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RESEARCH

Searching for New Possible Peripheral Biomarkers of Cognitive Decline in Down Syndrome: The Role of IL-18 Pathway and its Interaction with TGF-β1 and TNF-α

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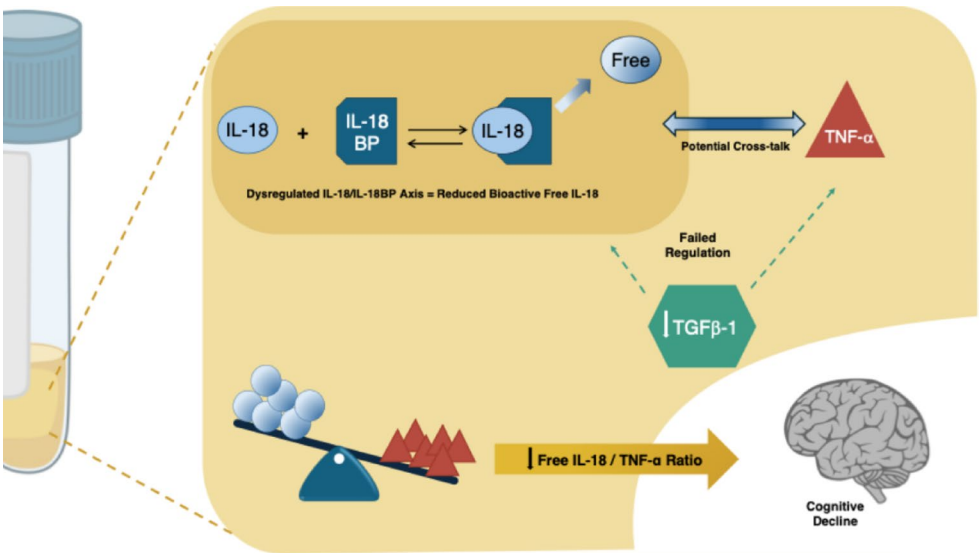
Abstract

Down syndrome (DS) represents one of the most common genetic disorders attributable to a partial or complete trisomy of chromosome 21 that affects about 1 in 700 individuals at birth. The diagnosis of Alzheimer's Disease (AD)-correlated cognitive decline in this population requires new approaches and new biomarkers that comprehensively assess health status and early cognitive decline. In this observational study, we explored for the first time the relation of IL-18, a cytokine member of IL-1 family involved in both innate and acquired immune responses, with DS associated cognitive decline. We observed that plasma total IL-18, in subjects with DS over 35 with and without AD-related cognitive decline, and plasma concentrations of its binding protein in subjects with DS (19–35 years) were correlated with lower plasma concentrations of Transforming Growth Factor (TGF-β1), which are linked to an increased rate of cognitive decline in adults with DS. In addition, we found a significant association between low baseline concentrations of Free IL-18, the active form of the cytokine, and an increased rate of cognitive decline at 12 months, calculated as delta of the Test for Severe Impairment (dTSI), in individuals with DS (19–35 years). Finally, we demonstrated a reduction of Free IL-18/TNF-α ratio, considered as a new possible double biomarker, in both young and older adult DS subjects without AD-related cognitive decline (area under the receiver operating curve (AUC) was 0.82 and 0.71, respectively), suggesting the advantage of the composite biomarkers in the discrimination of patients from healthy people over single biomarkers.



Keywords Down's syndrome · Cognitive decline · IL-18 · IL-18BP · Peripheral biomarkers

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Introduction

The age of onset of cognitive decline and the rate of progression of Alzheimer's Disease (AD) are highly variable in adults with Down's Syndrome (DS) (Larsen et al., 2024) (Iulita et al., 2022). AD in DS has a long preclinical phase, starting very early in their lifespan, during which, biomarkers such as cerebrospinal fluid (CSF) Aβ1–42/1–40 and plasma neurofilament light change predictably over more than two decades (Fortea et al., 2020). Research has focused on identifying new possible markers to predict the progression of AD-related cognitive decline in DS, integrating fluid-based biomarkers, neuroimaging, and the onset or progression of cognitive decline (Strydom et al., 2018), (Larsen et al., 2024). Blood-based biomarkers are especially promising because of their non-invasive nature, their availability and longitudinal utility. Different blood-based biomarkers related to neurodegeneration have been proposed in AD, although their validation in DS is still ongoing (Palmqvist et al., 2025), (Iulita et al., 2023). Furthermore, new blood-based biomarkers related to inflammation have been also proposed and examined (Grasso et al., 2024), (Iulita et al., 2016).

We recently showed in subjects with DS that lower plasma Transforming Growth Factor-beta1 (TGF-β1) concentrations, a well-known anti-inflammatory cytokine with neuroprotective activity, and elevated plasma concentrations of Tumor Necrosis Factor-alpha (TNF-α) a pro-inflammatory cytokine strongly correlated with the following cognitive decline at 12 months in DS individuals (Grasso et al., 2024) and AD-type dementia.

DS individuals show an irregular inflammatory response, with increased concentrations of serum pro-inflammatory cytokines, such as Interleukin-1beta (IL-1β), TNF-α and Interferon-gamma (IFN-γ) (Zhang et al., 2017), suggesting a state of chronic inflammation (Gensous et al., 2019). In people with DS, overexpression of IFN-related genes encoded in chromosome 21 may promote persistent inflammatory events (García & Flores-Aguilar, 2022).

In this context, IL-18—a member of the IL-1 family, originally named IFN-γ inducing factor—is of particular interest in DS. It regulates both innate and acquired immunity (Boraschi et al., 2011). IL-18 possesses a dual nature, being either beneficial or detrimental, depending on its cytokine milieu. In synergy with IL-12, it promotes Th1 response through IFN-γ production, while in its absence it can stimulate Th2 response (Alboni et al., 2010). IL-18 activity is modulated by a naturally occurring soluble inhibitor: IL-18 binding protein (IL-18BP). IL-18BPa, the most active of the four splice variants in humans, prevents excessive IL-18-mediated inflammatory responses by binding with an affinity comparable to most neutralizing antibodies and inhibiting its function (Kim et al., 2000). High IL-18 concentrations are not a hallmark of DS itself, though it is involved in other inflammatory conditions: elevated IL-18 and IL-18BP concentrations have been observed during chronic inflammation and neurodegenerative and autoimmune diseases. In AD, IL-18 production is correlated to cognitive decline and plasma concentrations are elevated in vascular dementia and mild cognitive impairment (Bossù et al., 2008).

IFN hyperactivity is a top hallmark of the molecular changes in DS and is associated with a distinct proinflammatory phenotype (Gensous et al., 2019), (Galbraith et al., 2023), (García & Flores-Aguilar, 2022), (Chung et al., 2021). Moreover, the overexpression of IFN-related genes encoded in chromosome 21 may activate the up-regulation of interferon-stimulated genes, like IL-18BP (Girard et al., 2016), (Alboni et al., 2010).

Against this background, we measured plasma IL-18 and IL-18BP in a cohort of DS individuals under and over 35 years at the time of the initial neuropsychological evaluation. We hypothesized that plasma concentrations of "Free IL-18"- the biologically active fraction not bound to IL-18BP- could have a good performance to early detect and follow AD-related cognitive decline in this DS cohort.

