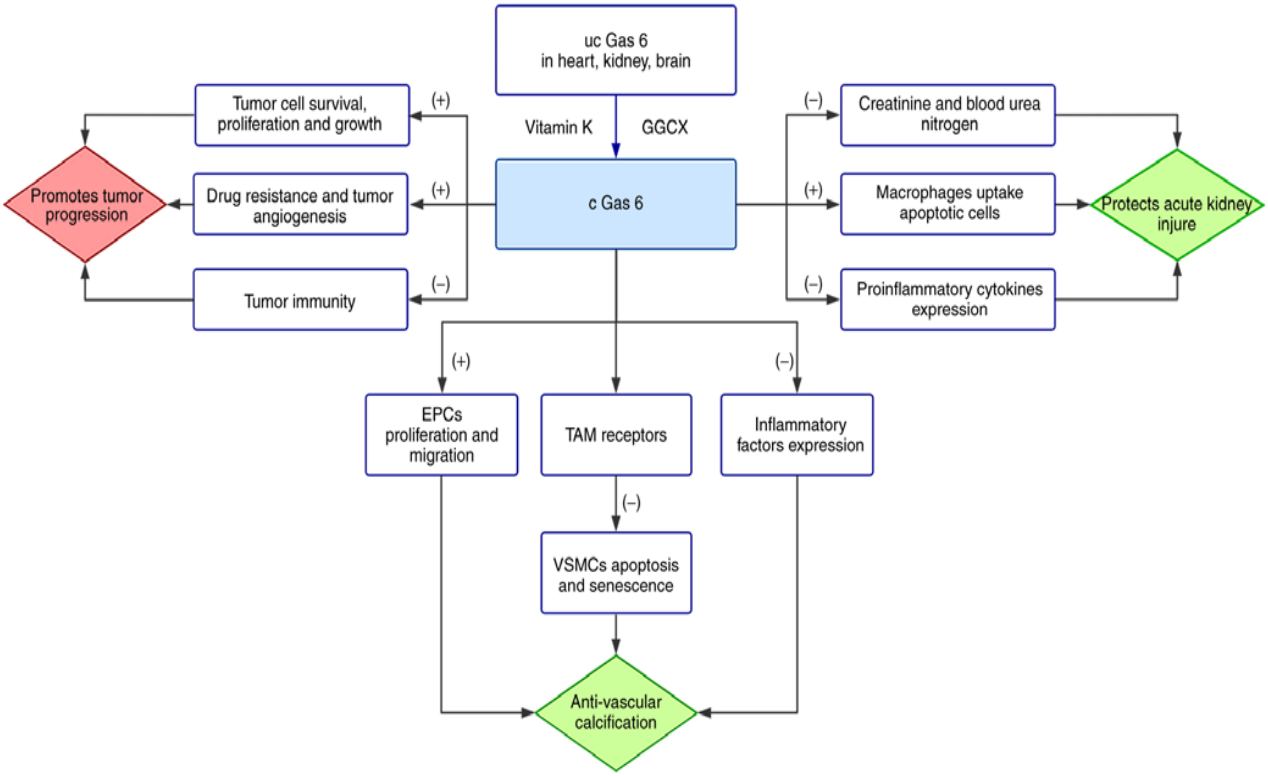


Figure 10*.Functional mechanisms of Gas6. Functional mechanisms of Gas6. The '+' refers to promotion and '-' refers to inhibition. Green represents Gas 6 physiological effects and red represents its pathological effects. Gas 6 is widely expressed in heart, kidney, brain, and other tissues. Abundant vitamin K ensures the activation from un uncarboxylated Gas6 (uc Gas6) to carboxylated Gas 6 (c Gas6) in the body. Gas 6 resists vascular calcification through three mechanisms: i) Gas 6 promotes proliferation and migration of endothelial progenitor cells (EPCs); ii) Gas 6 inhibits apoptosis and senescence of vascular smooth muscle cells (VSMCs) by binding Tyro3, Axl and Mer (TAM) receptors; iii) Gas 6 decreases expression of inflammatory factors, including TNF-α and ICAM-1. Similarly, Gas 6 protects from acute kidney injury: i) Gas 6 significantly reduces creatinine and blood urea nitrogen; ii) Gas 6 enhances macrophages to uptake apoptotic cells; iii) Gas 6 reduces the expression of pro-inflammatory cytokines, such as IL-1β. However, Gas 6 assists tumor progression: i) Gas 6 is necessary for survival, proliferation, and growth of tumor cells; ii) Gas 6 contributes to drug resistance and tumor angiogenesis; iii) Gas 6 negatively regulates tumor immunity (XIAO et al., 2021).*

1.4 TAM receptors

Tyro-3 (also called Rse, Sky, Brt, Tif, Dtk, Etk-2 and Resk), Axl (also called Ark, Ufo and Tyro-7) and Mer (also called c-Eyk, Nyk, Tyro-12 and MerTK) are the three members of the TAMs receptor family with reversible tyrosine kinase activity; the name TAM is obtained by the first letter of each receptor. They were discovered and cloned for the first time in the 1980s in a library composed by Schwann cell (Rothlin et al., 2015). As for PROS1 and Gas6, the three TAMs receptor share some homology in their structure (**Figure 11**), the extracellular portion of the receptor (amino terminal) consists of two immunoglobulin-like domains where the recognition of the LG domain of Gas6 and PROS1 occurs. The extracellular domain is made of two fibronectin type III (FNIII) repeats. The carboxyl-terminal domain which have the tyrosine kinase activity constitutes the intracellular portion of the receptor. The structure in composed by two homodimers (Linger et al., 2008a).

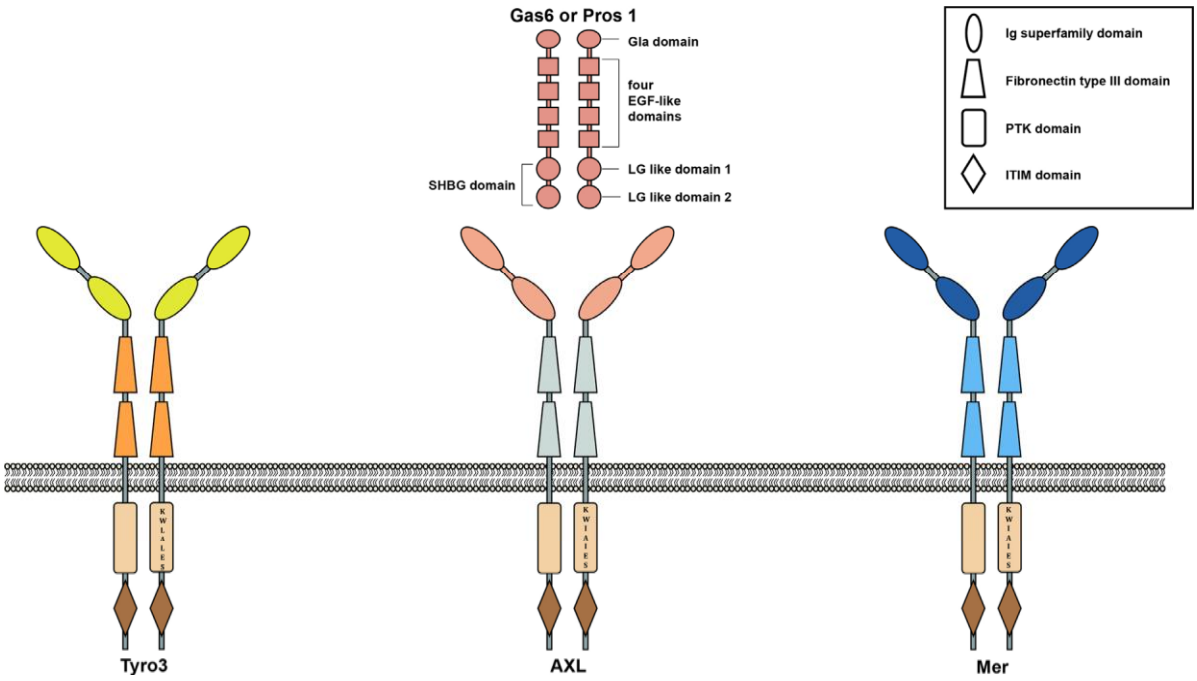


Figure 11. *Structures of TAM family receptors and their ligands. Ig superfamily domains of each TAM receptor recognize their ligands. The extracellular domain of each TAM family receptor contains two Ig superfamily domains and two fibronectin type III domains. The cytoplasmic tail of each TAM family receptor contains a well conserved "KW(I/L) A(I/L) ES" signature sequence. Also, the cytoplasmic tail of each TAM family receptor contains a conserved protein tyrosine kinase (PTK) domain and immunoreceptor tyrosine based inhibitory motif (ITIM) domain. Autophosphorylation sites of each TAM family receptor are located within PTK domain. Both Gas6 and PROS1 have γ- carboxyglutamate-rich domain (Gla domain) at their amino terminus. Also, both proteins contain four epidermal growth factor (EGF)- like domains and one sex hormone binding globulin (SHBG) domain which consists of two globular laminin G-like (LG) domain. Gas6 can bind to all three TAM family receptors with the highest affinity to Axl, whereas PROS1 can interact with Mer or Tyro3, but not Axl (Lee & Chun, 2019).*

Gas6 have a higher affinity for Axl but, anyway, can bind with lower affinity both Tyro-3 and MerTK; on the other hand, PROS-1 binds with higher affinity Tyro-3 and with lower affinity MerTK while can't be bound by Axl (Law et al., 2018b).

A crucial post-translational modification of TAMs receptors is the cleavage of the extracellular portion of the receptor. This process is operated by metalloprotease A disintegrin and metalloproteinase 17 (ADAM17) and A disintegrin and metalloproteinase 10 (ADAM10). The cleavage of MerTK in sMer (soluble Mer) is facilitated by MAPK p38 (**Figure 12**), and it occurs in two hotspot pro^{485} and ser^{468} (Vago et al., 2021). Researchers have suggested a similar mechanism for sAxl (soluble Axl), leading to the release of a soluble form of the receptor, weighting 65 kD. Since the extracellular portion of the receptor is still intact after the cleavage, sAxl and sMer are still able to bind Gas6 and can act as decoy receptors modulating the inflammatory response (Lemke & Rothlin, 2008).

