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ORIGINAL PAPER

C9orf72 Intermediate Alleles in Patients with Amyotrophic Lateral Sclerosis, Systemic Lupus Erythematosus, and Rheumatoid Arthritis

Micaela Fredi[1,2] · Ilaria Cavazzana[2] · Giorgio Biasiotto[3,4] · Massimiliano Filosto[5] · Alessandro Padovani[2,5] · Eugenio Monti[3] · Angela Tincani[1,2] · Franco Franceschini[1,2] · Isabella Zanella[3,4]

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Abstract

The commonest genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) is a large hexanucleotide expansion within the non-coding region of the *C9orf72* gene. The pathogenic mechanisms of the mutation seem toxic gain of functions, while haploinsufficiency alone appears insufficient to cause neurodegeneration. *C9orf72*$^{-/-}$ mice rather develop features of autoimmunity. Immune-mediated dysfunctions are involved in the pathogenesis of ALS and FTD and high prevalence of autoimmune disease has recently been observed in *C9orf72* expansion-positive patients. Since intermediate repeat expansions result in decreased transcription of the gene, we explored the hypothesis that *C9orf72* intermediate alleles could be a genetic risk for autoimmune conditions. We genotyped 69 systemic lupus erythematosus (SLE) and 77 rheumatoid arthritis (RA) patients, with 68 expansion-negative ALS patients, as control. A cut-off of ≥ 9 and ≤ 30 hexanucleotide units was chosen to define intermediate-length expansions. In the SLE and SLE + RA cohorts, both the number of patients with intermediate expansions and the overall number of intermediate alleles were significantly higher than in controls (23.2% vs. 7.4%, $p = 0.020$; 13.8% vs. 3.7%, $p = 0.006$, and 19.9% vs. 7.4%, $p = 0.033$, 11% vs. 3.7%, $p = 0.021$, respectively) and discernible although non-significant differences were found for the RA only cohort. Three SLE patients had intermediate-length expansions on both alleles, two of them harboring sequence variations within the hexanucleotide downstream region. However, no peculiar clinical features associated with the intermediate expansion were identified. Our results suggest that *C9orf72* intermediate alleles could be associated with systemic autoimmune diseases, indicating a role of *C9orf72* in immunity regulation.

Keywords *C9orf72* intermediate alleles · Amyotrophic lateral sclerosis · Frontotemporal dementia · Systemic lupus erythematosus · Rheumatoid arthritis · Autoimmunity

Background

Mounting evidence indicates that neuroinflammation and immunological mechanisms are involved in the pathogenesis of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), two neurodegenerative disorders characterized by several clinical, pathological, and genetic links (Biasiotto et al. 2016). Increased prevalence of autoimmune diseases has been observed in FTD patients (Miller et al. 2013, 2016) and several ALS- and FTD-associated genes play a role in immunity (Lall and Baloh 2017). A systematic investigation of genetic pleiotropy between ALS- and FTD-related disorders and immune-mediated diseases, performed through the analysis of large genome-wide association studies, has recently demonstrated immune-mediated genetic enrichment in FTD and, to a lesser extent, in ALS, further

Franco Franceschini and Isabella Zanella share senior authorship.

✉ Ilaria Cavazzana
ilariacava@virgilio.it

1 Rheumatology and Clinical Immunology Unit, ASST Spedali Civili di Brescia, Piazzale Spedali Civili 1, 25123 Brescia, Italy

2 Department of Clinical and Experimental Sciences, University of Brescia, Brescia, Italy

3 Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy

4 Clinical Chemistry Laboratory, Diagnostic Department, ASST Spedali Civili di Brescia, Brescia, Italy

5 Neurology Unit, ASST Spedali Civili di Brescia, Brescia, Italy

underscoring the contribution of immune dysfunctions in these neurodegenerative disorders (Broce et al. 2018).

A large G_4C_2-hexanucleotide repeat expansion within a non-coding region of the *C9orf72* gene is the most common genetic cause of ALS and FTD, accounting for several familial, but also sporadic cases (DeJesus-Hernandez et al. 2011; Renton et al. 2011). A repeat length of > 30 units is usually defined as pathogenic, with expansions of hundreds to thousand units typically found in ALS and FTD patients. Most healthy individuals have 2–30 repeat units on both alleles, commonly 2, 5, or 8 units. The exact cut-off to distinguish normal and pathogenic expansions has, however, not yet been determined. Intermediate expansions (> 8 but < 30 repeat units) are much less frequent in healthy individuals (Cacace et al. 2013), while expansions > 20 repeat units have been described in some ALS and FTD cases (Byrne et al. 2014; Gomez-Tortosa et al. 2013). Moreover, somatic instability has been reported and intergenerational changes have been observed occurring from a repeat number > 10 units (Beck et al. 2013; Van Mossevelde et al. 2017).