Materials and Methods

Study Design

The present study was conducted according to the Declaration of Helsinki, the Guidelines for Good Clinical Practice and it was approved by the Ethics Committee of the Oasi Research Institute-IRCCS (2021/02/16/CE-IRCCS-OASI/PA17, grant no.: GR-2019–12369983). This study is an observational study, in which the assignment of an intervention and its effect assessment, as well as health-related biomedical or behavioral outcomes are not investigated. Participants or their legal representatives gave written informed consent. The groups of DS participants were composed of 31 subjects under 35 years (DS NO AD (19–35 years), $n=17$ male, $n=14$ female), 17 subjects over 35 years (DS NO AD (35–60 years), $n=10$ male, $n=7$ female), and 12 subjects over 35 with AD-related cognitive decline (DS-AD (35–60 years), $n=6$ male, $n=6$ female) that were categorized according to the Dementia Scale for Down syndrome (DSDS) in early-onset, middle-stage, and late-stage dementia (Table 1A-B). This scale evaluates cognitive and behavioral symptoms with high sensitivity and specificity to identify dementia in people with intellectual disabilities, including those with DS (Wallace et al., 2021), (Iulita et al., 2016). The Italian version of the Leiter International

Table 1 Demographic and clinical information of DS participants (19–35 years old)

	HC (19–35 years)	DS NO AD (19–35 years)
N° of cases	27	31
Age (±SEM)		
	28,18 (±0,81)	28 (±0,72)
Sex (n°)		
Male	16	17
Female	11	14
Q.I. (mean)		
	N/A	44
TSI (mean)		
	N/A	22
DSDS Stage of Dementia		
	N/A	No Dementia

Number of cases, age (mean ± SEM) at baseline (T0), sex, I.Q. T0 (mean), TSI (mean) and DSDS classification at T0. I.Q.: Intellectual quotient. TSI: Test of Severe Impairment. DSDS: Dementia Scale for Down syndrome. N/A: not applicable. HC: Healthy Controls

Table 2 Demographic and clinical information of DS participants (35–60 years old)

	HC (35–60 years)	DS NO AD (35–60 years)	DS-AD (35–60 years)
N° of cases	22	17	12
Age (±SEM)			
	46 (±1,67)	44,82 (±1,44)	45,42 (±2,72)
Sex (n°)			
Male	11	10	6
Female	11	7	6
Q.I. (mean)			
	N/A	42	40
TSI (mean)			
	N/A	18,17	10,75
DSDS Stage of dementia (n°)			
No Dementia	N/A	17	0
Early	N/A	0	3
Middle	N/A	0	3
Late	N/A	0	6
Very Late	N/A	0	0

Number of cases, age (mean ± SEM) at baseline (T0), sex, I.Q. T0 (mean), TSI (mean) and DSDS classification at T0. I.Q.: Intellectual quotient. TSI: Test of Severe Impairment. DSDS: Dementia Scale for Down syndrome. N/A: not applicable. HC: Healthy Controls

Scale-Third Edition was used for the estimation of the intellectual quotient (IQ) in DS individuals. The Italian version of the Test for Severe Impairment (TSI), a valid and reliable test of cognitive function in patients with severe cognitive impairment, was administered at the time of plasma collection (baseline, T0) and at the follow-up period (12 months from T0) as previously described (Grasso et al., 2024). The rate of cognitive decline at the follow-up interval was evaluated as delta TSI (dTSI) in 23 DS NO AD subjects (19–35 years), in 10 DS NO AD (35–60 years) and 7 DS-AD (35–60 years), as previously described (Grasso et al., 2024), (Iulita et al., 2016), and was calculated by subtracting the cognitive score obtained in the TSI at the time of neuropsychological evaluation at baseline (T0) from the score obtained at the follow-up period (T1) and dividing it by the months (12 months) for each DS subject. Age- and sex-matched euploid volunteers with neither karyotype abnormalities nor neurological deficits were recruited as control groups (HC (19–35 years), $n=16$ male, $n=11$ female; HC (35–60 years), $n=11$ male, $n=11$ female) (refer to Tables 1 and 2).

Plasma Samples Collection

Fasting venous blood samples were collected in the morning in lavender EDTA-K2 BD Vacutainer tubes from all participants by healthcare professionals after informed consent. Samples obtained from DS participants at the time of the initial neuropsychological evaluation (T0) and from HC, were centrifuged at 1,900 rpm for 10 min to permit the separation of the plasma component from the cellular constituents. After a second centrifugation (3,900 rpm for 10 min), plasma samples were separated into aliquots and stored at − 80 °C until measurement.

Quantification of IL-18 and IL-18BP

Plasma concentrations of human total IL-18 were determined via enzyme-linked immunosorbent assay (ELISA) by using the commercially available Quantikine ® ELISA kit (R&D system, Catalog Number DL180), according to the manufacturer's instructions. Total IL-18 concentrations were measured in diluted plasma samples (1:3), and the optical density of each well was determined using a microplate reader Synergy HT (Agilent BioTek, Santa Clara, CA, United States) set to 450 nm, 540 nm, and 570 nm. The quantitative determination of human IL-18BP concentrations in plasma samples requiring a 10-fold dilution, was carried out using the ELISA kit (R&D system, Catalog Number DBP180), following the protocol detailed by the manufacturer.

Calculation of the circulating concentrations of Free IL-18 was determined from the concentrations of total IL-18 and IL-18BP using the law of mass action as per recent evidence (Girard et al., 2016). A single IL-18BP molecule binds a single molecule of IL-18 with a dissociation constant (K_d) of 0.05 nM, so the level of Free IL-18 was calculated from the equation:

$$x = (-b + \sqrt{b}^2 - 4c)/2$$

where x = [IL-18free], b =[IL-18BP] – [IL-18] + Kd, and c = -Kd×[IL-18] (Migliorini et al., 2010).

Statistical Analysis

Raw data for each group were examined to assess the normal or non-normal distribution by using the Kolmogorov-Smirnov and Levene tests. Non-parametric Mann–Whitney unpaired *t*-test or parametric unpaired *t*-test with Welch's correction were used to analyze data obtained from ELISA assays in plasma samples obtained from DS under 35 years and sex- and matched HC. Data obtained from the DS population over 35 years, and HC (35–60 years) were analyzed using one-way analysis of variance (ANOVA), followed by the non-parametric multiple comparisons (Kruskal–Wallis test) using Dunn's *post hoc* correction test or by ordinary one-way ANOVA followed by Bonferroni's multiple comparison test. Two-way ANOVA followed by Bonferroni's multiple comparisons test was used to analyze sex-related differences. Results are reported as means ± S.E.M, and only p values < 0.05 were considered statistically significant. The Spearman rank test and Pearson's correlation were used for bivariate correlation analyses. GraphPad Prism software ® 9.0 (GraphPad, La Jolla, CA, United States) was used to perform the analyses. Outliers were removed by applying the interquartile range (IQR) method.

To assess the discriminative ability of the Free IL-18/TNF-α ratio, TGF-β1 and TNF-α across different groups, Receiver Operating Characteristic (ROC) curve analysis was conducted. This established statistical method evaluates the performance of a continuous variable in distinguishing between two diagnostic categories. A ROC curve graphically represents the True Positive Rate (TPR), or Sensitivity, against the False Positive Rate (FPR), which is equivalent to 1 - Specificity, at various classification thresholds. For each analysis, study participants were categorized into two distinct groups. These comparisons included: (1) HC adults DS adults without dementia. (2) HC adults versus DS adults with dementia. (3) HC young versus DS young. (4) DS young vs. DS adults with dementia. (5) DS adults without dementia vs. DS adults with dementia. The ROC curve for each comparison was generated by systematically varying the threshold of the Free IL-18/TNF-α ratio, TGF-β1 and TNF-α, and calculating the corresponding TPR and FPR values. The overall discriminative power was quantified by the Area Under the Curve (AUC). An AUC value ranges from 0.5 (indicating no discriminative power, equivalent to random chance) to 1.0 (representing perfect discrimination). For each ROC curve, the optimal classification threshold was determined using Youden's J statistic. This metric is defined as J = TPR + Specificity − 1, or equivalently, J = TPR − FPR. The threshold that yielded the maximum Youden's J value was selected as the optimal point, representing the best balance between sensitivity and specificity for differentiating the respective groups. All calculations were performed using custom code written in Python 3.13.2, leveraging the matplotlib and scikit-learn libraries. The analyses were run on a system equipped with an Intel® Core™ Ultra 7 165U processor and 32.0 GB of RAM.