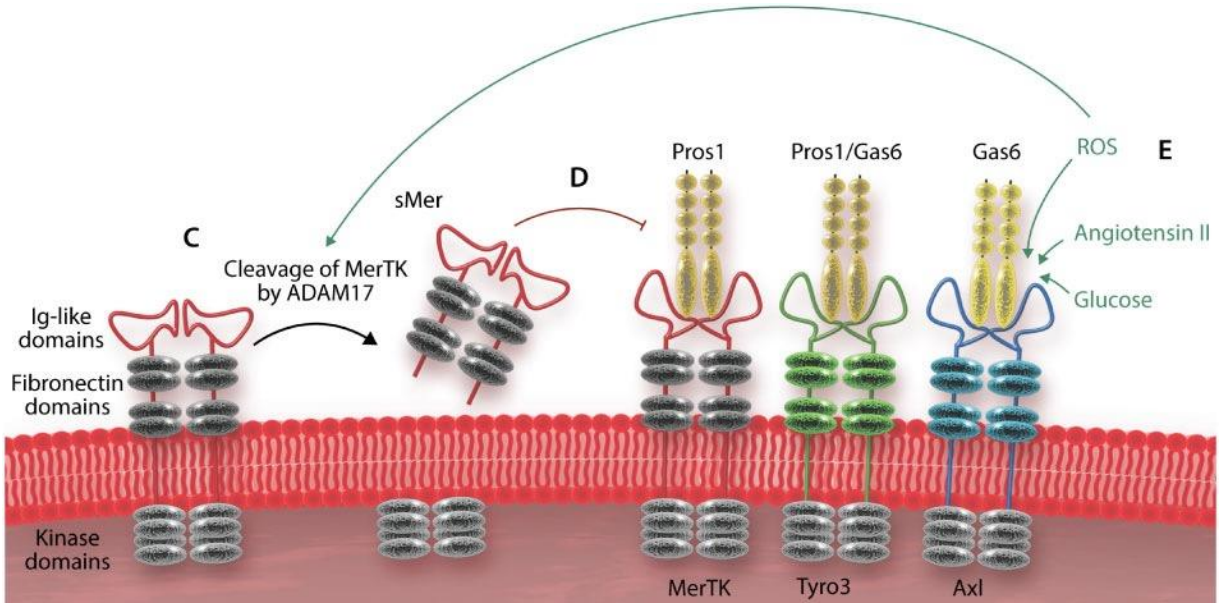


Figure 12. *Molecular regulation of the TAM receptors. The expression and activity of the TAM receptors is controlled by various factors at the transcriptional, post-transcriptional and protein levels. TAM receptor gene transcription can be up- or down-regulated by various factors, including cytokines. At the protein level, TAM receptors are rendered dysfunctional by cleavage of their extracellular domain by metalloprotease ADAM17. The soluble product released may act as a decoy for the receptor ligands, thus inhibiting TAM receptor activity. Activation of the receptors can also be enhanced by various environmental factors. Activation of the TAM receptors subsequently induce various molecular pathways affecting cell function (McShane et al., 2019).*

1.4.1 Function of TAM receptors

TAMs receptors and their ligands are the most highly expressed phosphatidylserine (Ptd-Ser) recognition system in macrophages. Ptd-Ser is one of the most expressed molecules in the membrane of cells and neurons, in physiological conditions it is not expressed in the out layer of the cell membrane. Conversely, when a cell undergoes to apoptosis, Ptd-Ser are exposed in the out part of the cytoplasmatic membrane and will act as an "*eat me*" signal, marking apoptotic cells for clearance. Moreover, TAMs are necessary for apoptotic clearance (AC) uptake by macrophage. The interaction between Ptd-Ser and TAMs is mediated by Gas6 and PROS1 that works as *"bridging molecules"*. The first link between a TAM signalling component and Ptd-Ser was revealed in 2003, identifying PROS1 as a serum protein which binds to Ptd-Ser (Anderson et al., 2003). After the identification of Gas6, the same functions were also assigned to it (Burstyn-Cohen & Maimon, 2019). TAM-mediated recognition and engulfment of Ptd-Ser-positive apoptotic cells is mediated by Mer and Gas6/PROS1, although in some cases, including infection, inflammation and tissue injury, this work is also mediated by Axl and Gas6. TAM receptors are furthermore involved in the infection by enveloped viruses. These viruses expose, on the envelope, Ptd-Ser and through a process called apoptotic mimicry can facilitate the entrance of a virus in the cells. As for AC, the link between TAMs and Ptd-Ser is mediated by Gas6 and PROS1. The activation of Axl suppresses the production of IFN type I in macrophage and dendritic cells, which is a powerful antiviral agent (Tutusaus et al., 2020).

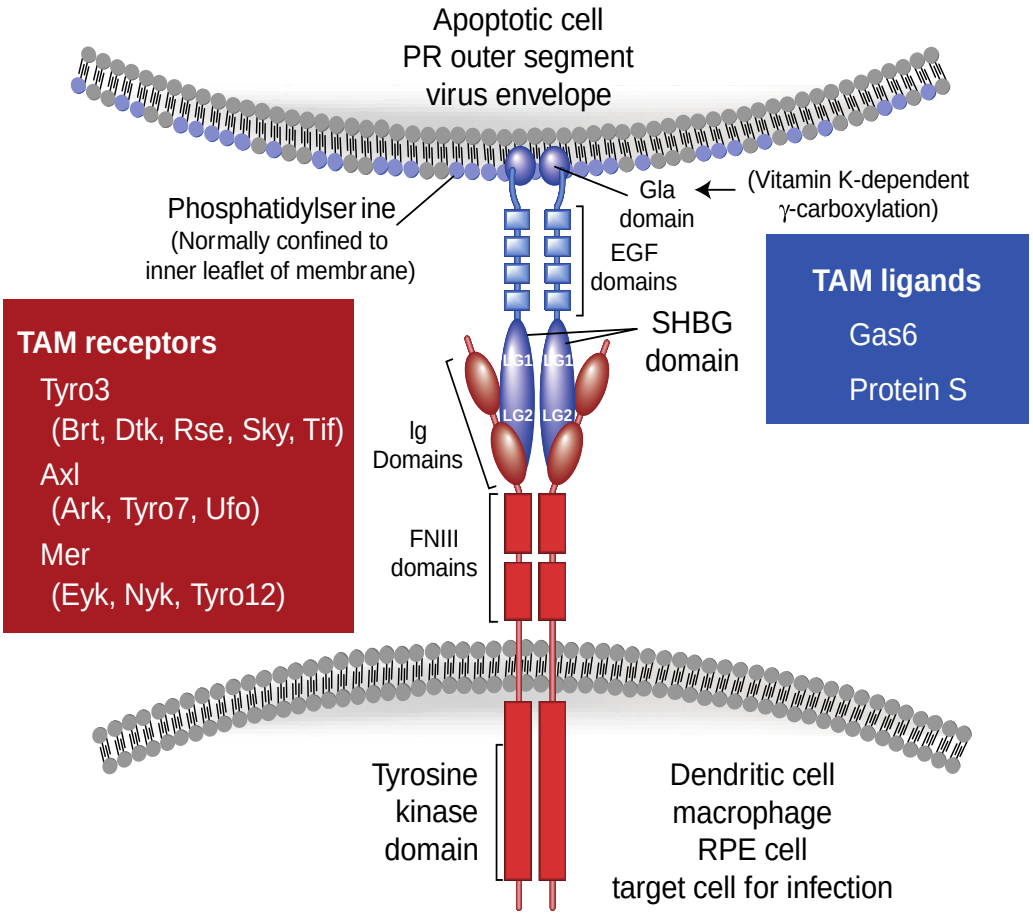


Figure 13. *Tam receptors and ligands. The TAMs are widely expressed by cells of the mature immune, nervous, vascular and reproductive systems. The TAM ligands (blue) are Gas6 and Protein S (PROS1). The carboxy-terminal SHBG domains of the ligands bind to the immunoglobulin (Ig) domains of the receptors, induce dimerization and activate the TAM tyrosine kinases. When g-carboxylated in a vitamin-K-dependent reaction, the amino-terminal Gla domains of the dimeric ligands bind to the phospholipid phosphatidylserine expressed on the surface on an apposed apoptotic cell or enveloped virus* ***(Lemke, 2013).***

A defective TAMs receptors activity seems to be implicated in the arise of autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Since the clearance of apoptotic bodies is one of the most relevant functions of the TAM receptors, a defect in this process and in the INF type I signalling leads to the development of autoimmune diseases (Rothlin et al., 2015). There are also associations between cancer and TAM receptors, since they are involved in several aspects in the development of a tumour. They regulate both the initiation and progression and in contraposition also play a role in anti-tumour response (Paolino & Penninger, 2016).

1.4.2 Tyro-3

Tyro-3 seems to have a role in physiological processes, such as immune regulation, phagocytosis and thrombosis (**Figure 14**). In the CNS, Tyro-3 gene is expressed in neurons, Schwann cells, oligodendrocyte, endothelial cells and in the hippocampus (*Prieto1999*, n.d.). It can also be found in monocytes, macrophages, NK cells, platelets and osteoclasts (Behrens et al., 2003). It is also expressed in other organs such as breast, ovary, testis, lung, kidney, and retinal epithelium. The human Tyro-3 protein incorporates 890 amino acids; the molecular weight is around 97 kD and due to post-translational modification, it can increase to a range of 120-140 kD. The Tyro-3 gene is expressed during CNS development, mainly in the piriform and hippocampal cortex but could be also found at the level of cortical neurons (Prieto et al., 2008). The expression of Tyro-3 in the CNS is regulated by the nerve growth factor (NGF) and is involved in the neuronal differentiation (Wang et al., 2011). The expression of Tyro-3 in axonal growth cone and dendrites suggests its possible implication in axonal pathfinding modulation of neuronal activity and plasticity (Thomas & Huganir, 2004). To exert this function, Tyro-3 has the capability of activating many signalling proteins involved in MAPK and PIK3 pathway. Moreover, Tyro-3 is co-expressed with other TAMs receptor in astrocytes, oligodendrocytes and glia. A model of Tyro-3 expression in the CNS also reported its possible implication in modulating postsynaptic neuronal excitability (Prieto et al., 2000).