In some repeat expansion disorders, intermediate-length alleles may be associated with pathological evidences of the same or distinct disorders (Lozano et al. 2014; Semaka and Hayden 2014). In the case of the *C9orf72* gene, as above mentioned, intermediate expansions have been described in some FTD and ALS patients, but no significant differences have been found in intermediate allele frequency in related or distinct neurological disorders, except for some reports concerning Parkinson's disease and atypical parkinsonisms (Nuytemans et al. 2013; Cannas et al. 2015; Ng and Tan 2017). To date, no data are available from clinical studies, specifically designed to evaluate the role of *C9orf72* intermediate expansions as genetic risk factor in apparently unrelated non-neurodegenerative disorders.

The mainly suggested pathogenic mechanism of the *C9orf72* dominant mutation is toxic gain of functions, through the production of repetitive transcripts and proteins (Lall and Baloh 2017). Nonetheless, reduced expression of *C9orf72* has been observed in post-mortem brains of mutated patients, because of epigenetic silencing through hypermethylation of the CpG islands near the G_4C_2-repeat expansion (Xi et al. 2014) or abortive transcription (Haeusler et al. 2014). Interestingly, methylation levels of homozygous intermediate repeat carriers (7–24 units) have been shown slightly but significantly increased in comparison with those of homozygous short repeat carriers (2–6 units), both in ALS/FTD patients and healthy individuals (Gijselinck et al. 2016). Moreover, decreased transcriptional activity with increasing number of repeats (7–24) has been clearly demonstrated in HEK293T and SH-SY5Y cells (Gijselinck et al. 2016). Interestingly, while *C9orf72* complete loss or haploinsufficiency alone seems insufficient to cause neurodegeneration in mice, *C9orf72*$^{-/-}$ knockout mice exhibit immune dysregulation and develop features of autoimmunity, like systemic lymphadenopathy, splenomegaly, high level of autoantibodies, and membrano-proliferative glomerulonephritis reminiscent of systemic lupus erythematosus (SLE), suggesting a protective role for the C9ORF72 protein against autoimmune diseases (Atanasio et al. 2016; Burberry et al. 2016; O'Rourke et al. 2016). This is not surprising, in view of the fact that C9ORF72 is highly expressed in myeloid cells, particularly in CD14$^+$ monocytes (Rizzu et al. 2016). Importantly, even hemizygous *C9orf72*$^{+/-}$ mice show altered inflammatory responses, suggesting that not only complete ablation but also haploinsufficiency could lead to unbalanced systemic immunity and autoimmunity in mice, under certain conditions of immunological stress (O'Rourke et al. 2016; Lall and Baloh 2017). In this respect, *C9orf72* haploinsufficiency could not only contribute to immune dysfunctions and neuroinflammation in ALS and FTD, but it could also be a genetic risk factor for autoimmune conditions.

In view of the above, we explored the hypothesis that normal, but in the upper range G_4C_2-hexanucleotide expansions in the *C9orf72* gene could be a risk to develop autoimmune diseases like SLE or rheumatoid arthritis (RA) or influence the phenotypic expression of these systemic autoimmune disorders.

Methods

Patients

All the consecutive adult SLE ($n = 69$) and RA ($n = 77$) patients, seen in the Rheumatology Unit of our Hospital from January to December 2017, were considered for the study. Clinical and laboratory data included in the SLICC criteria for SLE and EULAR/ACR criteria for RA (Petri et al. 2012; Aletaha et al. 2010) were collected from medical records and the occurrence of clinical features was considered at any time during the follow-up. A clinical and serological evaluation was performed according to recommendations (Aletaha et al. 2010; Petri et al. 2012). Since most studies found no significant differences in distribution, range and median repeat number between *C9orf72* expansion-negative ALS/FTD cases and healthy subjects (DeJesus-Hernandez et al. 2011; Renton et al. 2011; Byrne et al. 2012; Sabatelli et al. 2012; Ng and Tan 2017), as control group we included 68 patients with ALS, but without *C9orf72* pathogenic expansions. ALS patients were selected at the Centre for Neuromuscular Diseases and Neuropathies of our Hospital and, in part, have been previously described (Biasiotto et al. 2017). This study was performed according to the principles of the Declaration of Helsinki, with written informed consent from all subjects,

and was approved by the Ethic Committee of the Promoting Centre (reference no. 2918-Studio C9ORF72CTD).