Results

DS Under 35 Years Showed an Increase in IL-18BP Concentrations and a Reduction in the Circulating Concentrations of Free IL-18 at T0

To investigate the role of IL-18 and its binding protein in the DS population, we measured plasma concentrations of total IL-18 and the circulating concentrations of IL-18BP in DS individuals under 35 years at the time of the neuropsychological evaluation.

We found that DS subjects (19–35 years) showed a slight increase, which failed to reach statistical significance, of the total IL-18 plasma concentrations at T0 (Fig. 1A) and a significant increase of IL-18BP compared to sex and matched HC (** $p < 0.001$, Fig. 1B). Moreover, we observed a significant reduction of the circulating concentrations of Free IL-18 in DS (19–35 years) at T0 (* $p < 0.05$, Fig. 1C) consistent with the chronic hyperactivation of the IFN-mediated transcriptional response observed in DS individuals, in the cognitive decline AD-related asymptomatic stage. The same trends were observed when we analyzed sex-related differences (Fig. 1D, F), and we found a significant increase in male DS under 35 years of IL-18BP plasma concentrations (** $p < 0.01$, Fig. 1E) and a reduction of about 30%, even if not statistically significant, in both male and female DS individuals under 35 years compared to HC of the circulating concentrations of Free IL-18 (Fig. 1F).

DS Subjects Over 35 with or Without AD-related Cognitive Decline Showed an Imbalance of IL-18/IL-18BP Axis

In DS population over 35, we found a significant increase of IL-18BP plasma concentrations at T0 both in DS NO AD (35–60 years) and in DS-AD (35–60 years) compared to HC (35–60 years) (** $p < 0.01$, *** $p < 0.001$; Fig. 2B). In addition, DS subjects over 35 with AD-related cognitive decline showed an increase in baseline IL-18BP plasma concentrations compared to DS individuals of the same age range without cognitive impairment (* $p < 0.05$; Fig. 2B). We found that the circulating concentrations of Free IL-18, assumed to be the active form of the cytokine, were

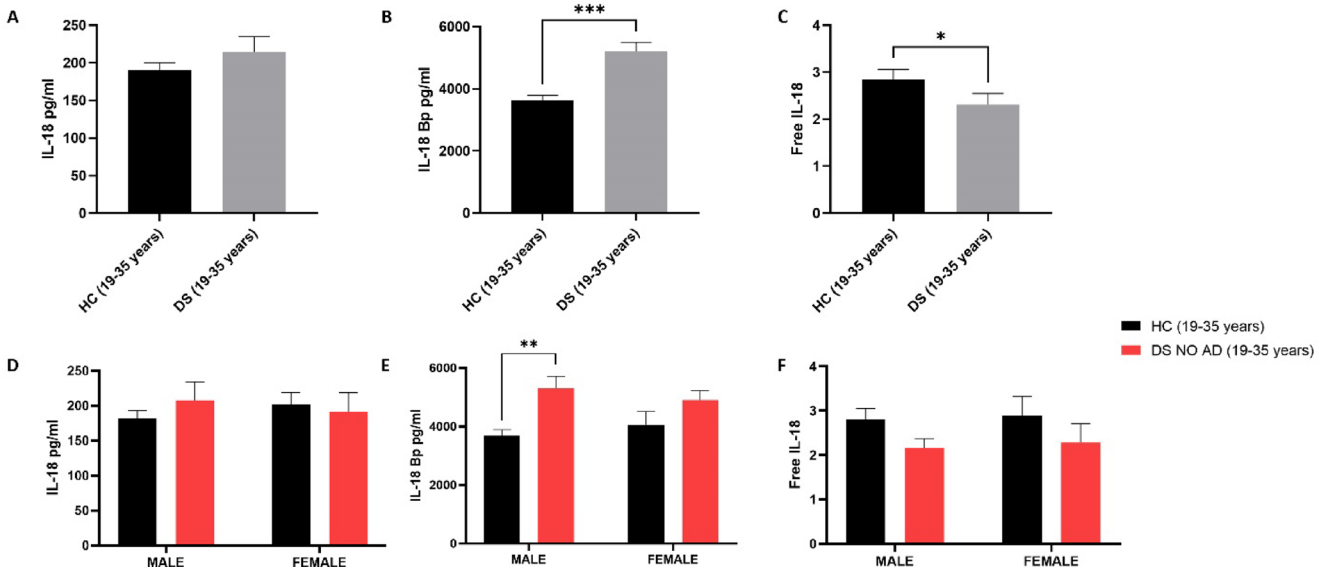

Fig. 1 Plasma concentrations of IL-18 pg/ml, IL-18BP pg/ml and Free IL-18 in DS (19–35 years) and HC (19–35 years). Plasma concentrations of **(A)** IL-18 pg/ml, **(B)** IL-18BP pg/ml and **(C)** Free IL-18 measured by ELISA assay in young adult individuals with DS (19–35 years) and HC (19–35 years). **(A)** T-test Unpaired Non-parametric test, Mann-Whitney test, $p=0.7$; **(B)** T-test Unpaired Parametric with Welch's correction, *** $p<0.001$; **(C)** T-test Unpaired Non-parametric test, Mann-Whitney test, * $p<0.05$. **D-E-F)** Plasma concentrations of IL-18 pg/ml, IL-18 BP pg/ml and Free IL-18, respectively, in male and female young adults with DS (19–35 years) and HC (19–35 years). Two-way ANOVA, Bonferroni's multiple comparisons test, ** $p<0.01$

