Autophagy and mitophagy elements are increased in body fluids of Multiple Sclerosis-affected individuals

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AUTHOR CONTRIBUTIONS

Simone Patergnani, Massimiliano Castellazzi, Carlotta Giorgi, Enrico Granieri and Paolo Pinton, conceived and designed the experiments. Simone Patergnani and Massimiliano Castellazzi conducted the experiments, analysis of samples, analysis and interpretation of data. Massimo Bonora, Saverio Marchi, Maura Pugliatti and Ilaria Casetta helped with patients' enrollment and data analysis. Simone Patergnani, Massimiliano Castellazzi, Enrico Granieri and Paolo Pinton wrote the manuscript

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DISCLOSURE

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CONFLICT OF INTEREST DECLARATION.

All authors declare no conflict of interest.

Background

Multiple sclerosis (MS) is a chronic multifaceted demyelinating and neurodegenerative disease of the central nervous system (CNS) of presumed autoimmune origin[1].

MS patients are characterized by a spatial and temporal dissemination of neurological sign and symptoms, by the presence of multi-focal lesions in the periventricular white matter on Magnetic Resonance Imaging (MRI) scans and by an immunoglobulin synthesis within the central nervous system[1]. Further diagnostic tools are desirable, and the use of blood and cerebrospinal fluid (CSF) biomarkers may contribute to the comprehension of the disease's pathogenesis and progression.

Autophagy is an evolutionarily conserved and genetically controlled cellular process where intracellular components are sequestered within double-membrane vesicles (autophagosomes), which then fuse with lysosomes where the material is degraded[2]. Autophagy also occurs as mitophagy, which is responsible for the removal of aberrant, aged and wasted mitochondria. Interestingly, auto-/mitophagic pathways have been found deregulated in various human diseases. In particular, it has been demonstrated how these catabolic pathways are implicated in several neurodegenerative diseases such Alzheimer's and Parkinson's diseases and amyotrophic lateral sclerosis[2]. Moreover, recent studies suggest a role of mitochondrial dysfunction in the neurodegenerative aspects of MS[3]. Despite these observations, the role of autophagy and mitophagy in MS is still elusive.

To tackle the question, we tried to verify the frequency of specific autophagic markers (ATG5 protein) and mitophagic markers (Parkin protein) in MS patients and neurological controls.

Methods

Forty consecutive untreated relapsing-remitting MS patients were included in the study (24 female and 16 male; mean age = 40.7 ± 10.5). Forty patients with other inflammatory neurologic diseases (OIND) (21 female and 19 male; mean age = 55.9 ± 17.5) and 40 subjects with non-inflammatory neurologic diseases (NIND) (21 female and 19 male; mean age = 64.8 ± 15.5) were included as

published before[4]. Paired CSF and serum samples collected from RRMS, OIND, and NIND patients were obtained for purposes of diagnosis. Serum samples obtained from 40 healthy donors (CTRL) (24 female and 16 male; mean age = 28.6 ± 6.5) were used as additional control. The study was approved by the Committee for Medical Ethics in Research of Ferrara and written informed consent was obtained from all subjects.

ELISA kits for ATG5 (MS7209535) and Parkin (MBS732278) were obtained from My Biosource, Inc. (San Diego, USA). TNFα ELISA kits (KHC3011) were purchased from ThermoFischer Scientific (Waltham, USA). All the assays were performed as instructed by the manufacturer. Statistical analysis was performed with Graph Pad Prism software. Parametric and non-parametric tests were used respectively for normally and non-normally distributed data.

Results.

Serum levels of ATG5 and Parkin were higher in MS patients than in OIND, NIND and CTRL ($p<0.0001$) (**Figure 1, panels A-B**). Similarly, CSF levels of ATG5 and Parkin were higher in MS patients than in OIND and NIND ($p<0.0001$) (**Figure 1, panels C and D**). A positive correlation was found between ATG5 and Parkin levels both in sera and CSF of MS patients ($p<0.001$ and $p<0.0001$) (**Figure 1, panels E and F**). In CSF of MS patients a positive correlations were described between TNFα and both ATG5 and Parkin levels ($p<0.0001$) (**Figure 1, panels G and H**). Serum and CSF titers of ATG5 and Parkin were not different in MS patients grouped according to clinical disease activity and did not correlate with age and sex in MS OIND and NIND (data not shown).