Genetic Analyses

Genomic DNA was extracted from peripheral blood using the Wizard Genomic DNA Purification kit, according to the manufacturer's instructions (Promega). Extracted DNA samples were quantified by the use of Qubit 2.0 Fluorometer (Thermo Fisher Scientific), with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). All samples were genotyped with a PCR-based two-step *C9orf72* analysis, including sizing PCR followed by repeat-primed-PCR, as previously described (Biasiotto et al. 2017). Amplicons were sized by fragment analysis on an automated ABI 3500 Genetic Analyzer (Thermo Fisher Scientific), using GeneScan-500LIZ (Thermo Fisher Scientific) as internal size standard. Data analysis was performed using GeneMapper v4.0 software (Thermo Fisher Scientific). Primers flanking the repeats were employed to amplify alleles suspected to have deletions or insertions within the GC-rich region immediately downstream the repeat (Biasiotto et al. 2017). Amplicons were sequenced by Sanger sequencing on an automated ABI 3500 Genetic Analyser (Thermo Fisher Scientific). The single nucleotide polymorphism (SNP) rs3849942, tagging the chromosome 9 Finnish founder ALS risk haplotype, was genotyped by PCR with specific primers, followed by Sanger sequencing (Biasiotto et al. 2017).

A cut-off of ≥ 9 repeat units was chosen to distinguish short (2–8) from intermediate (9–30) *C9orf72* hexanucleotide expansions, on the basis of the following criteria: (1) most healthy individuals harbor 2–8 repeats (DeJesus-Hernandez et al. 2011; Renton et al. 2011); (2) decreased transcriptional activity with increasing number of repeats (7–24) has been observed in healthy cases (Gijselinck et al. 2016); (3) somatic instability has been shown occurring from > 10 repeats and the unstable risk haplotype is more frequent above 8 repeats (Beck et al. 2013; Van Mossevelde et al. 2017).

Statistical Analyses

Categorical variables were reported as proportion and/or percentage, continuous variables as mean (± SD) values. Fisher's exact or Chi-square test for categorical variables and Student's *t* test for continuous variables were applied as appropriate. *p* values < 0.05 were considered significant. When significant, odds ratio (OR) with 95% confidence interval (95% CI) were indicated.

Results

In this study, we included 69 SLE and 77 RA consecutive unrelated patients. Sixty-eight ALS patients, without *C9orf72* large expansions, were considered as control subjects, since several studies in most countries found no differences in distribution, range and median repeat number between *C9orf72* expansion-negative ALS/FTD cases and healthy subjects (DeJesus-Hernandez et al. 2011; Renton et al. 2011; Byrne et al. 2012; Sabatelli et al. 2012; Ng and Tan 2017). Demographic data of patients are described in Table 1. As expected, SLE and RA patients were younger than control subjects affected by ALS and most of them were female.

Clinical evaluation of ALS patients did not reveal clear signs or symptoms of systemic autoimmune diseases.

Genetic analysis on SLE and RA patients did not reveal large *C9orf72* expansions (> 30 repeats). As previously reported in other populations, 2, 5, and 8 repeats were the most frequent expansions in our cohorts (Fig. 1). We identified *C9orf72* intermediate (9–30 repeat units) expansions in 16 (23.2%) SLE, 13 (15.6%) RA, 29 (19.9%) SLE + RA, and 5 (7.4%) ALS patients. Despite comparable average, median number of repeat units and interquartile range, the frequency of patients with intermediate alleles was significantly higher in the SLE and in the SLE + RA cohorts compared with the control cohort, while only a trend towards a higher prevalence of intermediate alleles was observed for RA patients (Table 1; Fig. 1). Intermediate expansions were present on both alleles in three SLE patients, while all RA and ALS and most SLE patients (81%) harbored only one intermediate allele. Comparing the overall number of intermediate alleles, we found it significantly higher in SLE (13.8%) and SLE + RA (11%) versus ALS (3.7%) patients. We also observed a trend in higher overall number of intermediate alleles in SLE compared with RA patients, but without reaching statistical significance ($p = 0.128$) (Table 1). Analyzing only female patients, we identified a more significant association for SLE and for SLE + RA patients, both considering the number of patients with intermediate *C9orf72* expansions (16/69 SLE vs. 1/32 ALS, $p = 0.011$, OR 9.35, CI 95% 1.19–198.3 and 27/130 SLE + RA vs. 1/32 ALS, $p = 0.017$, OR 8.12, CI 95% 1.102–167, respectively) and the overall number of *C9orf72* intermediate alleles (19/138 SLE vs. 1/64 ALS, $p = 0.005$, OR 10.05, CI 95% 1.37–206.3, 30/260 SLE + RA vs. 1/64 ALS, $p = 0.015$, OR 8.21, CI 95% 1.16–165.5, respectively).