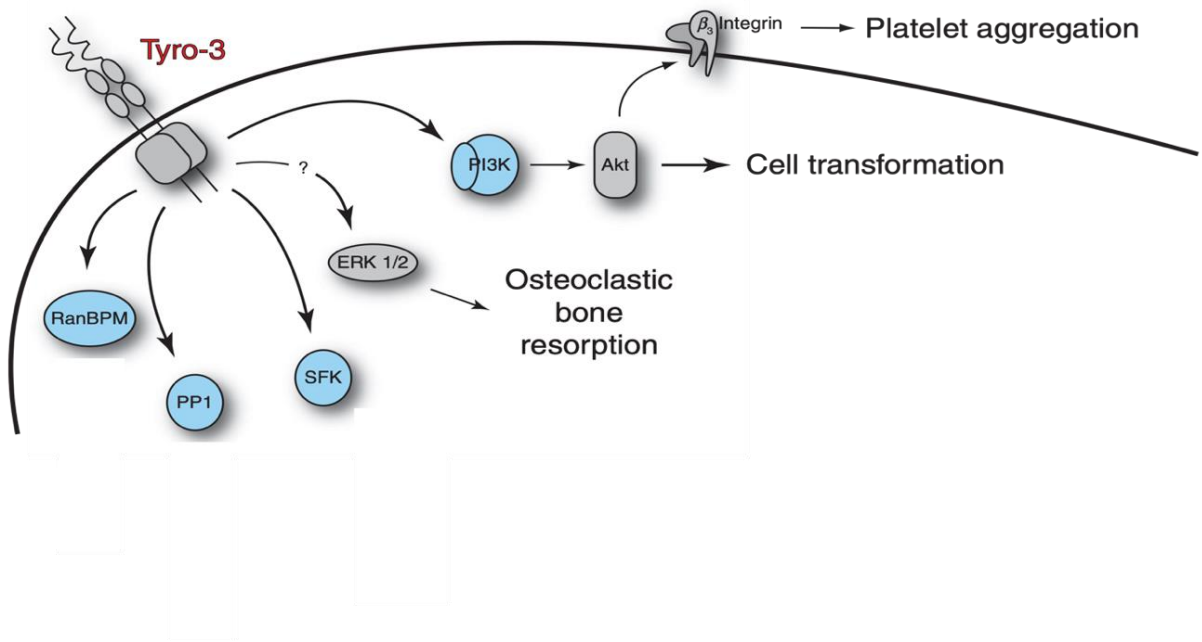


Figure 14. *Tyro-3 signaling pathways mediate platelet aggregation, cell transformation and osteo- clastic bone resorption (Linger et al., 2008b).*

1.4.3 Axl

Axl is the second member of TAM receptor family; it was firstly characterized in 1988 as a possible tumorigenic promoter in chronic myeloid leukemia (CML). The Axl gene is located in chromosome 19q13.2 and the origin of its name comes from the Greek word "anexelekto" (literary meaning “uncontrolled”) (Liu et al., 1988). Axl, as the other TAM receptor, is composed by an intracellular, transmembrane and extracellular domain. The Axl protein is composed by 894 amino acids and the molecular weight is around 100 kD, 140 kD after post-translational modifications and 85 kD for the soluble form of the receptor (Aplin, 2011). Axl is expressed in fibroblasts, endothelial cells, VSMCs and cardiac muscle cells and in the hemopoietic tissue of bone marrow, while it is not detectable in granulocytes ed lymphocytes. Most of the biological activity of Gas6 occurs through binding to the Axl receptor. The Gas6/Axl signalling pathway plays an important role in several biological processes like cells survival and proliferation, cells aggregation, cells migration and angiogenesis (**Figure 15**). In the CNS, Axl is extensively expressed by oligodendrocytes, microglia, neurons and Schwann cells, but its expression decreases with aging, while increases during the clinical course of certain diseases. Axl plays a crucial role in resolving inflammation; it reduces the expression of pro-inflammatory cytokines (Zhu et al., 2019). For example, by blocking the NFkB signalling pathway it reduces the production of TNFα. Moreover, Axl is able to stimulate the ACs by microglia; a defect in this process can contribute to the continuation of inflammation in the CNS (Weinger et al., 2011).

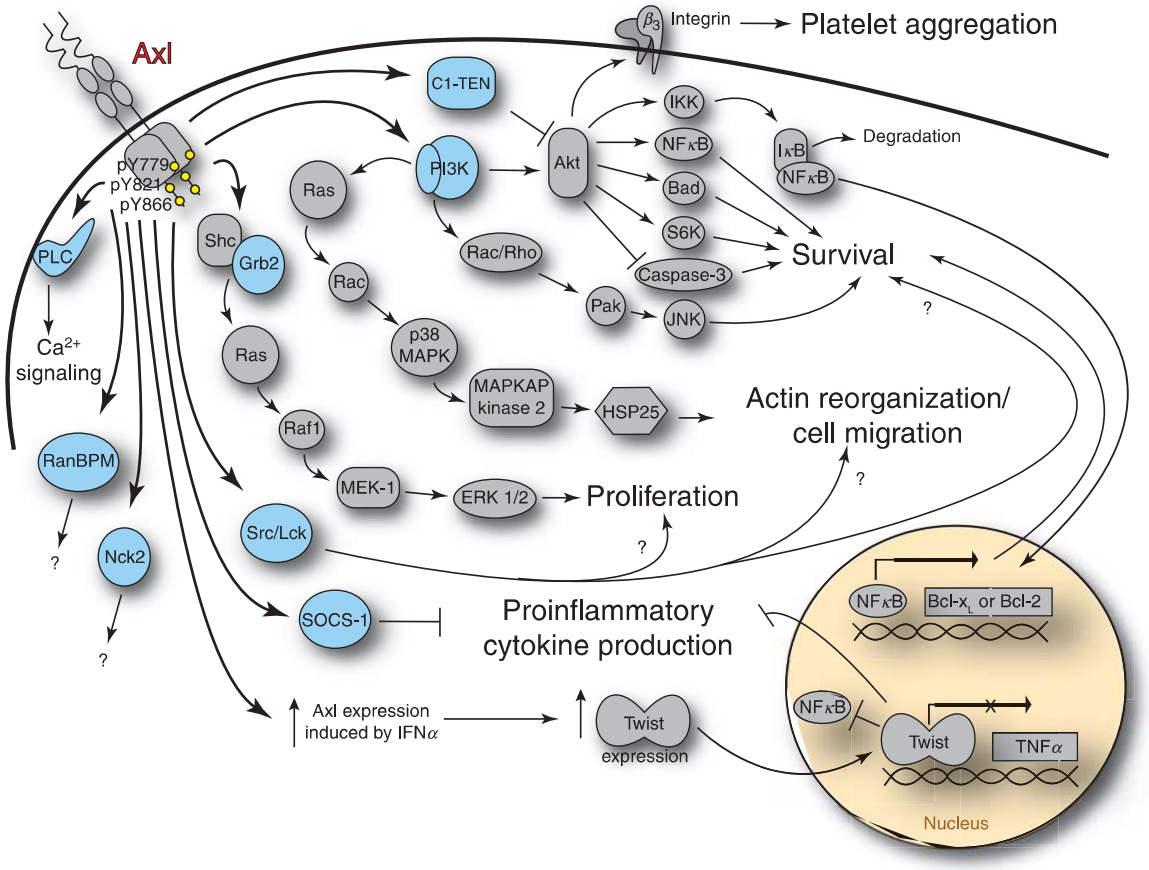


***Figure 15**. Axl signaling pathways lead to platelet aggregation, cell survival, proliferation, regulation of proinflammatory cytokine production and regulation of the actin cytoskeleton (Linger et al., 2008b).*

1.4.4 MerTK.

MerTK is the third member of the TAM receptor family; it was firstly identified, as an oncogene, in 1994 in cells and epithelium of reproductive origin. In 1999, through an in-situ hybridization assay (FISH) the gene was located in the chromosome 2q14.1 (Weier et al., 1999). As mentioned before, MerTK is mainly found in tissues belonging to the reproductive system like prostate, testes, ovary but also lungs, retina and kidney; while it is less expressed in brain, heart and skeletal muscle. One of the most important roles of MerTK is to enhance phagocytosis of damaged or dying cell, through the signalling of their surface of Ptd-Ser. MerTK is an essential regulator of homeostasis and has anti-inflammatory effect. MerTK signalling pathway in macrophages down-regulates proinflammatory signals which leads to the reduction of the secretion of the main pro-inflammatory cytokines such as TNF-α and IL-6. MerTK is an essential receptor in the context of innate immunity (**Figure 16**) (Behrens et al., 2003). Loss of MerTK alone confers susceptibility to autoimmunity. MerTK is also correlated with tumor processes; its over-expression is implicated in the oncogenesis of several cancers (Engelmann et al., 2022).