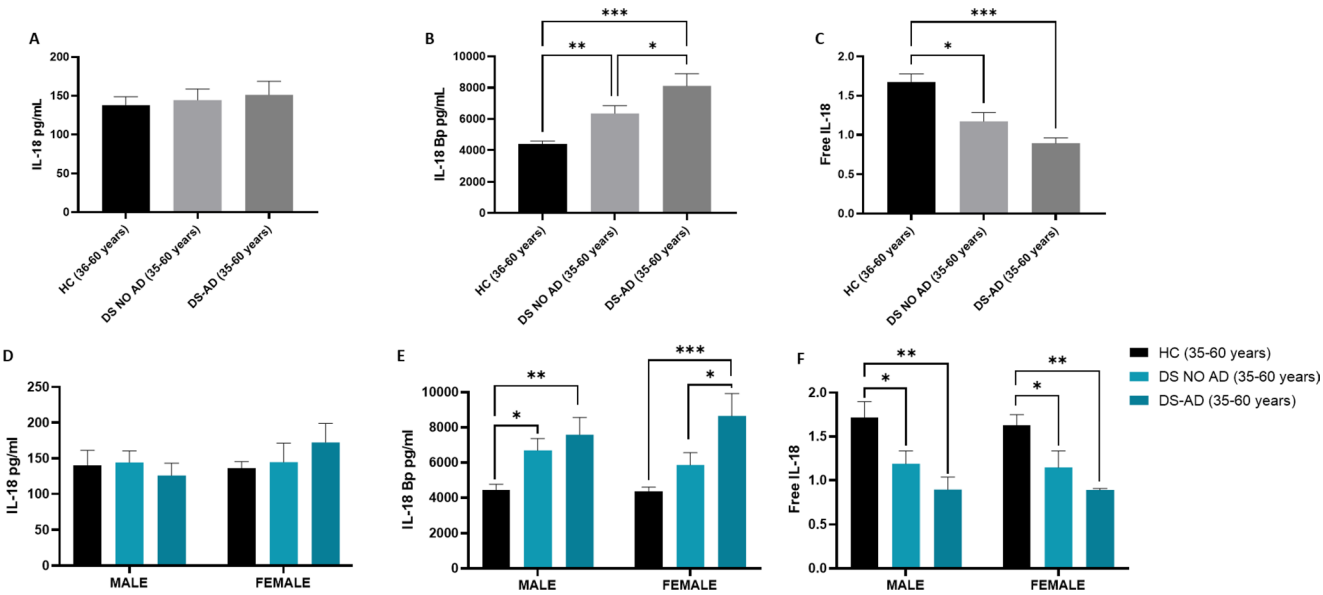

Fig. 2 Plasma concentrations of IL-18 pg/ml, IL-18BP pg/ml and Free IL-18 in older adult DS individuals with and without AD-related cognitive decline. Plasma concentrations of **(A)** IL-18 pg/ml, **(B)** IL-18BP pg/ml and **(C)** Free IL-18 measured by ELISA assay in DS older adults with or without AD-related cognitive decline (35–60 years) and HC (35–60 years). **A-B)** Ordinary One-way ANOVA, Bonferroni's multiple comparison test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **C)** One-way ANOVA, Kruskal-Wallis test, Dunn's post-hoc, * $p<0.05$; *** $p<0.001$. Plasma concentrations of **(D)** IL-18 pg/ml, **(E)** IL-18BP pg/ml and **(F)** Free IL-18 in male and female DS older adults with or without AD-related cognitive decline (35–60 years) and HC (35–60 years). Two-way ANOVA, Bonferroni's multiple comparisons test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

decreased in DS over 35 with or without AD-related cognitive decline compared to sex- and matched HC (* $p<0.05$, *** $p<0.001$; Fig. 2 C). Interestingly, we observed that male individuals with or without AD-related cognitive decline show an increase of IL-18BP plasma concentrations at T0, paralleling a decrease of the circulating concentrations of Free IL-18 compared to HC (35–60 years) (* $p<0.05$, ** $p<0.01$; Fig. 2E-F). In DS female subjects, we found a

significant increase in IL-18BP plasma concentrations at T0 in the DS-AD group compared to female HC (35–60 years) (*** $p<0.001$; Fig. 2E) and compared to female DS NO AD (35–60 years) (* $p<0.05$; Fig. 2E). Moreover, we observed a significant decrease in Free IL-18 concentrations, the fraction of cytokine not bound to IL-18BP, in female DS over 35 with or without AD-related cognitive decline (* $p<0.05$, ** $p<0.01$; Fig. 2F).

The Rate of Cognitive Decline Correlated with IL-18 System in DS Individuals

As showed in Fig. 3A and B, we found a positive correlation between the total IL-18 plasma concentrations and the circulating plasma concentrations of its binding protein predominant isoform in both young DS individuals (Fig. 3A, *$p<0.05$) and in older adult DS subjects with or without cognitive decline at T0 (Fig. 3B, ***$p<0.001$), suggesting that both parameters increase together and might regulate an inflammatory response. In addition, we used a bivariate correlation to investigate possible associations between baseline IL-18, IL-18BP and Free IL-18 concentrations in plasma and cognitive functions in the DS population. Although, in DS individuals under 35, there was a trend for a negative correlation between plasma IL-18 and dTSI at year 1 (Spearman r −0.3971, $p=0.07$) as well as a positive correlation between plasma concentration of IL-18 Bpa and dTSI (Spearman r 0.07431, $p=0.7$), we found a significant association between baseline Free IL-18 concentrations and the rate of cognitive decline according to the dTSI value (Fig. 3C, Spearman r −0.4390, * $p<0.05$) over 1 year in DS individuals under 35. Furthermore, in DS subjects over 35 years with or without AD-related cognitive decline, we found a significant correlation between baseline IL-18 plasma concentrations and dTSI at 12 months (Fig. 3D, Pearson r −0.5541, * $p<0.05$) as well as between the plasma concentrations of IL-18BP at T0 and the rate of cognitive decline according to the dTSI value over 1 year (Fig. 3E, Pearson r −0.5787, * $p<0.05$), while no significant association was found between baseline Free IL-18 concentrations and the rate of cognitive decline in DS subjects over 35 with or without AD-related cognitive decline (Spearman r 0,1823, $p=0.48$).

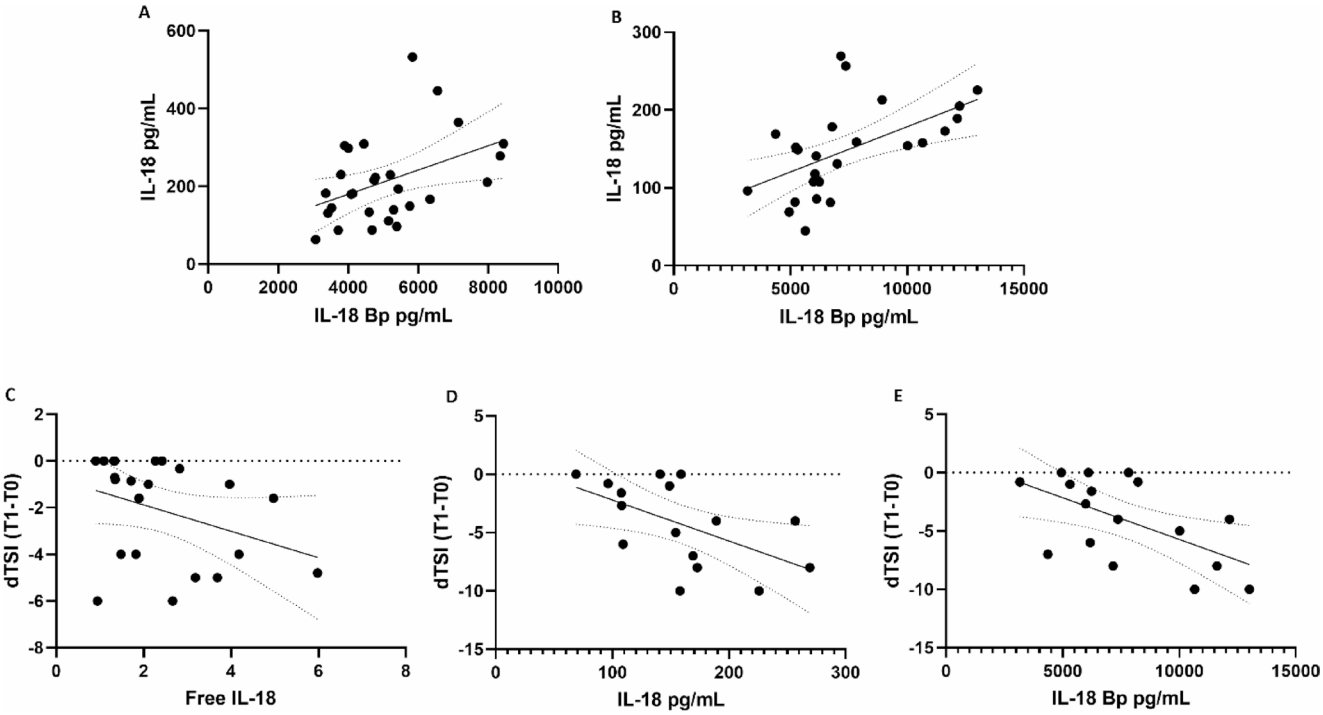

Fig. 3 Total IL-18 correlated to its binding protein plasma concentrations linking a strong correlation between the rate of cognitive decline and IL-18 system. Correlation between circulating IL-18BP concentrations and total IL-18 pg/ml in **(A)** young adult individuals with DS (19–35 years), Simple Linear Regression, Spearman r 0.4264, 95% CI: 0.05184 to 0.6958, *$p<0.05$; and in **(B)** older adults DS with or without AD-related cognitive decline (35–60 years), Simple Linear Regression, Spearman r 0.6526, 95% CI: 0.3522 to 0.8311, ***$p<0.001$. **(C)** Correlation between Free IL-18 at baseline (T0) and the rate of cognitive decline at the follow up period in young adult individuals with DS (19–35 years) calculated for each individual as dTSI (T1 TSI – T0 TSI/12 months); Simple Linear regression, Spearman r −0.4390, 95% CI: −0.7324 to −0.008071,* $p<0.05$; **D-E)** Correlation between the plasma concentrations of IL-18 pg/ml and IL-18BP pg/ml, respectively, at T0, and dTSI in DS older adults with or without AD-related cognitive decline (35–60 years). **(D)** Simple Linear regression, Pearson r −0.5541, 95% CI: −0.8236 to −0.08052, *$p<0.05$. **(E)** Simple Linear regression, Pearson r −0.5787, 95% CI: −0.8288 to −0.1359, *$p<0.05$