As expected, the analysis of the rs3849942 SNP in intermediate allele carriers showed the presence of at least one risk haplotype allele in all ALS and RA and all but one SLE patients (Table 1). Among SLE patients

Table 1 Demographic data and *C9orf72* hexanucleotide expansions in sytemic lupus erythematosus SLE, rheumatoid arthritis RA, SLE + RA, and amyotrophic lateral sclerosis ALS patients

	SLE $n=69$	RA $n=77$	SLE + RA $n=146$	ALS $n=68$
Sex, female (%)	69 (100%)	61 (79.2%)	130 (89%)	32
Caucasian ethnicity	63 (91.3%)	74 (96.1%)	137 (93.8%)	66
Mean age ± SD	41 ± 12	59 ± 12	51 ± 15	63 ± 11
Age range	20–68	25–71	20–71	30–86
Average number of repeats ± SD	5.05 ± 3.57	4.72 ± 3.52	4.9 ± 3.54	4.72 ± 2.92
Median number of repeats	5	4	5	5
Range	2–19	2–24	2–24	2–19
Interquartile range	6	5	5.75	6
Patients with ≥ 9 and ≤ 30 repeats (%)	16 (23.2%)[a]	13 (15.6%)	29 (19.9%)[c]	5 (7.4%)
Alleles with ≥ 9 and ≤ 30 repeats (%)	19 (13.8%)[b]	13 (7.8%)	32 (11%)[d]	5 (3.7%)
Patients with homozygous wild-type rs3849942 (%)[e]	1/16 (6.2%)	0/13 (0%)	1/29 (3.4%)	0/5 (0%)
Patients with heterozygous rs3849942 (%)[c]	13/16 (81.2%)	12/13 (92.3%)	25/29 (86.25)	4/5(80%)
Patients with homozygous mutated rs3849942 (%)[e]	2/16 (12.5%)	1/13 (7.7%)	3/29 (10.3%)	1/5(20%)
Patients with duplication (%)	2/69 (2.9%)	0/77 (0%)	2/146 (1.3%)	0/58 (0%)

[a]SLE versus ALS $p=0.020$, OR 3.84, CI 95% 1.19–12.85

[b]SLE versus ALS $p=0.006$, OR 4.18, CI 95% 1.41–13.24

[c]SLE + RA versus ALS $p=0.033$, OR 3.12, CI 95% 1.08–9.70

[d]SLE + RA versus ALS $p=0.021$, OR 3.22, CI 95% 1.16–9.65

[e]Calculated on patients with intermediate expansions

with intermediate expansions, we found two patients with a sequence variation within the GC-rich region immediately downstream of the repeat. Both patients harbored intermediate-length expansions on both alleles (9–10 and 10–10 repeats, respectively) and, in one allele, the same duplication variant (NC_000009.12:g.27573457_27573470dupGCGGTTGCGGTGCC) within the exon 1B (NM_018325.4) or intron 1 (NM_001256054.2 and ENSG00000147894) of the gene. The duplication was located at the beginning of the predicted CpG islands within the 3′ region of the expansion area, as reported by the UCSC Genome Browser (https://genome.ucsc.edu/). Bioinformatics analysis of the region (UCSC Genome Browser, https://genome.ucsc.edu/) also revealed the extremely high conservation of the sequence in mammals and primates (Fig. 2).

The three SLE patients with two intermediate alleles did not show similarities in their clinical history. Two of them had the newly described duplication: one is an Asian patient with a childhood SLE onset, recurrent skin manifestations, leukopenia, previous glomerulonephritis, persistent active serology with low complement level, elevated anti-DNA autoantibodies; the other one is an Afro-American patient, with polyarthritis, skin, and hematological manifestations. The latter one carries the wild-type form of rs3849942 SNP on both alleles. The third patient is Caucasian, with onset with arthritis and pleuropericarditis, without any flare during follow-up.

None of the SLE or RA patients with intermediate expansions experienced either signs/symptoms suggesting ALS or FTD or had first-degree affected relatives, although we cannot exclude that some patients with intermediate expansions, especially young patients, may develop ALS or FTD in the future, in spite of the fact that they were asymptomatic at the time of the study. Moreover, we divided our populations into two subgroups, considering the presence or absence of intermediate expansions and compared the two subgroups, analyzing demographical features (age at onset, gender, race) and the clinical manifestations included in the classification criteria for both the diseases. No peculiar features associated with the intermediate expansion were identified in patients with SLE or RA.

Discussion

In this study, we found that patients with SLE and SLE + RA, and to a lesser extent RA only, have a high frequency of intermediate *C9orf72* hexanucleotide expansions in at least one allele. We also found three SLE patients, harboring intermediate expansions on both alleles, two of whom also showing the same duplication variant within the downstream

