



types of age-related dementia. These include the second most common dementia (around 20%), namely vascular dementia (VD), caused by brain blood supply problems, leading to significant accumulated damage to the brain tissue and its functioning [6]. Additionally, it is important to point out that there can be mixed dementia (MD: around 10% of cases), in which both AD and VD characters are present in the same individual. Other forms of dementia are less common (around 10% altogether) [7]. Since AD and VD share many common pathological, symptomatic and neurochemical features [8] there are valid reasons to believe that ORO staining and CF rate may also be useful in early VD and MD diagnosis. In the present study, we determined ORO staining and CF rate in freshly isolated PBMCs from HC and from subjects with AD, VD and MD. In addition, we included samples from individuals with mild cognitive impairment (MCI), which is considered to be an intermediate stage between the expected cognitive decline of normal aging and the more serious decline of dementia [9]. Our aim was to verify whether changes in NLs and in the CF rate of naïve PBMCs might signal a neurological disorder other than AD, and whether they occur during the early stages of the disease.

A number of similarities between AD, VD pathology and several distinct age-related ocular degenerations has also been described in AD patients, in particular cataract, diabetic retinopathy, glaucoma and age-related macular degeneration, all leading causes of vision loss and blindness in the elderly [10]. All these are complex disorders, multifactorial degenerations of Central Nervous System (CNS) tissue, in which age is a primary risk factor, and specific areas of the brain are damaged over time [10]. Therefore, to further validate ORO staining and CF rate as potential biomarkers of CNS injury, we extended the study to these common age-related eye disorders.

## Materials and Methods

In this study, we determined ORO staining in 192 subjects 65 years or older. Demographic, clinical and comprehensive geriatric assessment (CGA) information was extrapolated from a previously computerized anonymous database, including 1,320 subjects attending the outpatient geriatric care unit services of the University of Cagliari for geriatric check-up from 2006 to 2014. During examination, either the patient or his/her legal guardian was requested to give written informed consent for these procedures, and also to state whether they agreed to anonymous use of data and blood samples for experimental purposes. The ethical committee of Cagliari University (Italy) approved this procedure. Detailed interviews about medical history and physical examinations were performed. Probable diagnosis of AD was made according to the criteria previously described in detail [11,12]. Diagnoses of possible/probable VD were made according to the criteria previously described in detail [13]. According to research by Health Quality Ontario [14], neuroimaging evidence (computed tomography scan and/or nuclear magnetic resonance) supported differential diagnosis between AD and VD. MCI is a syndrome defined by clinical, cognitive and functional criteria. Like dementia, it may be due to one or more aetiologies and cannot be diagnosed by a laboratory test. It was determined by changes in cognition faculties compared to the person's previous check-up results, impairment in one or more cognitive domains, preservation of independence in functional abilities and absence of dementia as previously suggested by Petersen [9]. Subjects without any form of dementia or MCI were taken as a control group (HC).

Dyslipidaemias, hypertension, diabetes, general atherosclerosis and the presence of the most common age-related eye problems were established by examining a careful and detailed medical history obtained by recording interviews, past medical problems and blood

test results. Medical records underlining the findings described in the manuscript are deposited in the public repository of the geriatric unit of the University of Cagliari (Italy).

## Comprehensive geriatric assessment (CGA)

### Functional assessment:

- Activities of Daily Living Scale (ADL) were performed as previously described in detail [15]. The summed score is from 0 to 100; 100 indicating a patient fully independent in physical functioning, and 0 representing a totally dependent bed-ridden state.
- Instrumental Activities of Daily Living scale (IADL) were assessed as previously described in detail [16], a score of 8 indicating total autonomy, and 0, total dependence.

### Comorbidities:

- Cumulative Illness Rating Scale (CIRS) was used to evaluate comorbidities as previously described in detail [17]. Severity of pathology in 14 categories was estimated assigning a scoring value from 1 (no impairment) to 5 (extremely severe) to each system. The final score, provided by the sum of the scores for each 14 categories, represents the CIRS-total (CIRS-T). CIRS-maximum organ impairment (CIRS-MI) reflects the highest degree of impairment assigned to one or more categories investigated in the CIRS (ranging 1-5). The total number of categories in which moderate or severe levels (grades 3-5) of disease are quoted (ranging 0-14) gives Comorbidity Index (CIRS-CI). Severity Index (CIRS-SI), reflects the overall severity of diseases and the average rating of 13 disease categories, excluding psychiatric and behavioural problems (ranging 1-5).

