1 Relationship between proinflammatory cytokines (Il-1beta, Il-18) and leukocyte telomere
2 length in mild cognitive impairment and Alzheimer's disease
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## Abstract

Inflammation plays a crucial role in Alzheimer's disease (AD). AD neurodegeneration and concurrent involvement of the peripheral immune system may promote leukocyte division and telomere shortening. We examined genotypes and plasma levels of two proinflammatory cytokines, IL-1beta and IL-18, and leukocyte telomere length (LTL) in patients with mild cognitive impairment (MCI) and AD. We wanted to determine whether changes in plasma IL-1beta and IL-18 levels, together with LTL shortening, could be diagnostic for disease progression from MCI to AD. Median plasma IL-1beta levels were in the order MCI patients (2.2 pg/mL) < AD patients (4.0 pg/mL), both of which differed significantly from the controls (0.0 pg/mL). In the AD patients, the lowest IL-1beta levels were associated with the presence of the C allele of IL-1beta rs16944 SNP. Median plasma IL-18 levels were in the order MCI patients (116.3 pg/mL) > AD patients (85.8 pg/mL), both of which were significantly higher than in the controls (17.6 pg/mL). Analysis of LTL showed a progressive reduction in the order controls > MCI > AD patients ($p<0.0001$). Overall LTL reduction was correlated with increased plasma IL-1beta levels, substantiating the hypothesis that inflammatory processes secondary to neuroinflammation may trigger telomere attrition. Changes in plasma IL-1beta and Il-18 levels, and LTL seem to reflect shifts in AD stage; they may have potential use as blood biomarkers to monitor disease onset and progression from MCI to AD.


**Key Words**: Alzheimer's disease, mild cognitive impairment, IL-1beta, IL-18, leukocyte telomere length

1 **1. Introduction**

2 The importance of inflammatory components in the pathogenesis of Alzheimer's disease (AD)
3 has been amply demonstrated.. Initial experiments in mouse and human brain reported the accumulation
4 of activated microglial cells around the amyloid deposits forming senile plaques, the histopathological
5 marker of AD (Haga et al., 1989). Such plaques are surrounded not only by activated microglia but also
6 by astroglial cells secreting several proinflammatory mediators that may contribute to the synaptic
7 rarefaction and the neuronal loss characteristic of AD. β-amyloid (Aβ) binding to the receptor for
8 advanced glycation end products (RAGE) (Cai et al., 2016) and toll-like receptors (TLR) induces NFkB
9 activation and transcription of inflammatory molecules in microglia and astrocytes, thus activating
10 signaling pathways involved in neurodegeneration (Bachstetter et al., 2011; Boche et al., 2013; Ricci et
11 al., 2012). Accordingly, a therapeutic strategy entails the use of anti-inflammatory protocols targeting
12 neuroinflammation (Ali et al., 2019; Businaro et al., 2018; Pinto et al., 2018). Moreover, the microglia
13 from aged mice were found to exhibit increased proinflammatory cytokine production, including IL-6, IL-
14 1beta, and TNF-α (Sierra et al., 2007), all of which are upregulated in senescent cells. Indeed, activation
15 of the peripheral immune status has been consistently reported during senescence (Coppé et al., 2008) and
16 especially in AD patients (Baker and Petersen, 2018; Flanary et al., 2007).

17 The increase in inflammatory mediators in the peripheral blood of AD patients may be caused by
18 neuroinflammation and cytokine release through the blood-brain barrier. Since its integrity is impaired in
19 AD (Gosselet et al., 2013) and such dysfunction depends on $A\beta_{25-35}$ -induced altered expression of tight
20 junction proteins (Cuevas et al., 2019) serum cytokine levels may reflect the changes in brain cytokines
21 released during neuroinflammation.

22 For the present study, our attention was focused on IL-1beta and IL-18, two proinflammatory
23 cytokines detected in close proximity to amyloid plaques in AD brains, on the basis of the following
24 observations. Single nucleotide polymorphisms (SNPs) of IL-1beta and IL18 genes have been found
25 associated with a greater probability of developing the disease (Tian et al., 2015; Yu et al., 2009). A
26 recent meta-analysis reported that these cytokines number among the specific peripheral inflammatory
27 biomarkers for AD (Su et al., 2019). In line with these observations, NOD-like receptor family pyrin
28 domain (NLRP3) inflammasome activation leading to IL-1beta and IL-18 production is recognized as a
29 central actor in the machinery underlying neuronal death in AD (Heneka et al., 2013; Lang et al., 2018).
30 TNF-α, IL-1beta, and IL-6 act directly on the neuron and induce apoptosis. Similarly, TNF-α and IL-
31 1beta can activate astrocytes, which release factors that activate microglia, giving rise to an amplification