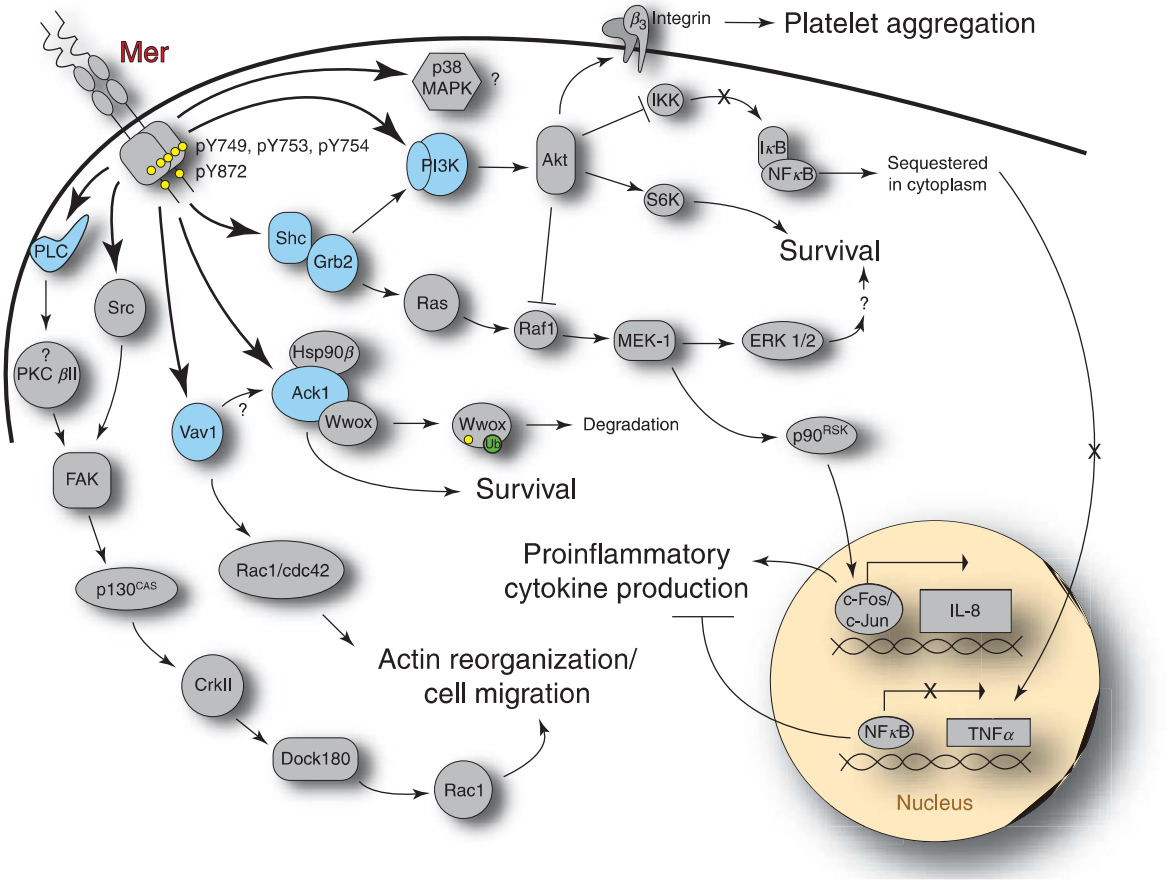


Figure 16. *Mer signaling pathways lead to platelet aggregation, cell survival, regulation of pro inflammatory cytokine production and regulation of the actin cytoskeleton (Linger et al., 2008b).*

1.5 Implication of Gas6/TAMs axis in MS

As mentioned before TAM receptors are widely expressed in the CNS, especially Tyro-3 and Axl while MerTK seems to be less expressed in brain. Their well-known activities comprise the regulation of innate immunity and the clearance of cellular and myelin debris. The failure of Gas6/TAM system has been linked to the development of autoimmunity (Shankar et al., 2003).

Nowadays, there are different studies suggesting an involvement of Gas/TAM axis in the pathogenesis of MS. A genome-wide association study (GWAS) provides evidence about an association between MS and Gas6/TAM (Ma et al., 2011). Twelve intronic single nucleotide polymorphism (SNPs) within the MerTK gene were associated with susceptibility to MS (Bahlo et al., 2009). Further evidence belongs from an autopsy study on MS patients: sAxl and sMer are upregulated in active lesions, while their membrane-bound form was expressed in physiological concentration (Weinger et al., 2009). In active and silent lesions, Gas6 has been found inversely correlate with sAxl and sMer. It is not known whether the low level of Gas6 is the result of a reduced secretion or it is due the decoy action of sAxl and sMer (Sather et al., 2007).

Moreover, confirmation of the involvement of the Gas6/TAM axis in the onset of MS is provided by studies in animal models (Procaccini et al., 2015). The two most used models are Cuprizone demyelination and experimental allergic encephalomyelitis (EAE) models. Cuprizone induces demyelination, loss of oligodendrocyte and microglial activation without inducing inflammation or altering the BBB(Hiremath et al., 1998). In these mice there is an altered expression of Gas6 and TAM. Gas6, Axl and MerTK are up-regulated while Tyro-3 is down regulated. A delayed remyelination together with a reduced number of oligodendrocytes has been demonstrated in $Gas6^{-/-}$ mice (Tsiperson et al., 2010). Gas6 increases remyelination in vitro in a dose-dependent manner, as proved by the observation that injection of Gas6 in the CNS improves the clarence of myelin and cellular debris, enhancing remyelination(Binder et al., 2011). The protective effects of Gas6 partly mediated by Axl (**Figure 17**); in $Axl^{-/-}$ mice the clearance of myelin debris and damaged cells are impaired. These evidence are confirmed in EAE mice models; in addition both $Gas6^{-/-}$ and $Axl^{-/-}$ have been shown an increased amount of pro-inflammatory cytokines inducing inflammation in the CNS and spinal cord (Weinger et al., 2011b).

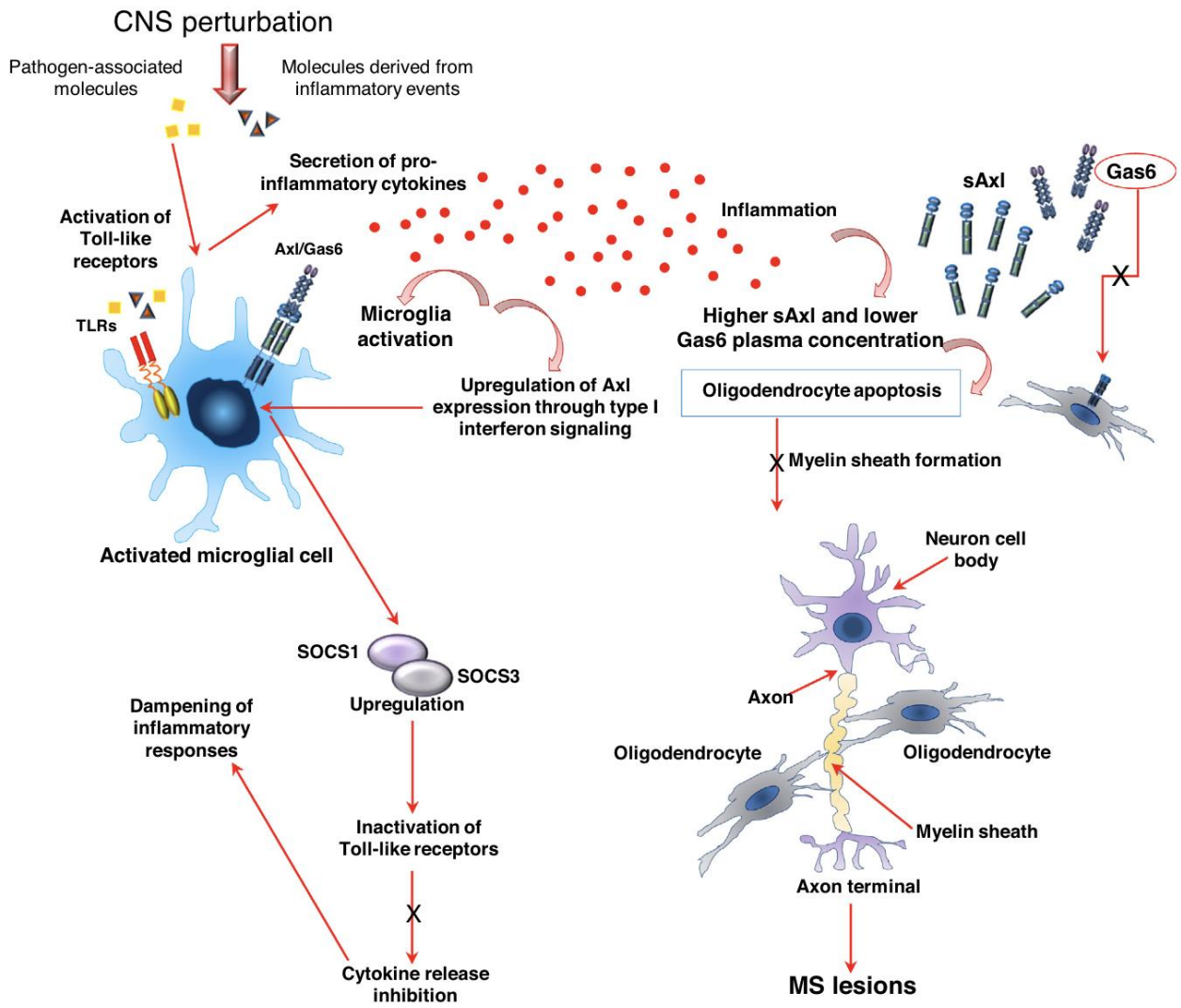


Figure 17*. Axl/growth arrest-specific protein 6 (Gas6) signalling in microglia innate immune system cells. Microglial cell stimulation involves the activation of Toll-like receptor (TLR) signalling, that, in turn, enhances proinflammatory cytokine secretion (red dots). Cytokine release upregulates Axl expression via type I interferon receptor signalling. Upon Gas6 binding, activated Axl induces intracellular pathways that work through a negative feedback loop involving the activation of suppressor of cytokine signalling 1 and 3 (SOSC1 and SOCS3), followed by the inactivation of TLR activity and cytokine receptor cascades. This results in the dampening of inflammatory responses. In the inflammation process that has been triggered by microglia activation, Axl receptor shedding increases, leading to high plasma levels of its soluble form, sAxl, which acts as a decoy receptor, sequestering soluble Gas6 and subtracting it from the full-length Axl on the cell surface. In this inflammatory environment derived from activated microglial cells, oligodendrocytes undergo apoptosis. The accumulation of cellular debris, together with the diminished ability to clear them because of the decreased activity of Axl/Gas6 signalling, causes demyelination of axon fibers in the central nervous system (CNS) and the onset of lesions in multiple sclerosis (MS) (Di Stasi et al., 2020).*

2. THE OBJECTIVE OF THE THESIS

MS is a disease characterized by inflammation and disruption of myelin. Gas6/TAM axis is involved in the regulation of innate immunity, inflammation and in the clearance of cellular and myelin debris. We hypothesized that Gas6 and its receptors might be potentially relevant in predicting patient's prognosis. On this basis, in the present work, we aimed to evaluate and investigate the possible role of Gas6 / TAM axis in longitudinal disease evolution in patients with early diagnosis of MS.