### Cognitive and mood status:

- Mini-Mental State Examination (MMSE), corrected for age and education, assessed as previously described in detail, was used for evaluating cognitive status [18,19]. Thirty correct-answer points indicate cognitive deficit absence, while a score of zero indicates maximum cognitive deficit. A score of less than 24 out of 30 points suggests cognitive deficit.
- Geriatric Depression Scale (GDS), assessed as previously described in detail [20], was used to assess psychological state. Scores of 0-4 are considered normal, depending on age, education and medical complaints; 5-8 indicate mild depression; 9-11 indicate moderate depression; and 12-15 indicate severe depression.

## ORO staining and CF

ORO staining was determined as previously described in detail [3]. Briefly, PBMCs were isolated by carefully layering 500 µl of anticoagulated whole blood onto the upper layer of an equal amount of Ficoll, which were put into 1.5 mL Eppendorf tubes. The tubes were then centrifuged at 5,000x g for 10 min. After centrifugation, PBMCs present as a white ring in the middle of the layer were taken out, with the help of micropipettes, and carefully plated in 6-well tissue culture plates. PBMCs were washed three times with phosphate-buffered saline (PBS), and fixed in 10% neutral-buffered formalin (10 min). Cells were then treated with isopropyl alcohol (60%), washed, stained with 1 ml of ORO working solution and incubated at room temperature for a few minutes. Haematoxylin counterstain was used to visualize the nucleus. ORO was then removed and cells washed 3 times with PBS, 3 min each. After ORO staining, cells were imaged using an inverted phase microscope fitted with a digital camera with magnification x400.

At least two different fields per sample were imaged and analysed. Red intensity was scored on a semi-quantitative scale (from 0-4) by two blinded observers: 0 indicates no staining; 1, rare positive cells or staining barely visible; 2, focal staining or faint diffuse staining clearly visible at low power; 3, multifocal staining or moderate diffuse staining; and 4, intense diffuse staining. Grades representing the proportion of clustered PBMCs were rated using a semi-quantitative scale 0-2, in which 0 indicates single cells or very few aggregated cells, 1, a moderate number of aggregated cells, 2, a high number of aggregated cells. Red intensity of ORO staining cells was also calculated as a percentage of positively stained total cell area by manually selecting one region of interest (ROI), utilizing Image J software (National Institutes of Health, United States).

### Statistical Analysis

Quantitative variables were expressed as means ± standard deviation (M ± SD). Data were analysed statistically by the Student t-test for independent samples. All variables were submitted to a Kolmogorov-Smirnov (K-S) goodness of fit test (normal probability test). Since data were normally distributed, we used one way analysis of variance (ANOVA), followed by a Bonferroni post hoc test to compare differences in parameters between various types of dementia and ocular diseases. Chi-square ( $\chi^2$ ) test was used for categorical data. Subsequently, we completed Spearman's Rank Correlation Coefficient to determine ORO staining, CF, MMSE and age correlations. Excel and

XLSTAT 2014 were used as software for other analyses and graphing.

### Results

This study includes 192 participants, of which 68 affected by different types of dementia (20 with AD, 14 with MD, and 34 with VD), 29 patients with MCI and 95 HC. We initially analysed age and CGA data. One-way ANOVA, followed by post-hoc Bonferroni test, showed that the average age between subjects with dementia, MCI and HC was not statistically different (Table 1). MMSE was significantly lower in demented patients than MCI and HC, and significantly higher in HC than in MCI (Table 1). GDS was significantly higher in demented patients than MCI and HC (Table 1). ADL were significantly lower in demented patients than MCI and HC (Table 1). IADL were significantly lower in demented patients than MCI and HC and significantly higher in HC than in MCI (Table 1). Other CGA parameters were not significantly different between demented patients, MCI and HC (Table 1). Furthermore, no significant differences were found about CGA parameters between AD, MD, and VD, except for CIRS-SI (Table 1). K-S test showed that, at the level of alpha significance=0.050, we cannot reject the null hypothesis of no difference between the empirical and theoretical cumulative distributions of CGA parameters in MCI, AD, MD, VD.