loop of the entire process (Meraz-Ríos et al., 2013; Nichols et al., 2019; Wang et al., 2015). The detection of high levels of IL-1beta peripherally in patients with MCI, before the development of established AD (Boza-Serrano et al., 2018; King et al., 2018), points toward early inflammatory changes in microglial cells even before the accumulation of Aβ. High levels of IL-18 in the cerebrospinal fluid of MCI patients and marked elevation of circulating sIL-1R2 and free IL-18, which is characteristic of MCI and disappears in AD, make them interesting markers for evaluating progression from MCI to AD (Italiani et al., 2018; Ojala et al., 2009). A further aim of our study was to clarify the possible role of the two cytokines as peripheral biomarkers for MCI and AD. In fact discrepancies exist in the data for the variation of plasma levels of IL-1beta and IL-18 as reported by Olson and Humpel (2010), being many reports controversial or simply inconclusive. Several studies reported a non-significant tendency of blood IL-18 levels to increase in MCI and AD patients, while others reported an increase in blood IL-18 levels only in the early stages of AD (mild AD) (Brosseron et al., 2014).

The neuroinflammatory state accompanying AD, and the concurrent involvement of the peripheral immune system, may promote leukocyte division and telomere shortening in relation to astrocyte and microglia proliferation (Liu and Chan, 2014; Liu et al., 2016). There is urgent need to develop a panel of biomarkers to predict the progression of MCI to AD. In this study, we examined the relationships between IL-1beta and IL-18 and leukocyte telomere length (LTL), with the aim to determine whether changes in plasma IL-1beta and IL-18 levels and shortening of telomere length could provide reliable predictive markers of progression from MCI to AD (Liu and Chan, 2014; Liu et al., 2016).

Human telomeres are composed of repeated TTAGGG nucleotide sequences located at the ends of each chromosome. Because telomere sequences are not fully replicated during DNA replication due to the inability of DNA polymerase to replicate the 3′ end of the DNA strand, telomeres shorten as cells divide. A cellular ribonucleoprotein enzyme complex, called telomerase, counteracts telomere shortening (Blackburn et al., 2015; Wong and Collins, 2003), but its activity, usually present in the early stages of embryonic development, is downregulated in several human somatic tissues relatively early (Ishaq et al., 2016; Ulaner et al., 1998; Ulaner and Giudice, 1997). Since telomerase is almost absent in adult tissues, including skin, kidney, liver, blood vessels, and peripheral leukocytes, the telomeres of replicating cells shorten progressively, and this mechanism is thought to underlie aging and age-associated diseases. Studies at the population level of leukocyte telomere length (LTL) have provided ample evidence that leukocyte telomere shortening is associated with aging (Cawthon et al., 2003; Kimura et al., 2008;

Lapham et al., 2015; Sanders and Newman, 2013) and with chronic diseases of advanced age (cardiovascular and metabolic disease, cancer), although some inconsistencies have been observed (Barrett et al., 2015; Codd et al., 2013; Sanders and Newman, 2013).When LTL was investigated in connection with neurodegenerative diseases, shorter leukocyte telomeres were frequently found in both monogenic (Huntington's disease, Mantuano et al., 2019; Scarabino et al., 2019) and complex disease (AD). The LTL association with cognitive decline/dementia and AD has been widely investigated (Boccardi et al., 2015; Cai et al., 2013; Forero et al., 2016), whereas the data on LTL in MCI patients are fewer and often contradictory (Movérare-Skrtic et al., 2012; Nudelman et al., 2019; Roberts et al., 2014). Recent studies have suggested that progressive LTL reduction may be a marker of the progression from MCI to AD (Jenkins et al., 2017; Scarabino et al., 2017).

We analyzed the link between changes in LTL and IL-1beta and IL-18 levels in MCI and AD patients to gain a better understanding of the relationships between telomere length, AD development, and inflammatory status. Serum IL-1beta and IL-18 levels and the genotypes of three IL-1beta and IL-18 SNPs were assayed in three samples (controls, MCI, and AD patients) and their relationship with LTL compared. The ultimate goal of the study was to investigate the pathogenetic mechanisms of AD development from its prodromal phases (MCI) to established disease and to evaluate the interconnections between inflammatory cytokines and LTL, two biomarkers that could provide a potentially useful diagnostic tool to identify patients who may switch from MCI to AD

## 2. Materials and Methods

### 2.1. Subjects

LTL was measured in two groups of patients (AD and MCI) and a control group recruited at the Alzheimer's Disease Center of Neurology Division of Verona Hospital (Table 1). All subjects were Caucasians, born in a limited geographical area in northern Italy (district of Verona and Veneto region).