Lower Concentrations of TGF-β1 Correlated with IL-18BP in DS Subjects Under 35 and To Total IL-18 in DS Over 35 Years

It is well known role of TGF-β1 in the pathophysiology of cognitive decline in AD and that rescue of TGF-β1 signaling impairment may be a new pharmacological strategy to prevent AD-related cognitive decline also in DS population (Estrada et al., 2018), (Grasso et al., 2024), (Caraci et al., 2018). We found that the lower plasma concentrations of TGF-β1 at T0 were inversely correlated to circulating concentrations of IL-18BP in young adult DS (19–35 years) (Fig. 4B, $*p<0.05$), while not significant results were found between the decreased concentrations of TGF-β1 and the increased plasma concentrations of total IL-18 at T0 (Fig. 4A, $p=0.5$), suggesting that deficit of TGF-β1, an early event in the pathophysiology of cognitive deficits in DS, can be linked to the dysfunction of IL-18-IL-18BP axis. In older adult DS individuals with or without AD-related cognitive decline, we found an inverse correlation between TGF-β1 and the total IL-18 plasma concentrations (Fig. 4C, $*p<0.05$) while not statistically significant correlation has been found between the anti-inflammatory cytokine and the increased concentrations of plasma IL-18BP at T0 (Fig. 4D, $p=0.54$), suggesting that it is possible that TGF-β1 can regulate different cytokines, including IL-18, and that its peripheral deficit might contribute to the uncontrolled inflammatory process driven by IL-18.

Free IL-18/TNF-α Ratio Plasma Level at T0: a New Possible Double Composite Biomarker?

To find a new peripheral tool for an early detection of cognitive decline in the DS population, we analyzed the ratio between the circulating concentrations of Free IL-18 and the plasma concentrations of TNF-α, a known pro-inflammatory

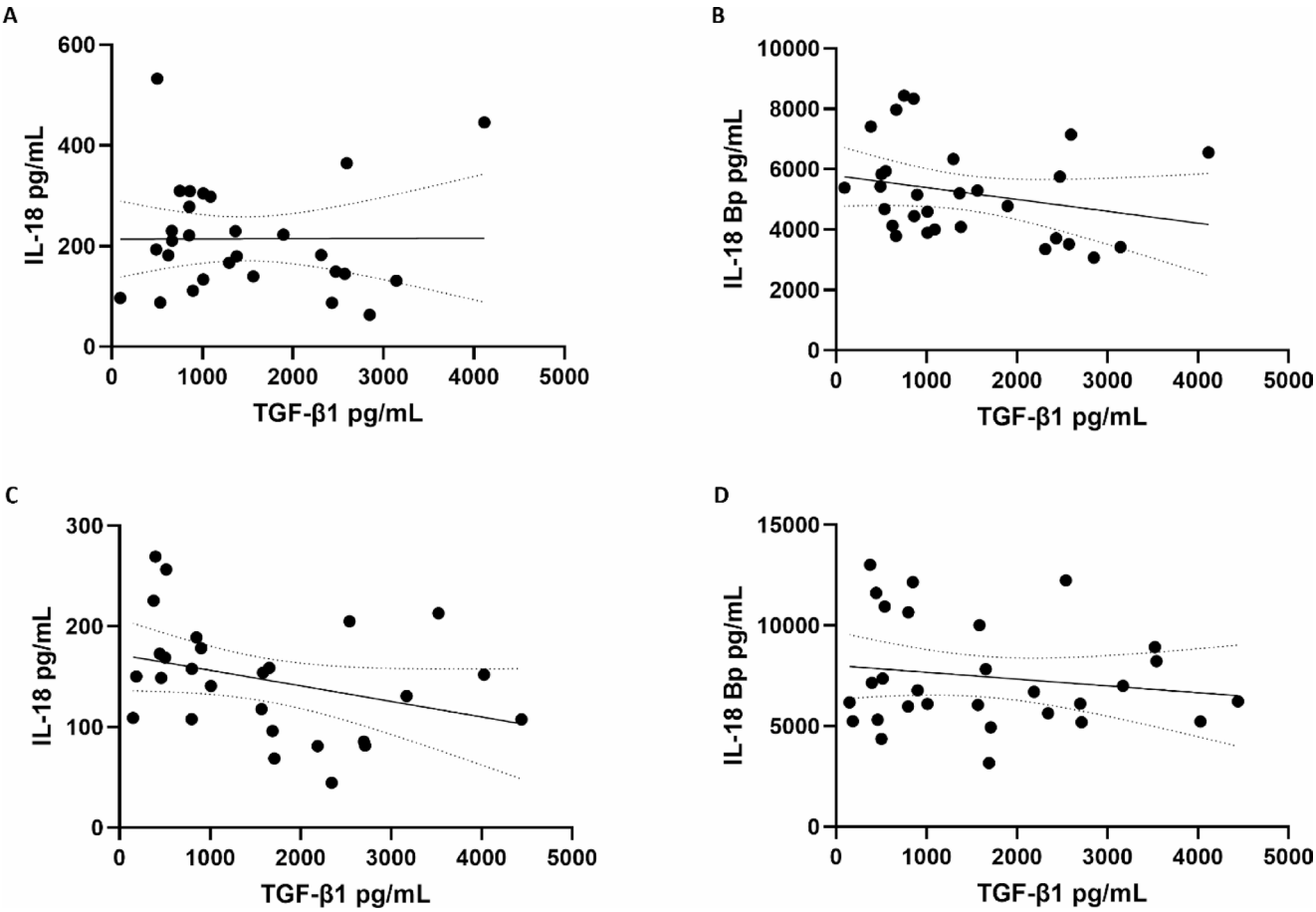

Fig. 4 Correlation between the lower concentrations of TGF-β1 with total IL-18 and its binding protein.(A) Correlation between TGF-β1 and total IL-18 pg/ml, Simple Linear regression, Spearman r −0.1275, 95% CI: −0.4868 to 0.2686, $p=0.5$ and **(B)** Correlation between TGF-β1 and IL-18 Bp pg/ml, Simple Linear regression, Spearman r −0.3683, 95% CI: −0.6539 to 0.009321, * $p<0.05$ in young adult individuals with DS (19–35 years). **(C)** Correlation between TGF-β1 and total IL-18 pg/ml, Simple Linear regression, Spearman r −0.3968, 95% CI: −0.6814 to −0.007972, * $p<0.05$ and **(D)** Correlation between TGF-β1 and IL-18 Bp pg/ml, Simple Linear regression, Spearman r −0.1177, 95% CI: −0.4731 to −0.2706, $p=0.54$ in older adults with or without AD-related cognitive decline (35–60 years)

cytokine with a key role in the AD-related cognitive decline in DS individuals (Iulita et al., 2016). We confirmed our previous results (Grasso et al., 2024) that demonstrated an increase of TNF-α plasma concentrations at T0 in DS individuals under 35 compared to sex- and matched HC as well as in DS-AD (35–60 years) compared to HC (35–60 years) (data not shown). Interestingly, we found a significant reduction of Free IL-18/TNF-α ratio in DS under 35 years compared to sex- and matched HC (Fig. 5A, *** $p<0.001$). In addition, the Free IL-18/TNF-α ratio was reduced in both DS individuals over 35 years with or without AD-related cognitive decline (Fig. 5B, ** $p<0.01$ DS NO AD (35–60 years), * $p<0.05$ DS-AD (35–60 years) vs. HC (35–60 years). In DS under 35 years, we did not find a significant correlation between Free IL-18/TNF-α ratio and dTSI (Simple Linear regression, Pearson $r= -0.4271$, $p=0.053$) as well as in DS over 35 with or without AD-related cognitive decline (Simple Linear regression, Pearson $r=0.06650$, $p=0.799$).