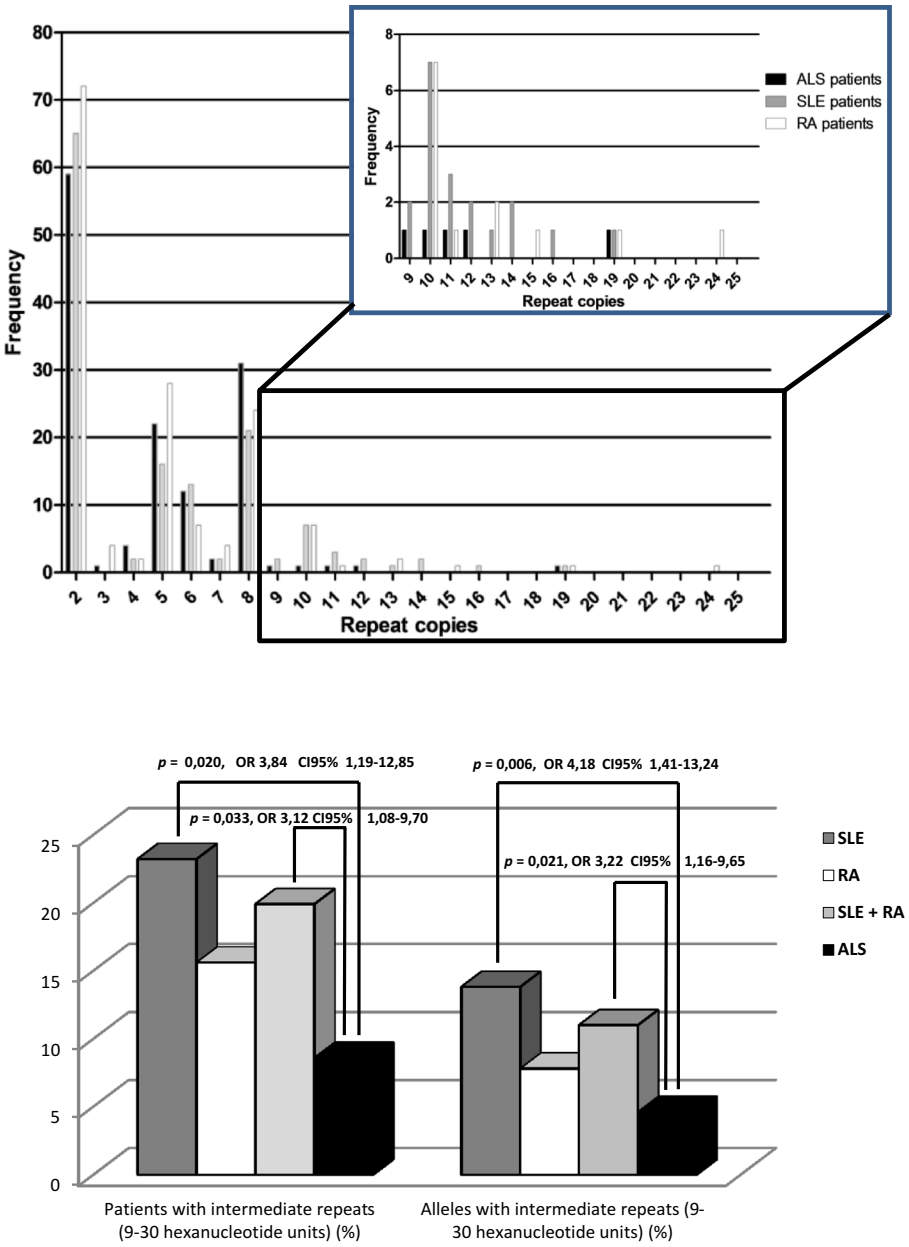

Fig. 1 **a** Histogram showing the distribution and frequency (%) (Y-axis) of the number of *C9orf72* hexanucleotide repeats (X-axis) in ALS, SLE, and RA patients' alleles. **b** Frequency (%) (Y-axis) of intermediate alleles > 9 and < 30 repeat units in SLE, RA, SLE + RA, and ALS patients and in patients' alleles. *SLE* systemic lupus erythematosus (dark gray), *RA* rheumatoid arthritis (white); SLE + RA (light gray); *ALS* amyotrophic lateral sclerosis (black)

GC-rich region. Our findings suggest that *C9orf72* intermediate alleles may represent a genetic risk factor, contributing to the occurrence of systemic autoimmune diseases. However, we identified no peculiar clinical features strictly associated with expansion length, co-existence of two intermediate alleles or presence of sequence variations.

The genetic association with ALS/FTD spectrum disorders of large *C9orf72* hexanucleotide expansions has been widely demonstrated (DeJesus-Hernandez et al. 2011; Renton et al. 2011; Byrne et al. 2012; Sabatelli et al. 2012). Nonetheless, a definite pathogenic cut-off for full mutations has not yet been established (Ng and Tan 2017). In some repeat expansion disorders, pathological evidences have been found in patients with intermediate premutation alleles (Lozano et al. 2014; Semaka and Hayden 2014). Full mutations and premutations can also result in clinically distinct phenotypes, like for intellectual disability/autism spectrum disorder related to Fragile X syndrome, due to full mutations in the *FMR1* gene, and Fragile X-associated tremor/ataxia syndrome (in males) or Fragile X-associated primary ovarian insufficiency (in females), observed in premutation carriers (Lozano et al. 2014). The presence of *C9orf72* intermediate-length alleles has not been found associated with a higher risk of neurodegenerative diseases, although it might predispose to neuropsychiatric symptoms (Ng and Tan 2017). More recently, *C9orf72* intermediate alleles > 20 repeat units have been found significantly increased in patients