3. METHODS AND MATERIALS.

3.1 Patients.

In this observational prospective cohort study, we recruited, between October 2017 and February 2022, 64 patients (43 females) in "Maggiore della Carità" Hospital in Novara, Italy. All patients had a follow up visit at least one year after their diagnosis, between July 2021 and December 2022. The clinical data were acquired twice, both at the diagnosis and at the last follow up visit. CSF and serum samples were acquired at diagnosis while the patients underwent in MS diagnostic work-up. The inclusion criteria of the study were the diagnosis of CIS, RIS or RR, according to McDonald 2017 (A. J. Thompson et al., 2018) at the end of the follow up.

3.2 Ethical committee.

All the participant signed an informed consent form. The study protocol was approved by the local Ethical Committee (CE 262/2022) and was conducted in accordance with the Declaration of Helsinki.

3.3 Clinical evaluation.

Demographic and clinical variables collected at diagnosis were sex, age at onset, clinical course, the presence of gadolinium-enhancing (Gad+) lesions and disability according to the expanded disability status score (EDSS) (Meyer-Moock et al., 2014b). Brain MRI was performed on 1.5 T with single dose of Gad within 3 months from the diagnosis. EDSS was used to assess disability and monitor changes over time. This score has been corrected by time-measure using the *MS* severity score (MSSS) (Kister & Kantarci, 2020) and by the age using the age-related MS severity (ARMSS) (Kister & Kantarci, 2020).

3.4 Sample collection and biomarker determinations

Cerebrospinal fluid (CSF) was collected through lumbar puncture at diagnosis. CSF was centrifuged at 1300 rpm for 10 min and stored at −80 °C until the time of the analysis. At the time of CSF collection, all patients were treatment-naïve (including disease modifying treatments and steroids). Serum was immediately collected by centrifugation at 3500 rpm for 15 min and stored at −80 °C until the time of analysis. CSF and serum NFL were measured with the Simple PlexTM fluorescence-based immunoassay by Bio-Techne with the Ella Simple PlexTM Platform (Bio-Techne s.r.l., Milan, Italy). NFL were measured using the Human NFL Simple PlexTM Cartridge Kit (Lot no. 21519). All kit

components (cartridge, sample diluent SD13, and Wash Buffer A) were provided ready to use, and they were allowed to reach room temperature before use. CSF and serum levels of Gas6 were determined by the ELISA technique by using a commercial kit (R&D Systems Duo Set Elisa DY6488, McKinley, MN, USA) and following the manufacturer's instructions. Samples were diluted 1:50 in sample diluent. The optical density at 450 nm was fitted versus a calibration curve that was prepared with a standard (0 ng/mL–1 ng/mL range), as suggested by the manufacturer. CSF and serum levels of sTyro-3 were determined by the ELISA technique by using a commercial kit (R&D Systems Duo Set Elisa DY6488, McKinley, MN, USA) and following the manufacturer's instructions. Samples were diluted 1:5 in sample diluent. The optical density at 450 nm was fitted versus a calibration curve that was prepared with a standard (0 ng/mL–4 ng/mL range), as suggested by the manufacturer. CSF and serum levels of sAxl were determined by the ELISA technique by using a commercial kit (R&D Systems Duo Set Elisa DY6488, McKinley, MN, USA) and following the manufacturer's instructions. Samples were diluted 1:25 in sample diluent. The optical density at 450 nm was fitted versus a calibration curve prepared with a standard (0 ng/mL–4 ng/mL range), as suggested by the manufacturer. CSF and serum levels of sMer were determined by the ELISA technique by using a commercial kit (R&D Systems Duo Set Elisa DY6488, McKinley, MN, USA) and following the manufacturer's instructions. Samples were diluted 1:2 in sample diluent. The optical density at 450 nm was fitted versus a calibration curve prepared with a standard (0 ng/mL–10 ng/mL range), as suggested by the manufacturer. Absorbance was recorded using a Victor X4 microplate reader (Perkin Elmer, Waltham, MA, USA).

3.5 Statistical analysis

For continuous variables, the measures of centrality and dispersion were medians and interquartile ranges [IQR] and comparisons between groups regarding these variables were performed using the Mann–Whitney U test and the Kruskal Wallis test. The Pearson χ^2 was used to analyse the association between categorical variables that are shown as frequencies (%). Correlations were performed with Spearman's rank correlation coefficient, and linear regression for significant predictors in the univariate model. Multivariable regressions were built to identify the variables independently associated with the severity score. The threshold for statistical significance was 0.05 (two-tailed). Statistical analyses were performed with Stata statistical software version 17.0 (Stata Corp, 4905 Lakeway Drive College Station, TX, USA) while graphs were created using GraphPad Prism version 9.4.0 (GraphPad Software, La Jolla, CA, USA).

4. RESULTS

In our prospective cohort study, we recruited 64 patients affected by MS. **Table 3** reports the main features of the study population.

Table 3. *General features of the study population and their clinical parameters. Continuous variables are presented as medians [IQR], and categorical variables as frequencies (%). Abbreviations: CSF = Cerebrospinal Fluid, OB = oligoclonal bands, EDSS = Expanded Disability Status Scale, MSSS = Multiple Sclerosis Severity Score, ARMSS = Age Related Multiple Sclerosis Severity, NFL = neurofilament*

Demographics parameters and clinical scores	**# of patients**
Sex (F / M)	43 (67.19) / 21 (32.81)
Age (years)	37 [19.0 - 61.0]
Age at onset (years)	32 [14.0 - 56.0]

Disease course	**# of patients**
Radiological Isolated Syndrome	2 (3.12)
Clinical Isolated Syndrome	3 (4.69)
Relapsing-Remitting MS	59 (92.19)

MRI features	**# of patients**
Gadolinium-enhancing lesions	39 (60.94)
Brain Lesions > 10	36 (56.25)
Spinal Lesion > 10	4 (67.19)

Disability measures	**# of patients**
Switch from first disease modifying treatments within 1 year	9 (5.7) *****
EDSS at diagnosis EDSS < 3 at diagnosis	1.5 [0.0 - 6.0] 55 (85.94)
EDSS at last follow up. EDSS < 3 at diagnosis	1.5 [0.0 - 6.5] 56 (87.5)
MSSS at last follow up	2.85 [0.24 - 9.59]
ARMSS at last follow up	3.22 [0.29 - 8.47]

Biomarkers at diagnosis	
NFL serum (pg/mL)	29.55 [12.1 – 262]
NFL CSF (pg/mL)	1590.5 [201 – 35824]
Gas 6 serum (ng/mL)	23.49 [12.26 - 54.65]
Gas 6 CSF (ng/mL)	7.76 [1.80 - 32.75]
sAxl serum (ng/mL)	29.22 [15.42 - 231.3]
sAxl CSF (ng/mL)	26.38 [7.9 - 48.19]
sMer serum (ng/mL)	2.54 [0.0 - 55.1]
sMer CSF (ng/mL)	0.0 [0.0 - 0.0]
sTyro-3 serum (ng/mL)	3.54 [1.77 – 9.63]
sTyro-3 CSF (ng/mL)	3.79 [1.71 – 6.56]

**3/9 patients stopped/changed the first DMT for side effects.*

4.1 Serum and CSF levels of Gas6 and TAM receptors

Initially, we investigated serum and CSF levels of Gas6 and its receptors. As shown in **Figure 17** (**A;B**) all molecules were detectable, except for CSF sMer. Gas6 (23.49 ng/mL in serum; 7.76 ng/mL in CSF), sTyro-3 (3.54 ng/mL in serum; 3.79 ng/mL in CSF), sAxl (29.22 ng/mL in serum; 26.38 ng/mL in CSF), NFLs (29.55 pg/mL in serum; 1590.5 pg/mL in CSF).

Subsequently, we investigated whether there was a correlation between serum and CSF levels of biomarkers. Our data show no statistically significant correlation between serum and CSF concentrations of Gas6, whereas serum and CSF levels of sTyro-3 ($p = 0.05$), sAxl ($p = 0.02$), and NFLs ($p = 0.0001$) were significantly related between the two fluids (sMer was not analysed since it was undetectable in the CSF) **Figure 17 (C-F)**