Surprisingly, when analyzing the ROC curve, drawn to determine the ability of the proposed plasma biomarker to distinguish patients with asymptomatic AD-related cognitive decline from HC, we found an AUC of 0.82 and a cutoff point of 0.51, with a sensitivity of 0.78 and a specificity of 0.80 for Free IL-18/TNF-α ratio in DS (19–35 years) versus sex- and matched-HC proving to be a good promising candidate to discriminate the asymptomatic AD-related cognitive decline in DS individuals from to euploid subjects (Fig. 5C). Moreover, when comparing DS without AD (35–60 years) versus HC (35–60 years), we found an AUC of 0.71 (Fig. 5D) that improved when we compared DS-AD (35–60 years) versus sex- and matched HC observing an AUC of 0.87 (Fig. 5E) with a sensitivity of 0.91 and a specificity of 0.75, suggesting that our proposed double biomarker could be a promising tool in dynamically predicting the risk of AD-related cognitive decline in its prodromal phase in the DS population. Our results suggest that Free IL-18/TNF-α ratio plasma level might be considered, in a large DS cohort, as a sensitive peripheral indicator able to track the progression of cognitive decline or to reflect in the periphery the inflammatory imbalance that preceded over time the cognitive decline development.

For the differentiation of the asymptomatic groups versus the AD-related cognitive decline group, we analyzed the ROC curve also for the other cytokines analyzed. While the Free IL-18/TNF-α ratio was not able to discriminate DS

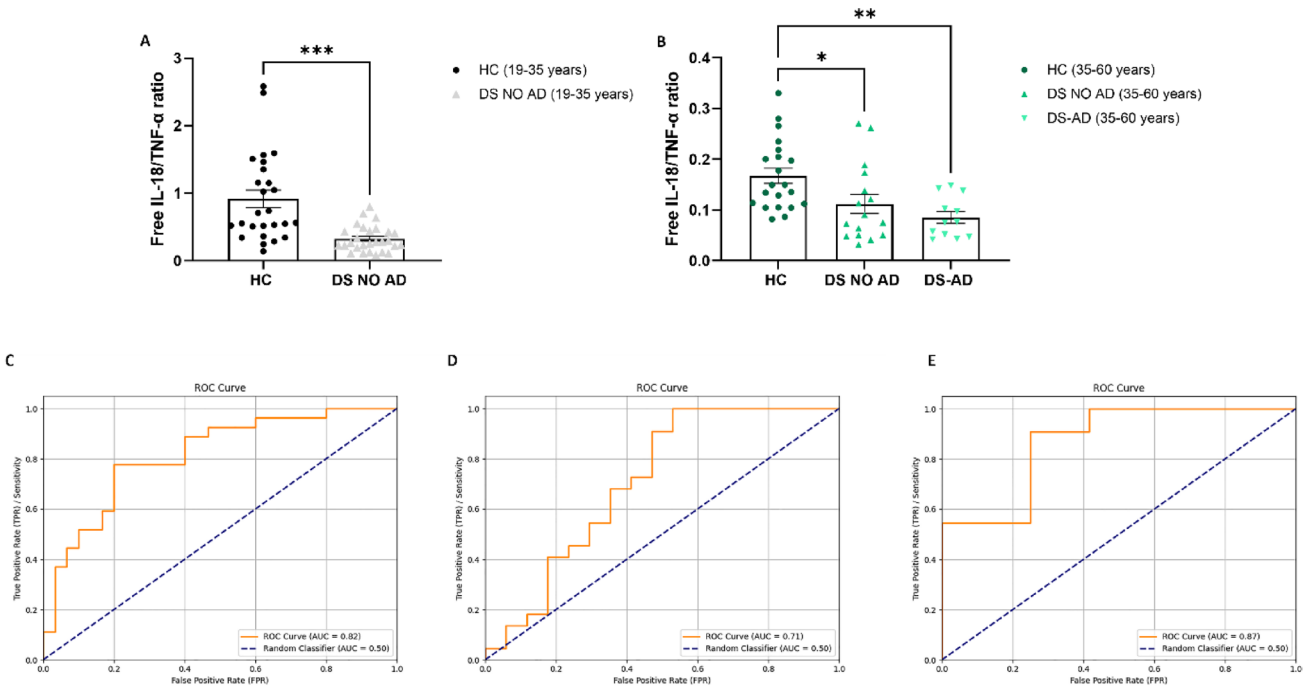

Fig. 5 Free IL-18/TNF-α ratio as a new double composite biomarker in DS. Free IL-18/TNF-α ratio in **(A)** young adult DS individuals (19–35 years, $n=30$), t-test unpaired, non-parametric test, Mann–Whitney test, *** $p<0.001$ DS NO AD (19–35 years) vs. HC (19–35 years, $n=27$); and in **(B)** older adult with or without AD-related cognitive decline DS individuals (35–60 years, $n=12$ and $n=17$, respectively) vs. HC (35–60 years, $n=22$), Ordinary One-way ANOVA, Bonferroni's multiple comparison test, ** $p<0.01$ DS NO AD (35–60 years) vs. HC (35–60 years), * $p<0.05$ DS-AD (35–60 years) vs. HC (35–60 years). **C-D-E)** ROC curve and AUC of Free IL-18/TNF-α ratio in **(C)** DS individuals (19–35 years, $n=30$) vs. HC (19–35 years, $n=27$), AUC = 0.82, Threshold: 0.51, FPR: 0.20, TPR: 0.78, Sensitivity: 0.78, Specificity: 0.80; in **(D)** DS NO AD (35–60 years, $n=17$) vs. HC (35–60 years, $n=22$), AUC = 0.71, Threshold: 0.08, FPR: 0.53, TPR: 1.00, Sensitivity: 1.00, Specificity: 0.47; and in **(E)** DS-AD (35–60 years, $n=12$) vs. HC (35–60 years), AUC = 0.87, Threshold: 0.10, FPR: 0.25, TPR: 0.91, Sensitivity: 0.91, Specificity: 0.75