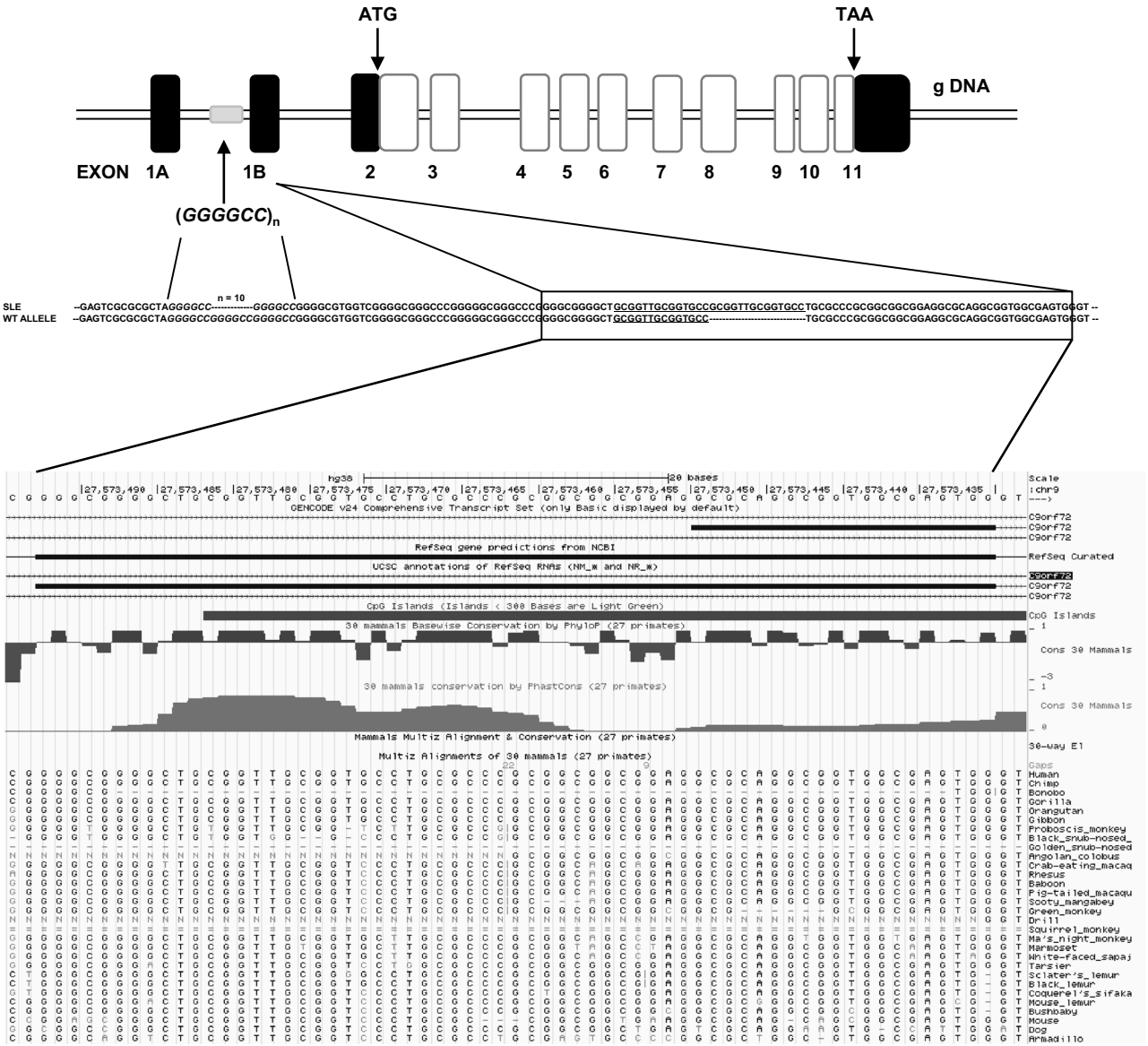

Fig. 2 The figure shows the structure of the *C9orf72* gene, with non-coding exons in black and coding exons in white. The position of the hexanucleotide repeat expansion is shown in gray, upstream of the non-coding exon 1B. Below, the sequence of the region encompassing the repeat (in italics) and the GC-rich low-complexity region, located immediately downstream of the repeat and comprising exon 1B (framed), is shown. DNA sequences for both intermediate alleles with duplication (number of hexanucleotide repeats, $n = 10$), found in two SLE patients, and wild-type (wt) allele, are shown. The duplicated sequence, located within exon 1B, is underscored. Below the DNA sequences, a bioinformatics analysis of the region (UCSC Genome Browser) shows the presence of predicted CpG islands in the duplicated region and the extremely high conservation of the sequence in mammals