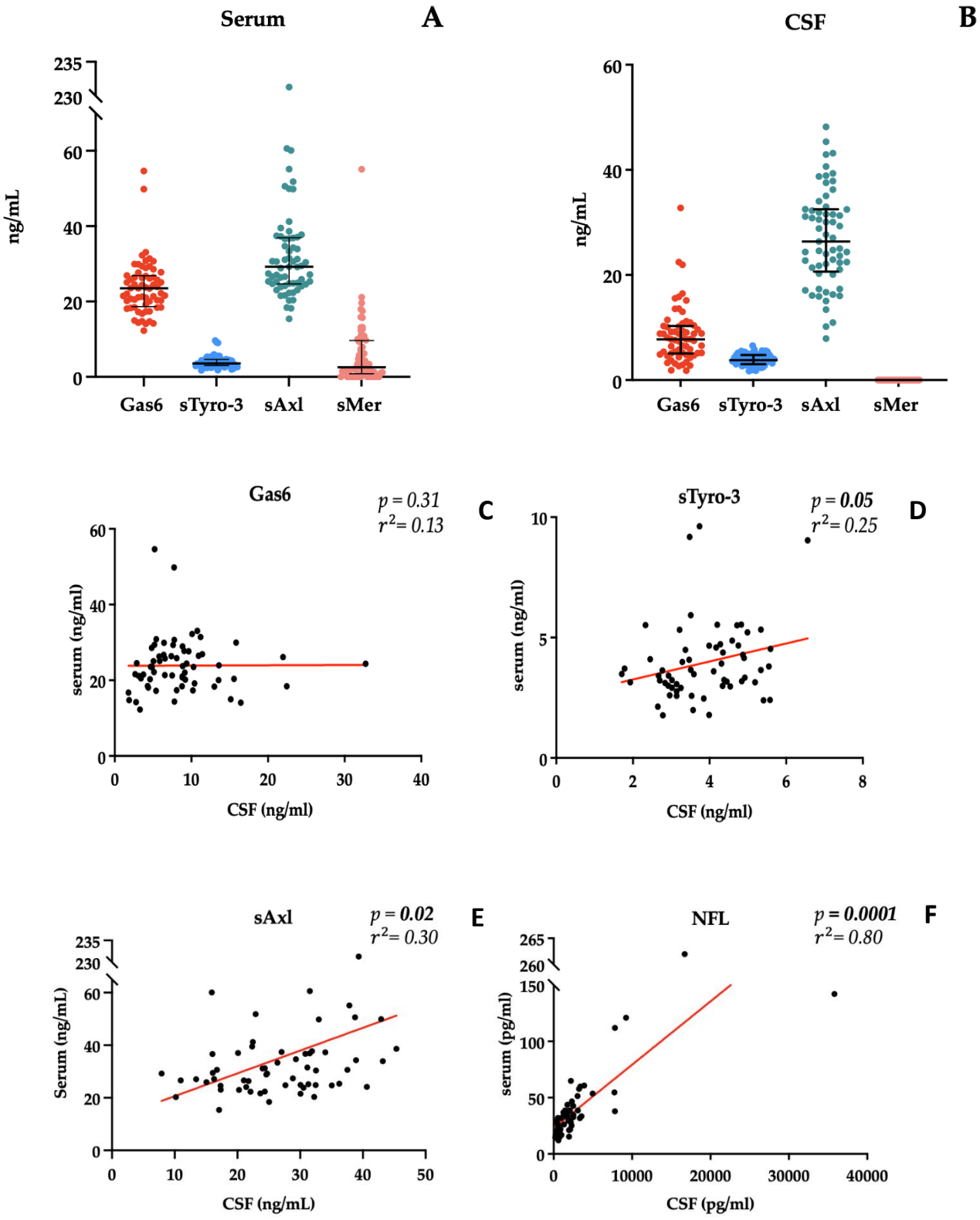
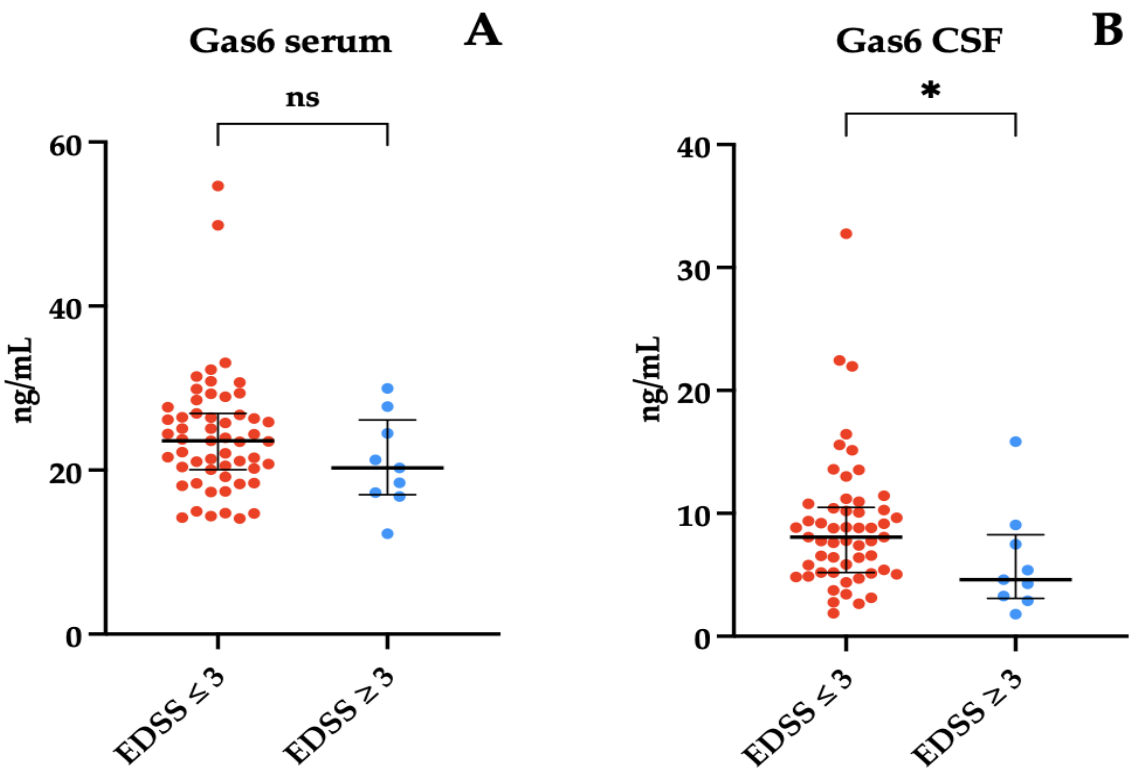


Figure 17. *Gas6 and TAM receptors distribution of the concentrations in serum (**A**) and CSF (**B**). Results are shown as medians [IQR]. Spearman's rank correlation between serum and CSF levels of Gas6 (**C**), sTyro-3 (**D**), sAXL (**E**) and NFLs (**F**) concentrations. R^2 = coefficient of correlation, p = p-Value.*

4.2 Gas6/TAMs and EDSS

To assess the association with disability measures we divided our patients according to them with EDSS score ≤ 3 or ≥ 3 at first and follow up visit. As shown in **Figure 18**, higher serum sAxl and CSF Gas6 levels related to an EDSS < 3 at diagnosis. As expected, higher NFLs levels in CSF and serum were associated with higher EDSS score at diagnosis in our patients. No associations were observed in the follow-up visit (data not shown). After the analysis sMer and sTyro-3 did not show a statistically significant correlation, thus, they are not show in the graph.