Dementia in the AD patients was diagnosed according to Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria (American Psychiatric Association, 2000). Probable AD was diagnosed according to National Institute of Neurologic and Communicative Disorders and Stroke and Alzheimer Disease and Related Disorders Association (NINCDS-ADRDA) Work Group guidelines (revised) (McKhann et al., 1984). Cognitive status was quantified using the Mini Mental State Examination (MMSE) (Folstein et al., 1975).

| | Controls | MCI patients | AD patients | p-value |
|---|---|---|---|---|
| Total | 162 | 56 | 254 | |
| Age (years) | 70.0 ± 8.9 | 76.1 ± 6.0 | 78.5 ± 8.2 | <0.0001 |
| Sex (females, %) | 61.1 | 66.1 | 71.1 | 0.10 |
| Age at onset | / | 69.4 ± 6.2 | 75.2 ± 8.1 | <0.0001 |
| MMSE score | / | 28.2 ± 2.0 | 18.7 ± 4.6 | <0.0001 |

Table 1. Demographic characteristics of Controls, MCI and AD patients

Amnestic MCI (aMCI) diagnosis was established according to internationally accepted criteria (Petersen et al., 2009; Albert et al., 2011): 1) subjective complaint of a memory (or other cognitive) deficit confirmed by a relative or caregiver; 2) objective evidence of impairment in cognitive domains (single or multiple cognitive domains) assessed using the Mental Deterioration Battery (MDB) (Carlesimo et al. 1996) which assesses attention, verbal memory, verbal fluency, and constructive praxis; 3) normal performance of activities of daily living as evaluated and excluded according to Clinical Dementia Rating (CDR) scale scores and two clinical interviews with both the patient and the informant (Instrumental Activities of Daily Living and Basic Activities of Daily Living) (Katz et al., 1970; Lawton and Brody, 1969); 4) absence of global deterioration (Mini Mental State Examination, according to Folstein et al., 1975 ) and dementia, as defined by the Diagnostic and Statistical Manual of mental disorders criteria (American Psychiatric Association, DSM-IV- 2000). Exclusion criteria were a history of head injury, psychiatric disorders, neurological diseases or severe sensorial deficits.

The control group was composed mostly of the patients' spouses, unrelated to any of the patients and not demented according to the interview. The protocol for the collection of biological material for the scientific studies was approved by the institutional ethics committees. Informed consent was obtained from all subjects.

2.2. Laboratory methods

Genomic DNA was extracted from venous blood drawn in $EDTANa_2$ as anticoagulant according to the salting out procedure described by Miller et al. (1988). IL-1beta(rs16944, -511) genotypes were determined according to Di Giovine et al. (1992). IL-18 (rs1946518 and rs187238) genotypes were determined with a tetra-primer amplification refractory mutation system PCR (ARMS-PCR). For IL18 rs1946518 ARMS-PCR, the external primers were forward5′ CTTTGCTAT CATTCCAGGAA 3′ and reverse: 5′ TAACCTCATTCAGGACTTCC 3′; the internal primers were forward 5′ GTTGCAGAAAGTGTAAAAATTATTAC 3′ and reverse 5' ACGGATACCATCATTAG ATTTTATT 3'. For IL18 rs187238 ARMS-PCR, the external primers were forward 5′

CCAATAGGACTGATTATTCCGCA 3′ and reverse R: 5′ AGGAGGGCAAAATGCACTGG 3′; the internal primers were 5′ CCCCAACTTTTACGGAAGAAAAG 3′ and reverse 5' CTTTTAATGTAATATCACTATTTTCATGAAATG 3'.

The average (of triplicate) telomere length in leukocytes was measured by real-time PCR quantitative analysis (qPCR) on a 7300 real-time PCR instrument (Applied Biosystems). This method allows the determination of the number of copies of telomeric repeats (T) compared to a single copy gene (S), used as a quantitative control ( T/S ratio) (Cawthon, 2002). The telomere and single-copy gene β-globin (HGB) were analyzed on the same plate in order to reduce inter-assay variability. DNA (35 ng) was amplified in a total volume of 20 µl containing 10 µl of SYBR Select Master Mix (Applied Biosystems); primers for telomeres and the single copy gene were added to final concentrations of 0.1 µM (Tel Fw), 0.9 µM (Tel Rev), and 0.3 µM (HGB Fw), 0.7 µM (HGB Rev), respectively. The primer sequences were: Tel Fw 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′; Tel Rev 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′; HGB Fw 5′-GCTTCTGACACAACTGTGTTCACTAGCAAC-3′; HGB Rev 5′-CACCACCAACTTCATCCACGTTCACCTTGC-3′ (Cawthon, 2002). The enzyme was activated at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. In addition, two standard curves (one for HGB and one for telomere reactions) were prepared for each plate using a reference DNA sample (Control Genomic Human DNA, Applied Biosystems) diluted in series (dilution factor = 2) to produce five concentrations of DNA from 50 to 6.25 ng in 20 µL. Measurements were performed in triplicate and are reported as T/S ratio relative to the calibrator sample to allow comparison across runs. . Replicate assays of same sample were carried out to calculate the interassay variation. The average standard deviation as calculated by measuring relative T/S ratios of a sample repeated over three different assays was 4.2%. Thus, assuming a normal distribution, samples differing in average telomere length by as little as 8.3% (1.96 X SD) should be distinguishable by this method at the 95% confidence interval (Cawthon, 2002). No amplification of the negative controls with both primer sets (HGB and telomeres) used was observed.