with primary progressive multiple sclerosis (PPMS), a neurodegenerative and autoimmune condition (Tiloca et al. 2018) and we have recently reported the finding of a multiple sclerosis (MS) patient with severe cognitive decline, harboring *C9orf72* alleles with 11 and 15 hexanucleotide repeats (Biasiotto and Zanella 2019), that could be defined as intermediate repeats (Cacace et al. 2013). No data are currently available regarding the influence of *C9orf72* intermediate alleles on conferring the risk to develop non-neurological diseases. The C9ORF72 protein is highly expressed in myeloid cells and has a role in autophagy-related pathways and immunity (Lall and Baloh 2017; Rizzu et al. 2016), acting as a suppressor of autoimmunity (Zhang et al. 2018). *C9orf72*$^{-/-}$ knockout mice exhibit an autoimmune phenotype and haploinsufficient hemizygous *C9orf72*$^{+/-}$ mice show enhanced cytokine responses to immune stimuli and partial disruption of myeloid cell functions (Atanasio et al. 2016; Burberry et al.

2016; O'Rourke et al. 2016). Liu et al. (2008) also identified polymorphisms in the *C9orf72* locus, associated with differential response to anti TNFα (tumor necrosis factor α) therapies in RA patients. Intermediate *C9orf72* alleles are hypermethylated in the CpG islands, located near the repeat, and decreased *C9orf72* transcriptional activity has been reported starting from an expansion > 7 units (Gijselinck et al. 2016). These observations prompted us to explore the hypothesis that *C9orf72* intermediate alleles, probably through decreased gene expression, might confer the risk to develop autoimmune diseases. In this regard, we have recently described reduced expression of *C9orf72* mRNA in peripheral blood cells of the above-mentioned MS patient with cognitive decline, who was found to be a homozygous carrier of *C9orf72* intermediate alleles (11 and 15 repeat units) (Biasiotto and Zanella 2019).

We did not find large *C9orf72* expansions in SLE and RA patients, none of them showed neurological signs or symptoms, suggestive of ALS, FTD or related neurodegenerative diseases, nor had first-degree relatives, affected by neurological disorders, although we cannot exclude that some patients, especially young patients, may develop ALS or FTD in the future. Indeed, as expected, the mean and median age of SLE and RA patients were lower than those of control subjects affected by ALS. Our results indicate that intermediate-length expansions > 9 and < 30 repeat units in the *C9orf72* gene are significantly more frequent in SLE patients than in a control ALS population without large expansions. We also observed a higher frequency of intermediate alleles in a cohort of RA patients, although the comparison did not reach the statistical significance; however, also the SLE + RA cohort showed a significantly higher prevalence of these alleles. Autoimmune manifestations frequently precede ALS symptoms and epidemiological and genetic studies have thoroughly documented the strict link between ALS/FTD and autoimmunity (Lall and Baloh 2017). A genetic overlap between immune-mediated diseases and ALS/FTD has been recently demonstrated (Broce et al. 2018) and increased rates of autoimmune manifestations have been observed in FTD and FTD–MND (motor neuron disease) patients, particularly non-thyroid diseases, like inflammatory arthritis, cutaneous conditions, and gastrointestinal disorders (Miller et al. 2013, 2016; Katisko et al. 2018), although *C9orf72* repeat expansion-positive FTD patients showed lower prevalence in those studies. To the best of our knowledge, this is the first study, analyzing *C9orf72* gene in patients with clinically defined systemic autoimmune diseases. Our findings suggest that *C9orf72* intermediate alleles may represent a genetic risk factor for these conditions. Together with the recent observation of *C9orf72* intermediate alleles in patients with PPMS (Tiloca et al. 2018) and MS (Biasiotto and Zanella 2019), our results further suggest a central role of the C9ORF72 protein in regulating autoimmunity.