Next, we estimated NLs in PBMCs from all enrolled subjects (Table 1 and Figure 1A). Figure 1A shows microphotographs of representative

Variables	All	HC	MCI	Dementia	AD	MD	VD
n	192	95	29	68	20	14	34
Gender (M/F)	68/124	43/52	15/14	10/58	4/16	4/10	2/32
Age, y	77.7 ± 7.0	78.4 ± 6.8	77.2 ± 8.6	77.0 ± 6.3	77.5 ± 6.8	73.7 ± 3.6	78.1 ± 6.4
MMSE, score	23.4 ± 5.8	27.3 ± 1.7	25.6 ± 1.8	17.7 ± 5.2	18.2 ± 4.3	16.1 ± 7.1	18.1 ± 4.6
GDS, score	8.0 ± 4.3	6.3 ± 3.8	7.8 ± 4.4	10.6 ± 3.5	11.1 ± 2.8	10.4 ± 4.3	10.3 ± 3.6
ADLs, score	75.9 ± 19.4	79.0 ± 20.6	81.4 ± 9.3	71.0 ± 19.9	66.9 ± 17.1	70.2 ± 19.1	75.5 ± 22.7
IADL, score	4.3 ± 2.4	6.2 ± 1.0	5.9 ± 0.8	1.9 ± 1.7	1.6 ± 1.4	1.9 ± 1.5	2.1 ± 1.9
CIRS-T, score	33.3 ± 4.4	32.6 ± 5.0	32.7 ± 4.4	34.2 ± 3.5	35.1 ± 3.3	33.3 ± 4.0	34.1 ± 3.2
CIRS-MI	3.7 ± 0.7	3.6 ± 0.7	3.5 ± 0.6	3.8 ± 0.8	3.9 ± 0.7	3.8 ± 0.8	3.8 ± 0.7
CIRS-SI	2.3 ± 0.3	2.3 ± 0.4	2.3 ± 0.3	2.4 ± 0.3	2.5 ± 0.2	2.3 ± 0.3	2.3 ± 0.2
CIRS-CI	7.5 ± 2.0	7.4 ± 2.4	7.1 ± 2.0	7.7 ± 1.7	8.4 ± 1.5	7.5 ± 1.6	7.4 ± 1.7
ORO score	1.7 ± 1.5	0.7 ± 0.8	2.0 ± 1.1	3.1 ± 1.1	3.1 ± 0.8	3.3 ± 1.2	3.1 ± 1.3
CF score	0.7 ± 0.9	0.2 ± 0.5	0.7 ± 0.7	1.5 ± 0.8	1.6 ± 0.7	1.4 ± 0.9	1.4 ± 0.9
% of total cell area	10.7 ± 6.2	7.2 ± 4.5	12.2 ± 5.4	14.9 ± 5.8	14.6 ± 4.9	18.3 ± 5.9	13.8 ± 5.9

Results are shown as mean ± standard deviation (SD). Statistically significant differences are indicated in bold type

#### HC, MCI, Dementia

ANOVA: Age,  $P=0.406$ ; MMSE,  $P<0.001$ ; GDS,  $P<0.001$ ; ADL,  $P=0.011$ ; IADL,  $P<0.001$ ; CIRS-T,  $P=0.064$ ; CIRS-MI,  $P=0.103$ ; CIRS-SI,  $P=0.175$ ; CIRS-CI,  $P=0.411$ ; ORO score,  $P<0.001$ ; CF score,  $P<0.001$ ; % of total cell area,  $P<0.001$

#### Bonferroni post-hoc test:

MMSE, significant differences ( $P<0.05$ ) in HC vs. MCI and Dementia and MCI vs. Dementia

GDS, significant differences ( $P<0.05$ ) in Dementia vs. HC and MCI

ADL, significant differences ( $P<0.05$ ) in Dementia vs. HC and MCI

IADL, significant differences ( $P<0.05$ ) in HC vs. MCI and Dementia and MCI vs. Dementia