Plasma IL-1beta and IL-18 levels were detected by ELISA according to the manufacturer's instructions (Immunological Sciences Cod. IK4152 Human IL-18 (interleukin 18) Elisa Kit, range 15.625-1000pg/ml , sensitivity < 1 pg/ml; Immunological Sciences Cod. IK4141 Human IL-1beta (interleukin 1 Beta) Elisa Kit, range 1.56-100 pg/ml, sensitivity < 0.15 pg/ml). IL-1beta and IL-18 Elisa kits intra-assay CV is <8% and inter-assay CV is <10%.

1 2.3 Statistical Analysis

2 Allelic frequencies were determined by the gene-counting method. Agreement between the
3 observed genotype distributions of the examined SNPs and those expected according to Hardy-Weinberg
4 equilibrium was verified using a chi square test. In the three groups (controls, MCI and AD patients) the
5 observed genotype frequencies agreed with those expected according to Hardy-Weinberg equilibrium .
6 The genotype distributions among the three groups were compared by a chi square test of independence.
7 Parametric (ANOVA) and nonparametric (Kruskal-Wallis or Wilcoxson rank-sum test) tests were used to
8 compare the distribution of quantitative variables across the three groups, and the distribution of mean
9 plasma IL-1beta/IL-18 levels and the mean T/S ratio across the genotypes of the SNPs examined.
10 Significance was set at $p < 0.05$.

11 **3. Results**

12 3.1 Plasma levels and genotypes of IL-1beta and IL-18

13 Plasma IL-1beta and IL-18 levels were measured in MCI, AD, and control samples. Prior to
14 further analyses, we examined the relationships of IL-1beta and IL-18 levels with age and gender by
15 multiple regression analysis, and no significant relationship was observed between IL1-beta and gender
16 (p=0.42) or age (p=0.40), and between IL-18 and gender (p=0.54) or age (p=0.83). Plasma IL-1beta
17 levels, compared by the Wilcoxson rank-sum test (Table 2, Fig. 1), were higher in the MCI (2.2 pg/ml,
18 p=0.02) and the AD (4 pg/ml p=0.001) patients than in the controls (0 pg/ml), and they were higher in the
19 AD patients than in the MCI patients (p=0.02).

20

21 Table 2. Median values of serum IL-1beta and IL-18 in Controls, MCI and AD patientt expressed as
22 pg/mL. In round brackets $1^{st}$ and $3^{rd}$ quartile values.

| | Controls [no.] | MCI patients [no.] | AD patients [no.] |
|---|---|---|---|
| IL-1beta | 0.0 (0.0 - 4.4) [21] | 2.2 (0.07 - 6.9) [54] | 4.0 (2.4 - 7.4) [35] |
| p | / | 0.02 | 0.001 |
| IL-18 | 17.6 (0.0 - 90.6) [15] | 116.3 (85.8 - 209.7) [20] | 85.8 (29.3 - 158.5) [20] |
| p | / | 0.0004 | 0.01 |

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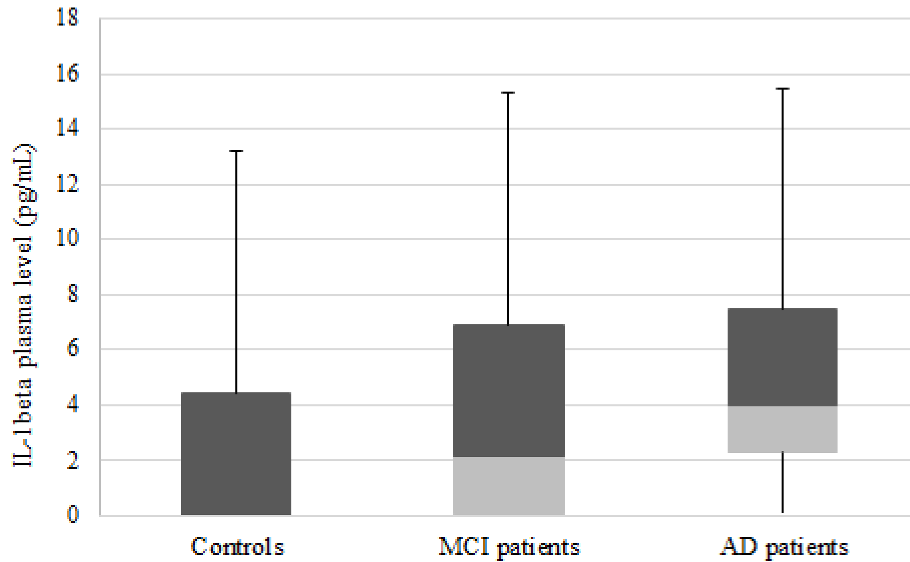