Discussion

The novelty of this study is the impressive increase of both catabolic markers in MS patients compared to controls, suggesting that elevated auto-/mitophagy seems to be specifically related to the disease. The role of auto-/mitophagic pathways in MS is not well clarified and it remains

doubtful whether they are protective or harmful processes. Some works suggest that autophagy is a protective homeostatic mechanism capable to influence synaptic growth and plasticity. For this reason, up-regulation of autophagy may prevent, delay or ameliorate neurodegenerative diseases[2]. Conversely, other studies consider these pathways a very dangerous condition in CNS. Indeed, inflammatory stimuli lead to a blockade of the differentiation of oligodendrocyte progenitor cell, resulting in a reduced myelination of axons *in vitro*, as a consequence of an increased autophagic activity[5].

The increases we found for both ATG5 and Parkin in CSF of MS patients confirm the last hypothesis and indicate a pathological role of auto-/mitophagy in CNS of MS patients[5]. In addition, the correlations between both ATG5 and Parkin levels and TNFα concentrations in CSF seems to confirm *in vivo* the association among auto-/mitophagic activity and inflammatory stimuli, as previously described in *in vitro* experiments[2,5]. Again, the positive correlation between ATG5 and Parkin concentrations suggests that autophagic and mitophagic mechanisms are reciprocally associated in CSF and serum of MS patients.

These results are particularly intriguing. In fact, if on the one hand CSF data should reflect a role of auto-/mitophagy in MS pathogenesis, on the other hand serum levels of these molecules could be used as biomarkers of MS. The main problem of lumbar puncture to obtain CSF is the invasive methods and special precautions should be taken during its execution. Thus, there is a pressing need for new biomarkers in more easily accessible body fluids such as peripheral blood.

The main limit of the present study is the lack of additional clinical information, such as disease duration and/or severity, and the lack of sex and age matching between MS patients and controls. However, we did not find any difference between male and female and there were not significant correlations between age and the concentrations of ATG5 and Parkin in all the four groups of patients and controls.

In conclusion, our study suggests, for the first time, that autophagy and mitophagy processes could play an important role in the pathogenesis of MS and introduces a fascinating conjecture. Further

studies on a larger population are needed to elucidate the molecular relationships between catabolic processes and MS ongoing and progression, as well as to elucidate the potential role of auto/mitophagic biomarkers in monitoring disease progression and/or the pharmacological response to therapy.

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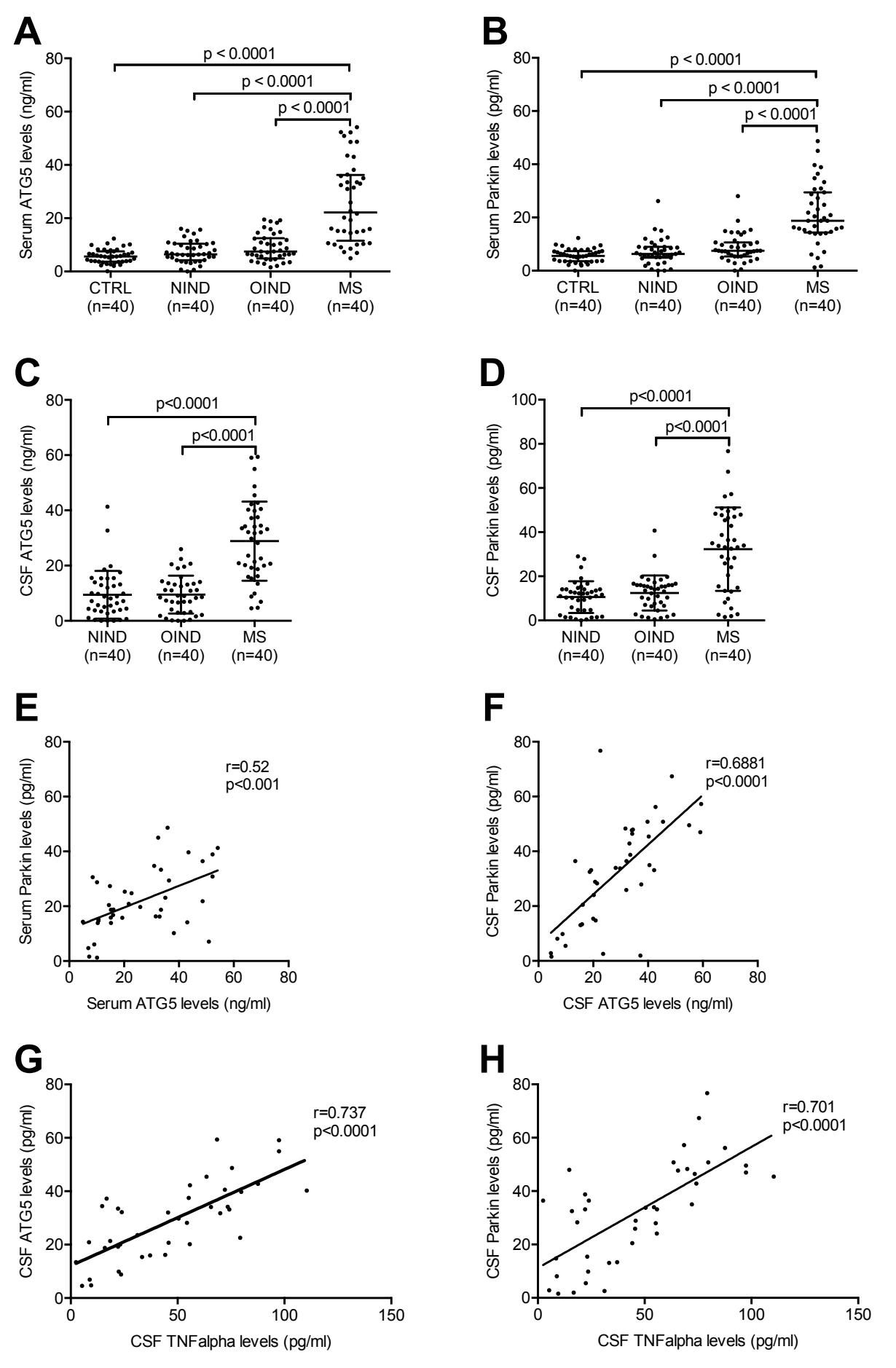
Figure Legend