ORO score, significant differences ( $P<0.05$ ) in HC vs. MCI and Dementia and MCI vs. Dementia

CF score, significant differences ( $P<0.05$ ) in HC vs. MCI and Dementia and MCI vs. Dementia

% of total cell area, significant difference ( $P<0.05$ ) in HC vs. MCI and Dementia

#### AD, MD, VD

ANOVA: Age,  $P=0.075$ ; MMSE,  $P=0.419$ ; GDS,  $P=0.715$ ; ADL,  $P=0.317$ ; IADL,  $P=0.579$ ; CIRS-T,  $P=0.307$ ; CIRS-MI,  $P=0.873$ ; CIRS-SI,  $P=0.005$ ; CIRS-CI,  $P=0.087$ ;

ORO score,  $P=0.847$ ; CF score,  $P=0.676$ ; % of total cell area,  $P=0.046$ .

#### Bonferroni post-hoc test:

CIRS-SI, significant differences ( $P<0.05$ ) in AD vs. MD and VD

% of total cell area, significant difference ( $P<0.05$ ) in MD vs. VD

**Abbreviations:** HC: Healthy Controls; MCI: Mild Cognitive Impairment; AD: Alzheimer's Disease; MD: Mixed Dementia; VD: Vascular Dementia; MMSE: Mini Mental State Examination; GDS: Geriatric Depression Scale; ADL: Activities of Daily Living; IADL: Instrumental Activities of Daily Living; CIRS: Cumulative Illness Rating Scale; CIRS MI: Maximum Impairment; CIRS-SI: Severity Index; CIRS-CI: Comorbidity Index; ORO score: Oil Red O Staining Intensity; CF score: Rate of Cluster Formation; % of total cell area: Percentage of Positively Stained Total Cell Area

**Table 1:** Comprehensive geriatric assessment parameters, ORO score, CF score and % of total cell area in controls, MCI and demented patients.

cells assigned to scores 0-4, based on red intensity of lipid droplet accumulation in PBMCs.

The rate of ORO score was significantly higher in PBMCs from demented patients than in those from MCI and HC, and significantly lower in PBMCs from HC than in those from MCI (Table 1 and Figure 1B). The percentage of ORO staining intensity in total cell area was significantly lower in PBMCs from HC than in those from MCI and demented patients (Table 1 and Figure 1C).

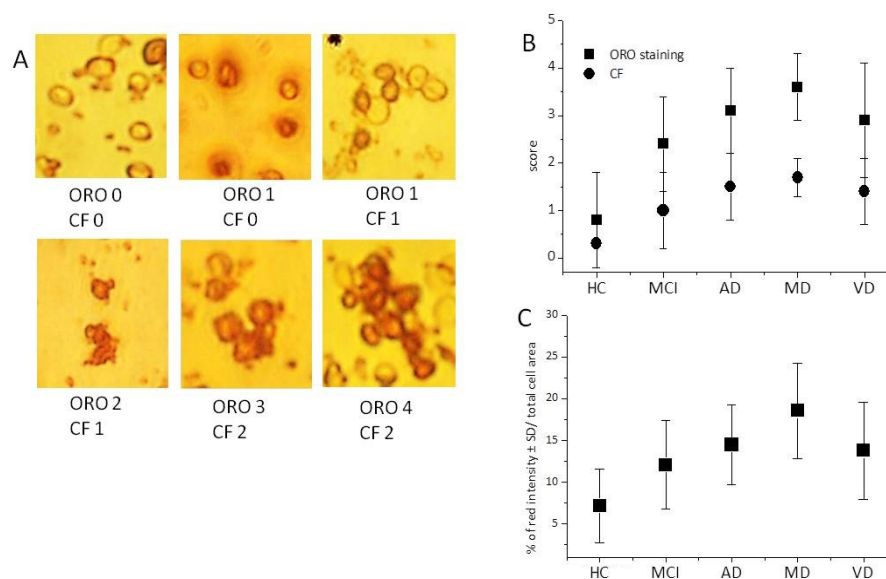
ORO staining did not differ significantly between AD, MD and VD groups (Table 1), but, the percentage of ORO staining intensity in total cell area was significantly higher in PBMCs from MD than in those from VD (Table 1). Furthermore, when ANOVA between HC, MCI, AD, MD and VD groups, followed by post hoc Bonferroni, were performed, significant differences ( $P < 0.05$ ) were observed in HC vs. MCI, AD, MD and VD, and MCI vs. AD, MD and VD (Figure 1). Same results were observed for CF rate (Figure 1). When the total cell area percentage was considered, post hoc Bonferroni showed significant differences in HC vs. MCI, AD, MD and VD and MCI vs. MD (Figure 1).