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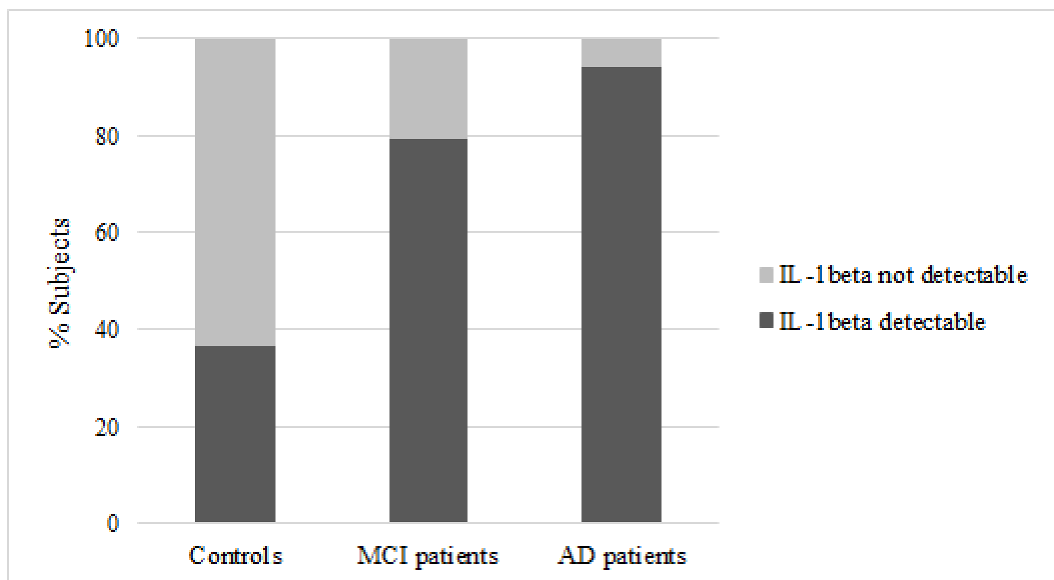
3 Fig. 1 Box and whisker plots showing the distribution of plasma IL-1beta levels in Controls, MCI , and AD
4 patients. Controls: median= 0.0, first quartile (q1) =0.0, third quartile (q3): 4.4; MCI: median=2.2, q1= 0.07, q3= 6.9;
5 AD patients: median=4.0, q1 = 2.4, q3: 7.4.
6
7
8 Plasma IL-1beta was detectable in 94.1% of AD patients, 79.6% of MCI patients, and only in
9 36.8 % of controls, a distribution which was statistically different (chi square=22.9, $p<0.0001$), mainly
10 because of the difference between AD patients and controls (Fig. 2).




11
12 Fig. 2 % detectability of plasma IL-1beta in controls, MCI and AD patients
13
14 On the contrary, plasma levels of IL-18 showed the highest values in MCI patients (116.3 pg/ml), which
15 were significantly different compared to both AD patients ( 85.8 pg/ml p=0.04) and controls ( 17.6 pg/ml
16 p=0.0004) (Table 2, Fig 3).

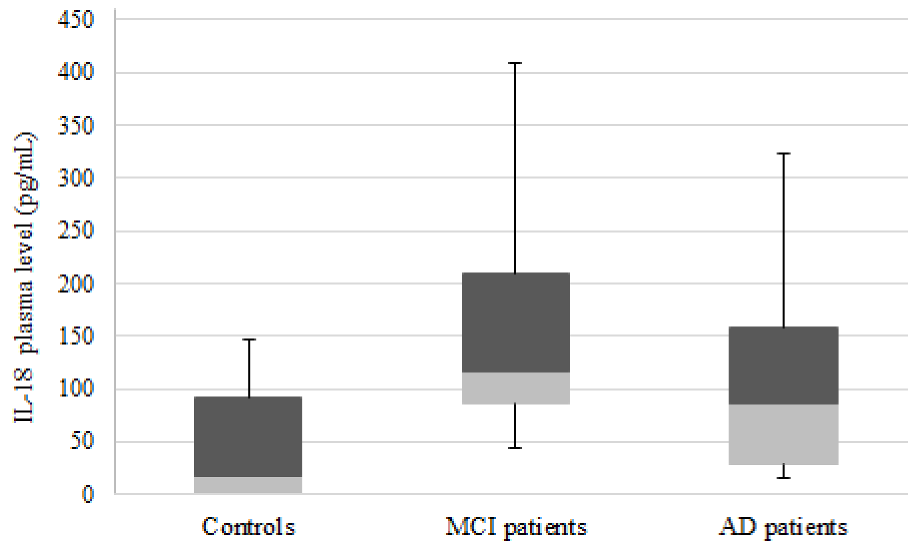



Fig 3- Box and whisker plots showing the distribution of plasma IL-18 levels in Controls, MCI , and AD patients. Controls: median= 17.6, first quartile (q1) =0.0, third quartile (q3): 90.6; MCI: median=116.3, q1= 85.8, q3= 209.7; AD patients: median=85.8, q1 = 29.3, q3: 158.5.