In our study, three SLE patients harbored intermediate *C9orf72* expansions on both alleles and two of them showed the same duplication variant. This duplication was not previously reported and in our cohort it was found in two patients with different non-Caucasian ethnicity. Sequence variations (insertions, duplications, and deletions) in the region are significantly more common in alleles with large expansions (22.5%) than in normal alleles (2.3%) (Nordin et al. 2017). These findings have been recently confirmed in two cohorts of Italian and Turkish ALS patients (Corrado et al. 2018), although in this study only deletions, and not insertions, were significantly more frequent in expansion carriers and the sequence variation frequency among expansion carriers was lower (8.6%). We found 2 out of 69 (2.9%) SLE patients carrying variants, that is 2 out of 16 (12.5%) with at least one intermediate allele, a frequency that is nearest that of largely expanded alleles (Nordin et al. 2017; Corrado et al. 2018). Conversely, we found no sequence variations in ALS control patients and in an ALS/FTD cohort, previously studied in our laboratory (Biasiotto et al. 2017). Homozygous intermediate repeat carriers are hypermethylated in the *C9orf72* promoter region in comparison with homozygous short repeat carriers and methylation degree is significantly higher in brain than in blood. Hypermethylation, although lower than that observed in large expansion alleles, is suggestive of reduced transcription (Gijselinck et al. 2016). Decreased transcriptional activity is further indicated by the presence of sequence variants in the GC-rich low-complexity region immediately downstream of the repeat (Gijselinck et al. 2016). In this respect, it is interesting to note that the duplication we found in two patients is located at the beginning of the CpG islands in the 3′ region of the repeat. The bioinformatics analysis also reveals the extremely high conservation of the region in mammals and primates (Fig. 2). We did not measure *C9orf72* transcription in our patients, and this is a limitation of our work. It is, however, noteworthy the fact that we have recently described reduced *C9orf72* mRNA expression in peripheral blood cells of a MS homozygous carrier of *C9orf72* intermediate alleles (Biasiotto and Zanella 2019). Furthermore, we cannot exclude that patients with intermediate alleles, found in this study, might have unchanged *C9orf72* expression in total blood cells, but decreased expression in cellular subsets, involved in the pathogenesis of the autoimmune disorder. In this regard, it is interesting to note that Rizzu et al. (2016) observed the highest expression of *C9orf72* in a particular subset of myeloid cells ($CD14^+$ monocytes) and differential expression of distinct transcriptional start sites of the *C9orf72* transcripts between myeloid cells and brain tissues. We cannot even exclude that patients with intermediate alleles might have somatic instability and larger expansions

in particular cells, involved in the disease. Indeed, the repeat may become unstable with increasing repeat units and we found the risk rs3849942 allele, defining a genetic background that could contribute to the expansion, in almost all patients with intermediate alleles. We did not observe instability in peripheral cells in most patients, however, in some of them we repeatedly observed one faint higher amplicon in sizing PCR, while no obvious instability was revealed by repeat-primed-PCR (not shown). We cannot exclude that the higher amplicon could be a PCR artifact, but it is tempting to speculate that it might derive from an extra-expansion in a subset of peripheral cells. Further studies are ongoing to verify this hypothesis on a larger sample of subjects with intermediate-length expansions in the *C9orf72* gene.

Our study has some further limitations. It was performed in a limited number of patients and patients included in this study were not matched to controls for sex/age, because we consecutively included regularly followed SLE and RA patients from January to December 2017. This can explain the different female:male ratio in the analyzed groups. Systemic autoimmune diseases mostly affect female, with a female:male ratio of 9:1 for SLE and 3:1 for RA, whereas ALS is slightly more prevalent in males (Curtis et al. 2017). However, previous studies did not report evident sex differences in the prevalence of *C9orf72* intermediate expansions (Ng and Tan 2017). Studies on larger cohorts of patients with SLE, RA, or other autoimmune disorders are needed to better define whether the presence of intermediate *C9orf72* alleles are associated with autoimmune conditions.

A further limitation is that we considered an ALS cohort as control, rather than a healthy cohort. Nevertheless, all published studies performed in ALS cases without pathological *C9orf72* expansions and healthy controls found no significant differences in distribution, range, and median number of repeats (DeJesus-Hernandez et al. 2011; Renton et al. 2011; Byrne et al. 2012; Sabatelli et al. 2012; Ng and Tan 2017).

It should also be stated that most of the intermediate expansions found in this study are minor expansions although >8 repeat units and that it is currently unclear what is the significance of these expansions (Biasiotto and Zanella 2019).

Finally, we cannot exclude that the high frequency of intermediate alleles and risk haplotype in SLE and RA patients could be linked to a genetic background defined by but unrelated to *C9orf72*, while possibly linked to other genetic loci within the region, as previously suggested (Liu et al. 2008).

In conclusion, in this study, we assessed the potential role of intermediate *C9orf72* alleles as genetic risk factor for the development of autoimmune conditions and found higher than expected frequency of these alleles in SLE, SLE + RA, and in a lesser extent RA only patients. Although our study has the limitation of sample size, this is the first report on *C9orf72* intermediate alleles in subjects with systemic and non-neurodegenerative autoimmune diseases, while the prevalence of autoimmune diseases in ALS/FTD patients with or without pathogenic *C9orf72* expansions is still a matter of debate (Miller et al. 2016; Katisko et al. 2018). Larger studies in different autoimmune conditions are warranted to clarify the role of *C9orf72* in autoimmune diseases in humans.

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Compliance with Ethical Standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical Approval This study was performed in accordance with the 1964 Declaration of Helsinki and its later amendments and was approved by the Ethic Committee of the Promoting Centre (reference no. 2918-Studio C9ORF72CTD).

Informed Consent Written informed consent was obtained from all individual participants included in the study.

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