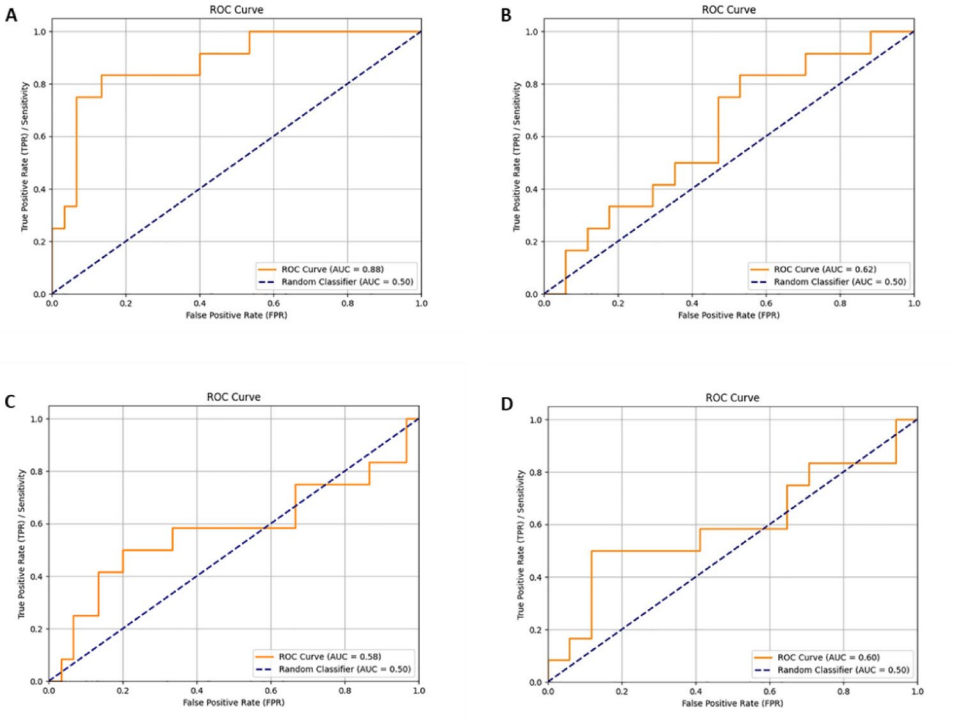

Fig. 6 ROC curve analysis of TNF-α and TGF-β1 in DS subjects. ROC curve and AUC of TNF-α in **(A)** DS individuals (19–35 years, $n=30$) vs. DS-AD (35–60 years, $n=12$), AUC = 0.88, Threshold: 10.59, Sensitivity: 0.83, Specificity: 0.87; and in **(B)** DS NO AD (35–60 years, $n=17$) vs. DS-AD (35–60 years, $n=12$), AUC = 0.62, Threshold: 10.59, Sensitivity: 0.83, Specificity: 0.47. In **C)** ROC curve and AUC of TGF-β1 in DS individuals (19–35 years, $n=30$) vs. DS-AD (35–60 years, $n=12$), AUC = 0.58, Threshold: 2.70, Sensitivity: 0.42, Specificity: 0.87; and in **D)** ROC curve and AUC of TGF-β1 in DS NO AD (35–60 years, $n=17$) vs. DS-AD (35–60 years, $n=12$), AUC = 0.60, Threshold: 2.54, Sensitivity: 0.50, Specificity: 0.88

subjects without AD from those with AD-type dementia (data not shown), the obtained AUC for TNF-α alone was able to distinguish both young and older adult DS individuals from older adult DS-AD subjects. As shown in Fig. 6, we found an AUC of 0.88 with a cutoff point of 10.59 obtaining a sensitivity of 0.83 and a specificity of 0.87 in DS (19–35 years) versus older adult DS with AD-related cognitive decline (Fig. 6A). Moreover, the high discriminative accuracy for TNF-α, was also found when we compared the DS-AD group with DS individuals of the same age range, but without AD-related cognitive decline, where we found an AUC of 0.62 with a sensitivity of 0.75 and a specificity of 0.53 (Fig. 6B).

As for TGF-β1 concentrations, ROC analysis revealed a value of AUC of 0.58 and 0.60 in DS (19–35 years) and in DS no AD (35–60 years), respectively, compared to DS-AD (35–60 years) individuals (Fig. 6C-D). When employed in distinguishing DS individuals from to euploid subjects of the same age range, we found an AUC of 0.62 in older adult DS-AD (Sensitivity: 0.86, Specificity: 0.42), and an AUC of 0.69 (Sensitivity: 0.89, Specificity: 0.47) in DS (19–35 years) individuals, respectively, versus their sex- and age-matched-HC (ROC curve not shown). AUC value increased when comparing DS NO AD (35–60 years) versus sex- and matched HC observing an AUC of 0.74 with a sensitivity of 0.64 and a specificity of 0.76 (ROC curve not shown).

Discussion

Recently, it has been demonstrated that significant differences in epigenetic age between DS individuals and HC groups highlight the profound impact of trisomy 21 on aging processes, supporting the key relevance of identifying new diagnostic and therapeutic strategies for age-related conditions in DS individuals such as cognitive deficits and the early onset of AD in this population (Cheishvili et al., 2025). The high prevalence of neurological comorbidities and cognitive decline development over 40 years highlights the need for specialized care for DS people during their adulthood (Grotteschi et al., 2025), and on the other hand, new and specific tools are needed to predict the onset of AD-type dementia at an early stage. In this context, blood-based biomarkers have gained attention as non-invasive scalable alternative to CSF and neuroimaging markers for tracking AD-related changes in this unique population (Petersen et al., 2025).

IL-18 is a pleiotropic cytokine possessing a dual nature being either beneficial or detrimental depending on the microenvironment. IL-18 is classified as both a Th1 and Th2 cytokine that in synergy with IL-12, induces IFN-γ production that in turn is able to modulate its production and activity via a negative feedback mechanism, allowing for a dynamic modulation of the immune responses and inflammatory processes (Novick, 2024). In physiological conditions, IL-18 plays a crucial role in the body's defense against infections

and in the regulation of immune responses. Elevated circulating concentrations of IL-18 have been reported in several infectious, autoimmune and autoinflammatory diseases, like multiple sclerosis, psoriasis, and Crohn's disease (Novick, 2024), while within the brain it has been demonstrated that the IL-18 system can either sustain neuroinflammation or act as modulator of memory and brain plasticity (Alboni et al., 2025).

A dysregulation in brain IL-18 may exacerbate neurodegeneration and cognitive impairments (Felderhoff-Mueser et al., 2005), which are hallmark features of the DS population. In particular, the dysregulation of the central IL-18 pathway can impact cerebral processes like synaptic plasticity, neuronal communication, and overall cognitive development, even though the exact link could depend on the specific biological context, the pathological condition, and how the immune system regulates the activity of IL-18 pathway (Sutinen et al., 2012), (Wu et al., 2020).

IL-18 activity is tightly regulated by the IL-18 binding protein, especially its predominant isoform IL-18 BPa, a glycosylated protein that binds the mature cytokine with high affinity and slow dissociation rate that has been proposed as a promising therapeutic agent (Novick, 2024).

The balance between IL-18 and IL-18BP is crucial in determining immune response and disease outcome and regulation of this fine-tuning tightly controlled. A significant positive correlation between expression concentrations of IL-18 and IL-18BP has been reported in several pathological conditions (Harel et al., 2022).

A large meta-analysis confirmed that children and adults with DS present increased circulating concentrations of pro-inflammatory mediators such as TNF-a, IL-1β and IFN-γ when compared with euploid controls (Zhang et al., 2017). IFN-related genes in circulating monocytes are activated in DS individuals (Sullivan et al., 2016). In our DS cohort, we found, for the first time, an increase in IL-18 and of IL-18BP plasma concentrations at the time of basal neuropsychological evaluation. Particularly evident was the increase of plasma IL-18BP concentrations in both male and female DS subjects, and this effect was consistent between the sexes and age groups considered, suggesting an effort by the immune system to modulate and/or limit the inflammatory process by the binding of total IL-18 to its binding protein in order to avoid chronic inflammation. Considering that IL-18BP concentrations are known to be upregulated by IFN-γ in vitro and in vivo (Wang, 2024), (Díaz-Pino et al., 2024), the increase in circulating IL-18BP may be linked to the interferon hyperactivity observed in DS (Sullivan et al., 2016).

An increase of these two molecules has been demonstrated during neuroinflammatory processes (Millward et al., 2010). Therefore, we hypothesize that the correlation between IL-18 and IL-18 BP concentrations found in our DS cohort, could suggest a regulatory mechanism with the aim of balancing the inflammatory processes in the periphery.