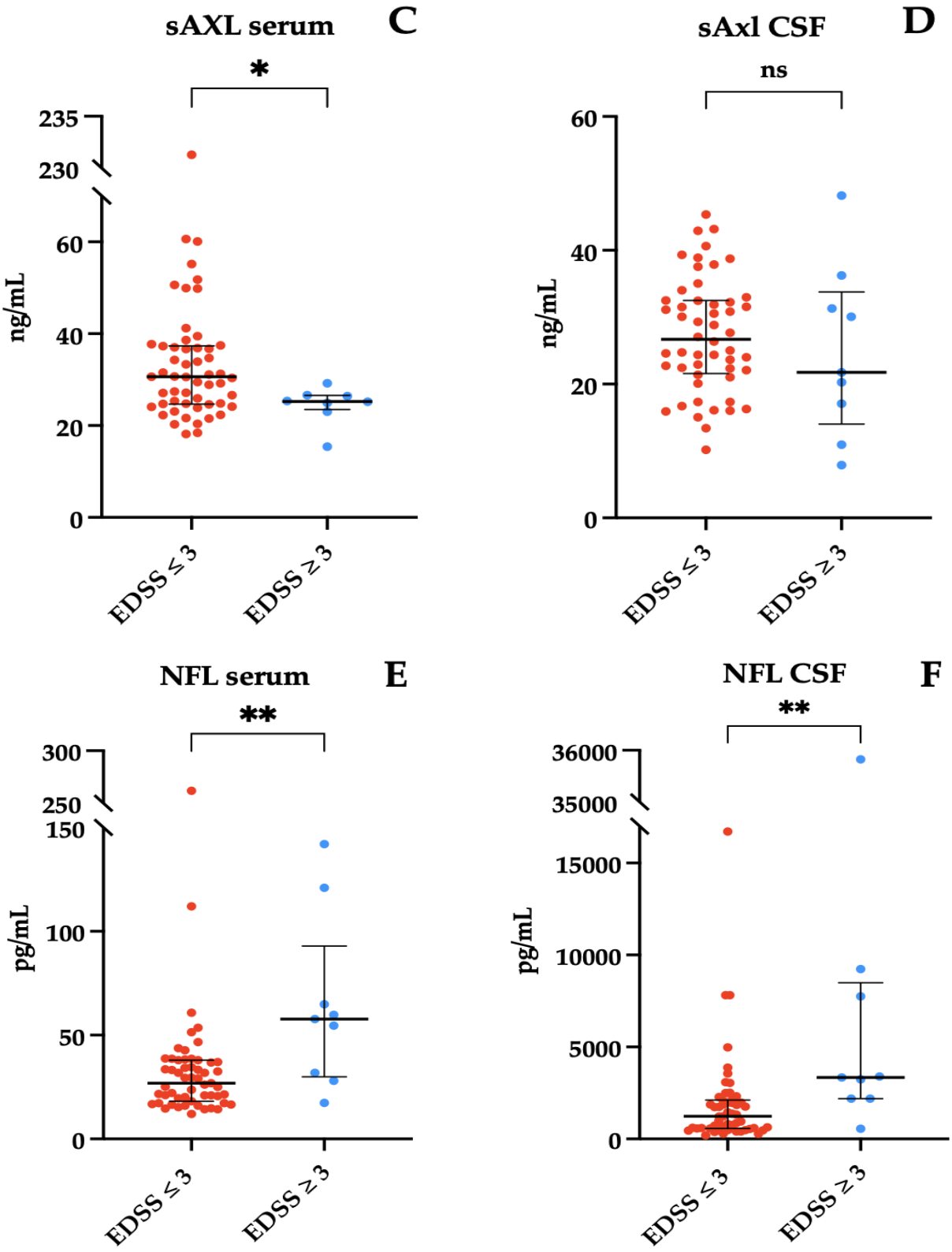
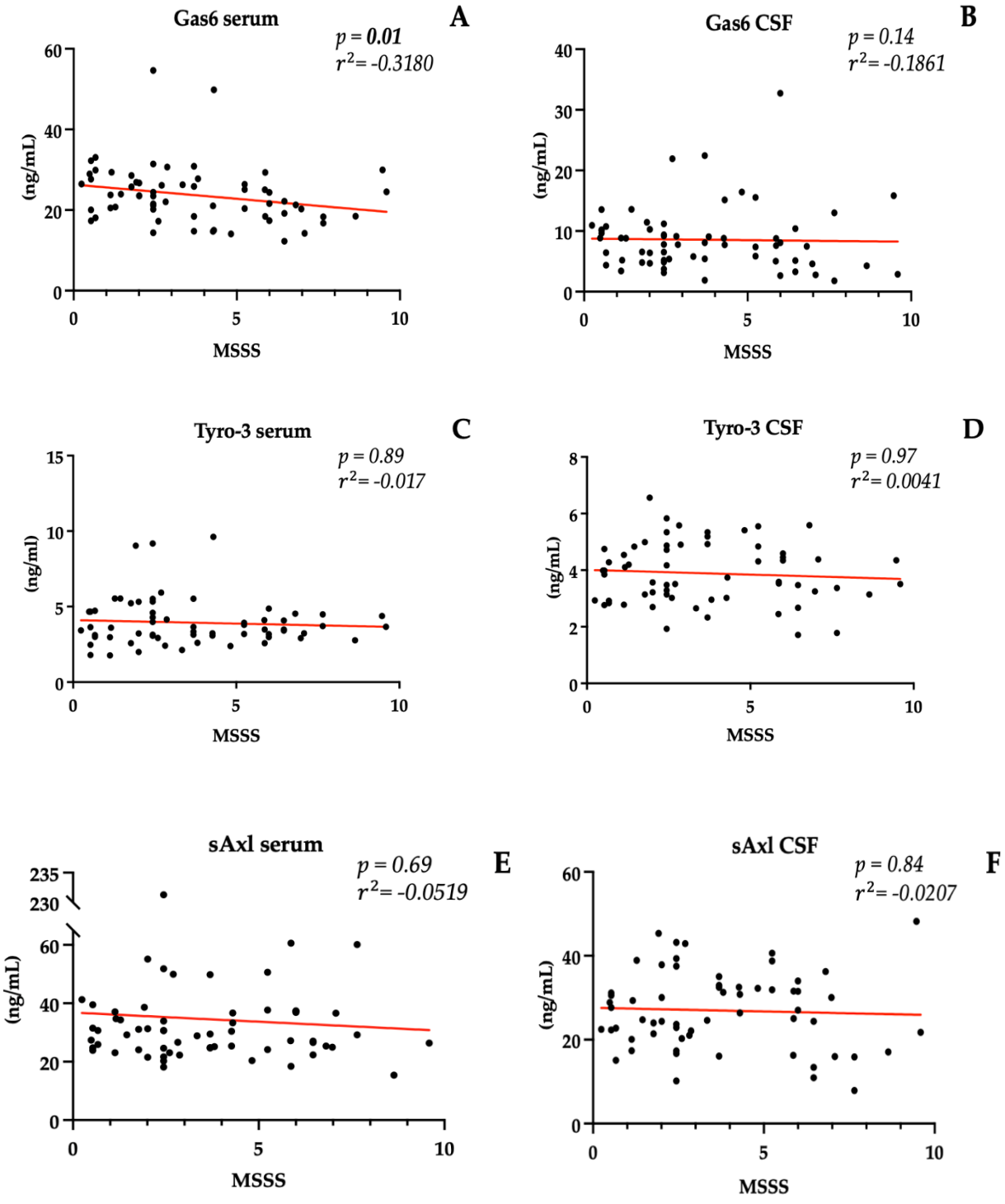


Figure 18. *Associations between Gas6 levels in serum and CSF (ng/mL) and ≥ 3 or ≤ 3 EDSS clinical scores in first visit. * p = 0.04 (**A**, **B**). sAXL levels in serum and CSF (ng/mL) in patients with ≥ 3 or ≤ 3 EDSS clinical scores in first visit. * p = 0.037 (**C**, **D**). Associations between NFL levels in serum and CSF (pg/mL) and ≥ 3 or ≤ 3 EDSS clinical scores in first visit 8 (**E**, **F**), ** p = 0.005, ** p = 0.002. Results are shown as medians [IQR]. Ns = not significant.*

4.3 Gas6/TAMs and MSSS

Finally, we evaluated the possible correlations of Gas6/TAMs and the disability scores at last follow up, MSSS and ARMSS. As shown in **Figure 19**, a negative correlation was found only for serum Gas6 and MSSS. As expected, NFLs levels in serum and CSF had a correlation with MSSS score.



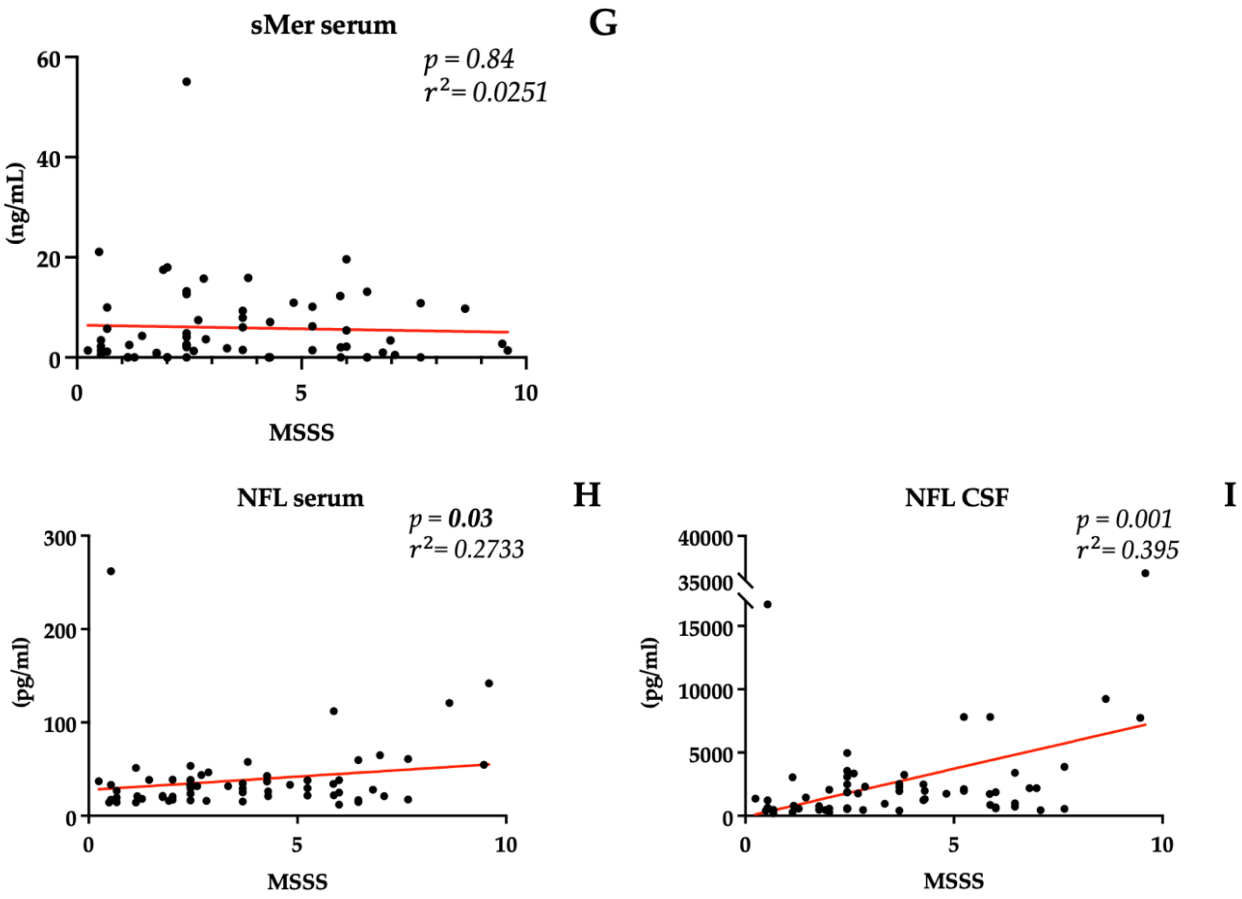


Figure 19. *Spearman's correlation between MSSS and Gas6 levels in serum and CSF (**A**, **B**), TAM receptors levels in serum and CSF (**C**, **G**) and NFLs levels in serum and CSF (**H**, **I**). R^2 = coefficient of correlation, p = p-Value.*

4.4 Multivariate analysis.

We finally performed multivariate regression models to predict MS outcome according to EDSS at diagnosis (**Table 4**) and MSSS score (**Table 5**). The included independent variables were sex and age, number and types of lesions, and the other serum biomarkers.

***Table 4**. Multivariate regression model of serum Gas6 predicting a lower MSSS score including demographic and severity variables.*

Predictor	Coefficient	Standard error	p - value	95% confidence interval
Gas6 serum (ng/ml)	-0.1577	0.0503	**0.003**	-0.2588 – -0.0565
sAxl serum (ng/ml)	0.0317	0.0285	0.27	-0.0256 – 0.1227
sMer serum (ng/ml)	0.0192	0.0515	0.71	-0.0843 – 0.1205
sTyro- 3 serum (ng/mL)	0.0687	0.1983	0.73	-0.3298 – 0.4673
NFL serum (pg/mL)	0.0098	0.0078	0.21	-0.0059 – 0.0257
Age	0.0688	0.0305	**0.029**	0.0074 – 0.1302
Sex	-0.7116	0.6308	0.26	-1.9793 – 0.5560
N° brain lesion >10	0.2788	0.5978	0.64	-0.9226 – 1.4803
N° spinal lesion >10	1.3529	0.6075	**0.035**	0.0970 – 2.6088
Gadolinium-enhancing	0.7653	0.6075	0.41	-0.4555 – 1.9861

After the multivariate analysis, serum Gas6 (p = 0.003) retained its prognostic role resulted as predictors for MSSS.