Genotypes of IL-1beta and IL18 SNPs, were determined in larger samples of controls, MCI and AD patients (Table 3). It should be noted that genotyped subject samples did not always match serum sample numbers. No difference was observed in the IL1 beta and IL18 genotype distribution between MCI/AD patients and controls.

Table 3. Genotype distribution of IL-1beta and IL-18 genotypes in controls, MCI and AD patients

| Gene/genotypes | | Controls | MCI patients | AD patients |
|---|---|---|---|---|
| IL-1beta rs16944 | TT | 16 (9.9) | 8 (14.3) | 22 (8.7) |
| | TC | 76 (43.2) | 22 (39.3) | 121 (47.6) |
| | CC | 70 (46.9) | 26 (46.4) | 111 (43.7) |
| tot | | 162 | 56 | 254 |
| Chi square p | | | 0.68 | |
| IL-18 rs187238 | CC | 82 (54.7) | 31 (58.5) | 140 (55.1) |
| | CG | 57 (38) | 20 (37.7) | 91 (35.8) |
| | GG | 11 (7.3) | 2 (3.8) | 23 (1.1) |
| tot | | 150 | 53 | 254 |
| Chi square p | | | 0.76 | |
| IL-18 rs1946518 | CC | 52 (33.1) | 19 (34.5) | 73 (28.7) |
| | AC | 86 (54.8) | 28 (50.9) | 139 (54.7) |
| | AA | 19 (12.1) | 8 (14.5) | 42 (16.5) |
| tot | | 157 | 55 | 254 |
| Chi square p | | | 0.68 | |

When we analyzed the relationships of IL1beta genotypes with IL1 beta plasma levels, a trend showing an association of lower IL1beta levels with CC genotype was observed, mainly in AD subjects, although the difference among the mean IL-1beta level and genotype (Table 4) was not significant. A comparison of IL-1beta levels associated with CC genotypes (3.5±3.5 pg) and genotypes carrying T allele (4.7±5.0 pg) carried out across the overall sample of controls, MCI and AD patients, showed a similar trend, although not significant (p=0.55).

| IL-1beta genotypes | Controls | MCI patients | AD patients |
|---|---|---|---|
| TT | 0.0 ± 0.0 (2)* | 6.2 ± 4.7 (8) | 9.5 ± 8.4 (2)* |
| TC | 3.6 ± 4.9 (9)* | 3.6 ± 4.5 (21) | 6.3 ± 4.8 (12)* |
| CC | 1.5 ± 2.3 (9) | 3.4 ± 3.6 (25) | 4.6 ± 3.5 (21) |
| p | 0.65 | 0. 42 | 0.24 |

*these genotypes were pooled for the analyses

Table 4. Relationships of IL1beta genotypes with plasma IL1 beta levels

A similar analysis for plasma IL-18 levels and IL-18 genotypes could not be performed due to the small number of IL-18 assays in the MCI and AD groups.

### 3.2 Leukocyte telomere length

Leukocyte telomere length was determined in the subjects in which serum IL-1beta and IL-18 levels were assayed. By using Kruskal-Wallis test, significant LTL differences were found between the controls, aMCI and AD patients ($p<0.0001$), with mean LTL values (±SD) in the order AD patients (0.71T/S ± 0.14) < aMCI patients (0.80T/S ± 0.13) < controls (0.98T/S ± 0.03).

The relationships between LTL and serum IL-1beta and IL-18 levels were analyzed. Overall a significant negative linear relationship (p=0.02) was observed between serum IL-1beta levels and LTL (adjusted for age) ($y= -0.009x +0.83$). In accordance with this finding, significantly higher mean LTL values (0.88 ±0.13 T/S) ) were observed in the subjects in which IL-beta was not detectable compared to those in which IL-1beta was detected (0.78 ±0.16 T/S, p=0.01).