Different studies in IL-18pathies or autoimmune diseases demonstrated that higher concentrations of circulating IL-18 are paralleled by increased circulating IL-18BP, most likely as an attempt to counteract the excess of IL-18 (Millward et al., 2010). Usually, despite this parallel increase, the antagonistic potential of IL 18BP often falls short and an increase in Free IL-18 is reported in these conditions. Free IL-18 can represent a more effective biomarker to correlate clinical and biological signs of autoimmune and inflammatory conditions (Harel et al., 2022) and could serve as a standard for the identification of patients who are likely benefit of IL-18 pathway negative modulation (Novick, 2024).

On the contrary, in our cohort of DS individuals, we found an impairment of the IL-18 system characterized by reduced concentrations of Free IL-18 that seems to contribute to the onset of cognitive decline. Our results demonstrated a decrease in Free IL-18 at T0 in both DS under 35 and in older adult DS subjects with or without AD-related cognitive decline, and more interestingly, we found for the first time a significant association between reduced baseline Free IL-18 concentrations and the following rate of cognitive decline according to the dTSI value at 12 months in DS individuals under 35 years, suggesting that the estimation of circulating Free IL-18 concentrations could have a good diagnostic performance to detect prodromal AD-related cognitive decline in the DS population.

While in vivo and in vitro data suggest that IL-18-IFN-γ-IL-18BP may interact in a self-regulation loop, the increase in IFN stimulation reported in DS population may interfere with this regulatory mechanism causing a lifelong imbalance in IL-18 signaling and a dysfunction of the IL-18 pathway. Given the double-edged role of this cytokine in homeostatic regulation, autoimmune diseases, infection, cancer, and neuroinflammation, future studies in animal and translational models of DS may help to elucidate its role in DS pathology.

Finally, to account for different immune dysregulations observed in DS, we also considered the ratio between Free IL-18, assumed to be the not neutralized fraction of secreted IL-18, and the pro-inflammatory TNF-α, a cytokine whose dysregulation contributes to an abnormal immune and inflammatory response, that is linked to increased susceptibility to infections, higher rates of autoimmune conditions, and a propensity for chronic inflammation, which may also influence the increased risk of AD-like pathology in DS people (Ahmed et al., 2021).

When we compared DS NO AD (35–60 years) versus HC (35–60 years), we found an AUC of 0.71 that improved when we compared DS (19–35 years) versus sex- and

matched-HC where we found an AUC of 0.82 with a cut-off point of 0.51, and a sensitivity of 0.78 and a specificity of 0.80 for Free IL-18/TNF-α ratio. A recent study explored the ratio between TNF-α and IL-18 demonstrating a diagnostic and predictive potential in orthopedic surgery patients (Papatheodorou et al., 2025), but this study did not analyze Free IL-18, the biologically active fraction that better describes the functional activity of IL-18 pathway. Therefore, their combined assessment in DS people could represent a more accurate tool to describe the immune system dysregulation underlying DS-associated cognitive decline, considering the role played by Free IL-18, the fraction biologically active of the IL-18 cytokine, a major regulator of the immune activation, and the well-known pro-inflammatory TNF-α mediator. Overall, our results suggest that our proposed double biomarker could be a new promising tool for the assessment of AD-related cognitive decline in its prodromal phase in the DS population, supporting the advantage of employing composite biomarkers rather than single biomarkers in the discrimination of patients from healthy people (Liu & Zhou, 2013), although the full potential of Free IL-18/TNF-α remains to be demonstrated and validated in a larger DS cohort. We believe that Free IL-18/TNF-α ratio plasma level might be considered in future prospective observational studies as a new peripheral indicator that could contribute to improve both the early diagnosis and longitudinal assessment of AD-related cognitive decline in the DS population.

It is well known the neuroprotective role of TGF-β1 in AD and that rescue of TGF-β1 signaling impairment may be a new pharmacological strategy to counteract AD-related cognitive decline also in DS population (Estrada et al., 2018), (Caraci et al., 2016), (Grasso et al., 2024). We therefore analyzed the possible interaction between TGF-β1 and the IL-18 axis. In our DS cohort, we found that lower plasma concentrations of TGF-β1 at T0 were inversely correlated to circulating concentrations of IL-18BP in young adult DS and to the total IL-18 plasma concentrations in older adult DS individuals, suggesting that the deficit of TGF-β1 can be linked to the dysfunction of IL-18/IL-18BP axis in an early phase of cognitive decline in DS. We cannot exclude that TGF-β1 production might be regulated by IL-18 or IFN-γ, and that its peripheral deficit might contribute to the both the uncontrolled inflammatory process or the imbalance in IL-18 activity. Moreover, TGF-β1 plasma concentrations at T0 showed a discrete performance to discriminate DS individuals from euploid controls of the same age range, but we believe that further long-term studies are needed to better understand the link between the deficit of TGF-β1 concentrations and the reduced Free IL-18/TNF-α ratio in the pathophysiology of cognitive decline in DS individuals (Grasso et al., 2024).

We know that the monocentric and observational design of the study, along with the limited size of our cohort, may limit the generalizability of our findings to validate our results through large-scale studies as well as with a longer follow-up of 24 months. Nevertheless, our findings support the utility of the measurement of plasma proposed cytokines to an early detection of prodromal AD-related cognitive decline in DS individuals to evaluate the presence of an early and evolving neuroinflammatory phenotype across the lifespan in DS individuals that represent a unique population at an increased risk to AD-type dementia development (Flores-Aguilar et al., 2020).

Although further studies are required to assess the clinical utility of our proposed blood-based biomarkers in evaluating cognitive decline in DS population, we believe that the identified changes in plasma concentrations of cytokines analyzed hold promise for the early detection of cognitive decline reliably.

Conclusions

In this observational study, we found for the first time an imbalance of IL-18/IL-18BP axis in DS individuals with or without AD-related cognitive decline. We found that lower plasma concentrations of TGF-β1, an anti-inflammatory cytokine known to be reduced in DS, were correlated with plasma total IL-18 in DS subjects DS over 35 and with plasma concentrations of its binding protein in DS subjects under 35 years, suggesting that the deficit of TGF-β1 can be linked to the dysfunction of IL-18/IL-18BP axis in an early phase of cognitive decline in DS. Finally, we demonstrated for the first time a reduction of Free IL-18/TNF-α ratio, considered as a new possible double biomarker, in both young and older adult DS subjects without AD-related cognitive decline, suggesting the advantage of the composite biomarkers in the discrimination of patients from healthy people over single biomarkers, as a new peripheral indicator of the inflammatory imbalance that precedes over time the development of cognitive symptoms in DS individuals at the higher risk of dementia.

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[Grasso Margherita], [Fidilio Annamaria], [Benatti Cristina], [Caraci Filippo]; Writing - review and editing: [Grasso Margherita], [Fidilio Annamaria], [Francesca L'Episcopo], [Marilena Recupero], [Concetta Barone], [Marta Lovino], [Alboni Silvia], [Maria Giulia Bacalini], [Caruso Giuseppe], [Greco Donatella], [Buono Serafino], [De La Torre Rafael], [Tascedda Fabio], [Johanna M. C. Blom]; Funding acquisition: [Grasso Margherita], [Caraci Filippo], [Maria Giulia Bacalini]; Resources: [Grasso Margherita], [Caraci Filippo], Supervision: [Caraci Filippo], [Benatti Cristina]. All authors read and approved the final manuscript.

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Data Availability The data supporting the results of this study are available upon reasonable request to the corresponding author.

Declarations

Competing Interests The authors declare no competing interests.

Ethical Approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the Oasi Research Institute-IRCCS (2021/02/16/CE-IRCCS-OASI/PA17, grant no.: GR-2019-12369983).

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Consent to Publish Not applicable.

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