Figure 1. Serum levels of ATG5 and Parkin from healthy donors (CTRL), subjects with non-inflammatory neurological disorders (NIND), other inflammatory neurological disorders (OIND) and from patients with multiple sclerosis (MS). Concentrations of ATG5 and Parkin were different among CTRL, NIND, OIND and MS (Kruskal–Wallis; $p < 0.0001$). Serum values of ATG5 (Mann–Whitney with Bonferroni correction): MS versus OIND ($p < 0.0001$), MS versus NIND ($p < 0.0001$) and MS versus CTRL ($p < 0.0001$) (panel **A**). Serum levels of Parkin (Mann–Whitney with Bonferroni correction): MS versus OIND ($p < 0.0001$), MS versus NIND ($p < 0.0001$) and MS versus CTRL ($p < 0.0001$) (panel **B**). CSF levels of ATG5 and Parkin were different among MS, OIND and NIND (ANOVA; $p < 0.0001$). CSF values of ATG5 (t-test with Bonferroni correction): MS versus OIND ($p < 0.0001$) and MS versus NIND ($p < 0.0001$) (panel **C**). CSF levels of Parkin (t-test with Bonferroni correction): MS versus OIND ($p < 0.0001$) and MS versus NIND ($p < 0.0001$) (panel **D**). There were positive correlations between ATG5 and Parkin levels in sera (Spearman; $p < 0.001$) and CSF (Pearson; $p<0.0001$) of MS patients (panel **E** and **F** respectively). Additional correlations were found in CSF for both ATG5 and Parkin levels and TNFα among MS subjects (Spearman; $p < 0.0001$) (panel **G** and **H** respectively). Parametric and non-parametric tests were used respectively for normally and non-normally distributed data. Accordingly, results are presented as mean and standard deviation for normally-distributed variables and as median and interquartile range for non-normally distributed data. A value of $P <0.05$ was accepted as statistically significant.


A
Serum ATG5 levels (ng/ml)
p < 0.0001
p < 0.0001
p < 0.0001
CTRL (n=40)
NIND (n=40)
OIND (n=40)
MS (n=40)
B
Serum Parkin levels (pg/ml)
p < 0.0001
p < 0.0001
p < 0.0001
CTRL (n=40)
NIND (n=40)
OIND (n=40)
MS (n=40)
C
CSF ATG5 levels (ng/ml)
p<0.0001
p<0.0001
NIND (n=40)
OIND (n=40)
MS (n=40)
D
CSF Parkin levels (pg/ml)
p<0.0001
p<0.0001
NIND (n=40)
OIND (n=40)
MS (n=40)
E
Serum Parkin levels (pg/ml)
r=0.52
p<0.001
Serum ATG5 levels (ng/ml)
F
CSF Parkin levels (pg/ml)
r=0.6881
p<0.0001
CSF ATG5 levels (ng/ml)
G
CSF ATG5 levels (pg/ml)
r=0.737
p<0.0001
CSF TNFalpha levels (pg/ml)
H
CSF Parkin levels (pg/ml)
r=0.701
p<0.0001
CSF TNFalpha levels (pg/ml)