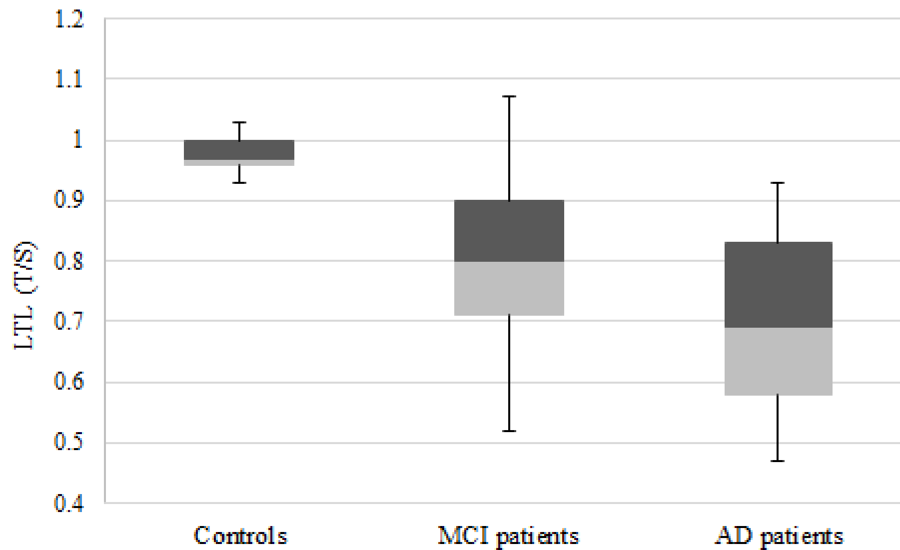



Fig. 4 Box and whisker plots showing the distribution of LTL (T/S ratio) in Controls, MCI , and AD patients. Controls: median= 0.97, first quartile (q1) =0.96, third quartile (q3): 1.0; MCI: median=0.80, q1= 0.71, q3= 0.90; AD patients: median=0.69, q1 = 0.59, q3: 0.83.

When we tested the possible association between IL-1beta rs16944 genotypes and LTL in the three groups, we observed no association for the control and MCI groups (Table 5). Differently, significantly higher LTL values were found associated with CC genotype in the AD patients compared to the LTL values associated with genotypes carrying the IL-1 beta T allele. Finally, no significant linear relationship was detected between plasma IL-18 levels and LTL in the whole sample. The effect of IL-18 genetic variation (rs1946518 and rs187238 SNPS) on serum Il-18 levels could not be tested due to the small sample size of serum IL-18 measurements.

Table 5. Relationships of IL1beta genotypes with LTL

| IL-1beta genotypes | Controls | MCI patients | AD patients |
|---|---|---|---|
| TT | 0.97±0.11 (6) | 0.78 ± 0.13(6) | 0.64± 0.07 (3)* |
| TC | 0.90± 0.13(31) | 0.80± 0.10 (18) | 0.64 ± 0.12 (27)* |
| CC | 0.92±0.12 (33) | 0.81± 0.16 (21) | 0.77 ± 0.14 (29) |
| | 0.45 | 0. 88 | 0.001 |

*These genotypes were pooled for the analysis

**4. Discussion**

Inflammatory mechanisms play a key role in the pathogenesis of AD (Glass et al., 2010). Because the course of the disease takes several years, even decades, before clinical hallmarks such as loss

1 of memory and alteration of cognitive processes become manifest, there is a need to identify biomarkers
2 that, besides discriminating different stages of the disease, can work as prognostic indicators of the
3 transition from a healthy status to MCI and from MCI to AD. To date, however, studies measuring serum
4 inflammatory cytokine levels (Italiani et al., 2018; Lai et al., 2017) to identify possible biomarkers
5 associated with AD stages or prognostic indicators of the transition from MCI to AD (Brosseron et al.,
6 2014) have yielded discrepant results. Inflammation is thought to underlie leukocyte telomere attrition, a
7 phenomenon frequently associated with cognitive decline and AD, which probably reflects the activation
8 of leukocyte proliferation induced by the inflammatory status. .Accordingly, LTL has been investigated
9 as an additional indicator of the neuroinflammatory processes accompanying neurodegeneration as it
10 progresses from a prodromal state (MCI) to full-blown AD (Forero et al., 2016; Hochstrasser et al., 2012;
11 Honig et al., 2006; Movérare-Skrtic et al., 2012; Panossian et al., 2003; Scarabino et al., 2017).
12 Here, we examined IL-1 beta and IL-18, two proinflammatory cytokines, as markers of the
13 inflammasome activation that accompanies the neurodegenerative process, and we sought to determine
14 their relationship with LTL in MCI and AD patients. Analysis of peripheral IL-1beta concentrations
15 showed higher levels in the MCI compared to the control group, and an even higher level in the AD
16 compared to the MCI group. This pattern is consistent with previous studies that reported an upregulation
17 of IL-1beta in AD patients. Published data on IL-1beta levels in MCI patients are scarce and less
18 homogeneous, however (Brosseron et al., 2014; Heneka et al., 2015; Lee et al., 2009; Licastro et al.,
19 2000). In the current sample, IL-1 beta was detectable in 80% of the MCI patients, with levels falling
20 between those of the controls and the AD patients. This finding suggests that the increase in IL-1 beta
21 was present from the early stages of disease. It has been suggested that the increased IL-1beta levels in
22 AD patients may be linked to Aβ formation (Meraz-Ríos et al., 2013). It follows then that the detection of
23 serum IL-1beta levels in patients with MCI could signal early neurodegeneration and progressive
24 deposition of amyloid plaques.
25 Analysis of IL-1beta SNP (rs16944) revealed no association between IL-1beta genotype and
26 susceptibility to MCI or AD. Previous studies have produced highly heterogeneous results (Su et al.,
27 2016), and a meta-analysis reported a modest association for the TT genotype only when the Caucasian
28 studies with the highest statistical power were included in the subgroup analysis (Di Bona et al., 2008).
29 Based on the genotype frequencies we observed, a very much larger sample would be needed to reach the
30 same statistical power. Since the IL-1beta rs16944 SNP is located in the promoter region of the IL-1beta
31 gene, we wanted to explore the possible association between IL-1 beta genotypes and serum Il-1beta

1 levels. We found that, in line with previous observations (Hall et al., 2004; Su et al., 2016), the presence
2 of the T allele was associated with higher IL-1beta levels, mainly in the AD group, whereas the C allele
3 was associated with the lowest IL-1beta levels. This association was not significant, however, and was
4 seen only in the AD group, i.e., in the sample with the highest IL-1beta levels. Nonetheless, together with
5 evidence from previous studies (Hall et al., 2004; Su et al., 2016) our finding does support the hypothesis
6 that genetic variation at the IL-1beta locus may influence serum IL-1beta levels.