***Table 5**. Multivariate regression model of CSF and serum Gas6 predicting a < 3 EDSS score including demographic and severity variables.*

Predictor	Coefficient	Standard error	p - value	95% confidence interval
Gas6 serum (ng/ml)	-0.0169	0.0075	**0.029**	-0.0320 – -0.0018
Gas6 CSF (ng/mL)	-0.0192	0.0089	**0.037**	-0.0372 – -0.0012
sAxl serum (ng/ml)	-0.0026	0.0044	0.55	-0.0112 – 0.0063
sMer serum (ng/ml)	0.0067	0.0081	0.41	-0.0097 – 0.0231
sTyro- 3 serum (ng/mL)	0.0002	0.0290	0.99	-0.0582 – 0.0586
NFL serum (pg/mL)	0.0020	0.0011	0.21	-0.0026 – 0.0044
Age	0.0025	0.0045	0.58	-0.0066 – 0.0116
Sex	-0.060	0.0936	0.52	-0.2487 – 0.1279
N° brain lesion >10	0.1444	0.0907	0.11	-0.0381 – 0.3269
N° spinal lesion >10	0.0985	0.0900	0.28	0.0819 – 0.2871
Gadolinium-enhancing	0.7653	0.6075	0.41	-0.0826 – 0.2796

After the multivariate analysis, only serum and CSF Gas6 levels (p = 0.029; p = 0.037) resulted as predictors for the EDSS at the first visit, sAxl didn't retain its prognostic role after demographic and severity correction.

5. DISCUSSION

MS is a long-lasting inflammatory condition affecting the CNS, leading to neurodegeneration and neurological impairments. There are different evidence proving that the disease's cause is influenced by multiple factors including both genetic and environmental factors (Reich et al., 2018). Among the environmental factors, EBV infection, obesity, smoking and vitamin D deficiency have shown a greater association with MS (Morgenstern & Goerbig, 2022). TAMs are transmembrane tyrosine kinase receptor and together with their ligand Gas6 are expressed in several tissues and cells, including in CNS. In order to exert its functions, Gas6 needs to bind its receptors, playing a role in cell survival, growth, aggregation and migration, angiogenesis and control of inflammatory responses, apoptotic cell and membrane engulfment and phagocytic elimination (Burstyn-Cohen, 2017). TAM receptors can be cleaved into their soluble forms (named sTyro-3, sAxl, and sMer) by specific metalloproteases, ADAM 10 and 17. These soluble receptors can still bind Gas6 protein retaining their functions in the modulation of inflammation.

The neurofilaments (NFs) are the major components of the mature neurons and are the gold standard biomarkers in MS since, after the damage to the neurons, NFLs are released in the interstitial fluid in CNS and then in CSF, if the BBB is damaged, they could be scattered in bloodstream (Williams et al., 2021).

In our prospective cohort study, we have evaluated if the proposed biomarkers were detectable in both serum and CSF in MS patients at diagnosis. All biomarkers were detectable in both fluids, except sMer that was not detectable in CSF, consistently with the results of Prince and colleagues (Pierce & Keating, 2014). Then we investigated whether there was a correlation between serum and CSF levels of Gas6 and TAMs receptor. Serum and CSF levels of sTyro-3 ($p = 0.05$), sAxl ($p = 0.02$), and NFLs ($p = 0.0001$) showed a statistical correlation between the two fluids, whereas Gas6 showed no statistically significant correlation between serum and CSF levels. Since sMer was not detectable in the CSF it was not included in the statistical analysis. There is no work in the literature with which to compare the results obtained, except for a study conducted by Sainaghi and colleagues in which they have investigated the levels of Gas6 in serum and CSF and try to evaluate a possible role of Gas6 in predicting the relapse in RRMS patients. Regarding the quantification of Gas6 in serum and CSF, their results were in agreement with ours, they did not find a significant correlation between Gas6 levels in the two fluids (Sainaghi et al., 2013).

After this first analyses, we searched for any associations between Gas6 and TAM receptors and the clinical presentations at the diagnosis. EDSS is a clinician-administered assessment scale useful to

evaluate the functional systems of the CNS; this score is used to describe disease progression in patients (Meyer-Moock et al., 2014c). We divided our patients by EDSS score ≤ 3 or > 3 at first and follow up visit (Şen, 2018b). Those patients with EDSS score ≤ 3 showed significantly higher CSF Gas6 levels and serum sAxl concentrations if compared to those with an EDSS > 3. Moreover, as expected, serum and CSF NFLs was higher in patients with an EDSS ≥ 3 at diagnosis (Benkert et al., 2022).

Finally, after investigating the correlation between Gas6 and sAxl with EDSS at diagnosis, we have tried to evaluate whether they also had prognostic value over time. As an indicator of disease course, we used MSSS, which analyses the progression of the disability in MS patients (Pachner & Steiner, 2009). A prognostic role over time emerged only for serum Gas6, since higher levels of this biomarker are correlated to a lower MSSS, suggesting a possible protective role in the disease course. As expected, on the contrary, higher CSF and serum NFLs levels are correlated to higher MSSS. These data have confirmed the trend that high NFLs values are associated to a worse clinical course, while also suggesting a possible protective role of Gas6 in disease progression.

A possible pathogenic hypothesis is related to the Gas6 and TAM receptors expression in several cell types in the nervous system, including ODs. (Goudarzi et al., 2016) Activation of the Axl receptor by Gas6 induces an intracellular response that promotes oligodendrocyte survival and stimulates the myelination process (Goudarzi et al., 2016). Nonetheless, hyperactivation of the immune system also contributes to impaired remyelination. This condition is demonstrated in several mouse model of experimental autoimmune encephalomyelitis (EAE), in which the loss of Axl increases inflammation in CNS, delaying the removal of myelin debris (Gruber et al., 2014). Moreover, studies with Gas6 knockout mice showed impaired remyelination due to increased activation of microglia. Other studies in Gas6 and Axl knockout mice demonstrated the specific contribution of Gas6/Axl signalling in remyelination processes. In some of these studies it is suggested that Gas6/Axl signalling may have an important role in reducing CNS inflammation and maintaining axon integrity after demyelinating/proinflammatory stimuli, as demonstrated in a study in which exposure to toxic cuprizone caused extensive damage to axons in mutant mice, associated with an abnormal inflammatory response due to reduced SOCS expression (Ray et al., 2017a; Shafit-Zagardo et al., 2018; Tsiperson et al., 2010).

Supporting our hypothesis of the protective role that Gas6 could have in the disease, there are some evidences in the work of Li and colleagues (Li et al., 1996). They have identified Gas6 as a growth factor for human Schwaan cells in an in vitro study. In this study they have demonstrated that in the presence of recombinant human Gas6 (rhGas6), there was an increase in mature oligodendrocyte

proteins respect to cells grown in the absence of Gas6. More in detail rhGas6 sustained human oligodendrocyte viability by Gas6 activation of TAM receptors and downstream signalling via the PI3-kinase/Akt pathway (Weinger et al., 2008). Regarding a possible role of Axl, Shankar and colleagues have discovered that Gas6/Axl interactions may enhance adhesion between axons and oligodendrocytes during myelination where high levels of both ligand and receptor are observed (Shankar et al., 2006). Another work of Ray e colleagues as shown as Axl and Gas6 are elevated in MS tissue, and strongly support a functional and physiologic role for signalling through this family of receptor tyrosine kinases, likely in reducing inflammation, debris clearance and restoring cellular homeostasis (Ray et al., 2017b). Few studies in literature elucidated the possible role of sAxl in MS. In line with our results, the work of Brosseron and colleagues in which they dosed sAxl in Alzheimer's disease. They found that sAxl has a protective role with regard to the course of the disease, in fact patients with higher levels of sAxl have less deterioration in cognitive function than patients with low levels of sAxl over a five-year time interval. Overall, the role of sAxl is complex and further studies are needed to fully understand its mechanisms of action.

Another possible evidence of the involvement of Gas6/TAMs pathway in the disease is its key role during viral infection (Zhang et al., 2017a). Gas6 may act as a modulator of inflammation, regulating the immune response and limiting the inflammation and tissue damage associated with viral infection (Morizono & Chen, 2014). As said before, the activation of TAM receptors by Gas6 could influence the response of immune cells like macrophage and dendritic cells (Grabiec et al., 2018; Morizono & Chen, 2014). If the infection takes place, it can influence the expression of Gas6 and TAM receptors (Tonello et al., 2022). For instance, during EBV infection, it has been observed that Axl expression can increase in infected cells. From this observation it could be deduced that Gas6 and TAM receptors could play a response role in EBV infection (Zhang et al., 2017b). However, the direct link between Gas6 and EBV still needs several studies to be proven.

In conclusion, this observational prospective cohort study has been focused on the possible role of Gas6/TAM axis as a prognostic marker for multiple sclerosis. To investigate the possible role of these biomarkers, we focused on clinical scores indicating the course of the disease, such as EDSS and MSSS. We had seen that high levels of Gas6 in CSF and sAxl in serum correlated with a lower EDSS at diagnosis, indicating them as possible protective factors. Moreover, Gas6 could also have prognostic value as we observed that high levels of this biomarker in serum correlated with a lower MSSS at the end of follow-up. Taken together we can say that the Gas6-TAM axis showed a tendency to identify patients with more favourable prognosis.

Considering the lack of literature and the complex system of interaction between soluble and membrane forms, further studies are needed to fully understand the role of the Gas6/TAMs axis in the disease.

We are aware that this study may have several limitations, like the number of patients involved and the monocentric nature of the recruitment; however, the results are promising and could be extended by the Gas6/TAM levels follow-up during the entire evolution of the pathology.

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