7 A different picture emerges from the analysis of serum IL-18: cytokine levels were higher in the
8 MCI group and lower in the AD group, but still higher than in the controls. This observation is at variance
9 with previous investigations, as no previous study analyzing MCI patients reported elevation of IL-18
10 (Brosseron et al., 2014). Nevertheless, in a study involving AD patients in which disease severity was
11 classified by MMSE score (Motta et al., 2007), IL-18 levels were higher in the patients with mild-AD and
12 progressively lower in the subgroups with moderate and severe AD. Our results seem to reflect these
13 observations: elevated IL-18 levels in very early stages (in the MCI group), followed by a drop in IL-18
14 levels in the AD group, but still significantly higher than in the controls. An explanation for this is that
15 the increase in free IL-18 in MCI may imply early IL-18-dependent inflammation-driven alterations in the
16 blood-brain barrier and neuronal damage preceding the development of AD (Ojala et al., 2017). In AD,
17 increased expression and production of IL-18 in the brain may be the cause of a general and systemic
18 increase in its inhibitor (Bossù et al., 2010; Ojala et al.,2009) in a feedback mechanism resulting in a
19 reduction of free IL-18 levels. We observed no association between IL-18 genotype and susceptibility to
20 MCI or AD. This contrasts with previous studies (Zhang et al., 2016), that reported a protective
21 association of the C alleles of both polymorphisms with AD. The absence of any association in our
22 sample may have resulted from the inclusion criteria in sample collection or the sample size.

23 Overall, the most significant findings are the results of the serum IL-1 beta and IL-18 assay:
24 compared to the controls, both interleukins showed a distinctive behavior in the MCI group, in which IL-
25 1beta was detectable and IL-18 was very high probably due to NLRP3 inflammsome activation by
26 canonical (involvement of caspase 1) and non canonical pathway (involvement of caspase-11) (He et al.,
27 2016; Heneka et al., 2018). Their combined presence is indicative of MCI and provides support for
28 clinical diagnosis.

29 In a previous study (Scarabino et al, 2017), we observed a progressive reduction in LTL with
30 AD development: the mean LTL values were highest in the controls, lower in the MCI, and lowest in the
31 AD group. Also, we observed a positive relationship between LTL and cognitive performance (as

measured by MMSE). The present study showed a difference in LTL between controls, MCI and AD patients, strengthening previous observations ( Scarabino et al., 2017) that LTL could be a useful marker of progression of cognitive impairment. In addition, we observed a relationship between shorter LTL and higher serum IL-1beta levels: LTL was longest in the subjects in which IL-1beta was not detectable and IL-1beta levels were lowest in the AD patients carrying the IL-1beta rs 16944 CC genotype, found to be associated with the lowest IL1beta levels. These results provide an *in vivo* link between telomere shortening and serum IL-1beta levels, an inflammation biomarker. The general picture suggests that activation of peripheral inflammatory processes secondary to neuroinflammation may trigger active leukocyte proliferation and result in telomere shortening.

## 5. Conclusions

The use of biomarkers based on cerebrospinal fluid (CSF) and neuroimaging has markedly improved the diagnostic accuracy of MCI and AD. Nonetheless, such biomarkers still have only a partial role in clinical practice owing to limitations in the assay methods or the invasive procedure necessary to obtain CSF. Hence, novel biomarkers that can be used to monitor additional important molecular mechanisms of AD and that can be obtained through noninvasive procedures are needed (Blennow et al., 2016; Patel et al., 2011). Researchers and clinicians increasingly acknowledge that it is not only important to define a clinically and biologically well-characterized diagnosis of AD but also to identify individuals in the prodromal phases of the disease or at increased risk of developing AD so as to implement prevention, early interventions, and monitoring to improve clinical outcomes (Jongsiriyanyong and Limpawattana, 2018). Here we provide evidence that the combined assay of IL-1beta and IL-18, two cytokines, and LTL measurement may be used as blood-based biomarkers for changes in relation to disease stages and to monitor disease onset and progression from MCI to AD. Further investigations in larger samples are desirable to confirm present results, and studies with a follow-up design would be suitable to determine whether and to what extent LTL and/or IL-1beta and IL-18 assays may predict the clinical course of cognitive decline in MCI and the transition to AD.

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3
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