We also found that freshly isolated (unstimulated) PBMCs from demented patients and MCI groups tend to aggregate *in vitro* in low, medium, or high clusters (Figure 1A), hence resembling cultured PBMCs after mitogen activation with PHA [3,4]. By rating CF using a semi-quantitative scale 0-2, we found that CF follows a similar pattern to ORO staining (Table 1 and Figure 1B).

Since the groups were gender-imbalanced, we also analysed gender distribution by comparing age, CGA, ORO staining (ORO score and % of total cell area) and CF rate in all males and all females and in HC, demented and MCI males and females (Table 2). We found that MMSE score was significantly higher in males than in females, while GDS, ORO score, CF rate and total cell area percentage were significantly lower in males compared to females (Table 2). These changes are probably due to the greater prevalence of dementia in the female group rather than the male one (46.8% vs. 14.7%). There were no differences regarding other parameters considered. Regarding data for HC, we found that GDS, CIRS-T and CF scores were significantly lower in males compared to females (Table 2).

In MCI subjects, we found that GDS and IADL scores were significantly lower in males compared to females, while CIRS-T score was higher in males rather than females (Table 2). ORO score, CF rate and total cell area percentage do not differ regarding MCI in males and females, supporting the hypothesis that changes in these three parameters are present not only in full-blown dementia, but probably also in the early stages of the disease (Table 2).

No significant differences were found between demented men and women in regard to all CGA variables (Table 2). However, we observed, in demented females rather than demented males, a trend to reach greater ORO staining intensity (ORO score and % of total cell area) and CF rate, although only in CF rate it resulted statistically significant (Table 2). These changes are probably due to the imbalance in the number of males and females affected by dementia.



**Figure 1:** Visualization and quantification of NLs by ORO analysis and cluster formation (CF) in PBMCs from HC and MCI subjects and from patients with dementia. Freshly isolated PBMCs were stained with ORO to demonstrate NLs and counterstained with haematoxylin for nuclei. Cells were then examined by light microscope; two different fields per sample were imaged. Percentage (%) of red intensity total cell area was measured in these two fields using NIH Image J software. **(A)** Red intensity was scored on a semi-quantitative scale (from 0-4) by two blinded observers: 0 indicates no staining; 1, rare positive cells or staining barely visible; 2, focal staining or faint diffuse staining clearly visible at low power; 3, multifocal staining or moderate diffuse staining; and 4, intense diffuse staining. Grades representing the proportion of clustered PBMCs were rated using a semi-quantitative scale 0-2, in which 0 indicates single cells or very few aggregated cells, 1, a moderate number of aggregated cells, 2, a high number of aggregated cells. Representative images of ORO stained PBMCs and their corresponding CF. Original magnifications: x400. **(B)** Means ± SD of ORO and CF scores. **(C)** Red intensity of ORO staining cells was also calculated as percentage of positively stained total cell area by manually selecting one region of interest (ROI), utilizing Image J software (National Institute of Health, United States). One-way ANOVA revealed statistically significant differences between groups ( $P < 0.001$ ). For ORO score, post hoc Bonferroni showed significant differences ( $P < 0.05$ ) in HC vs. MCI, AD, MD and VD and MCI vs. AD, MD and VD. Same results were obtained for CF. When the percentage of total cell area considered, post hoc Bonferroni was showed significant differences in HC vs. MCI, AD, MD and VD and MCI vs. MD

n	All Males 68	All Females 124	HC Males 43	HC Females 52	MCI Males 15	MCI Females 14	Demented Males 10	Demented Females 58
AGE	78.6 ± 6.7	77.3 ± 7.0	77.9 ± 6.1	78.7 ± 7.3	79.3 ± 9.3	75.8 ± 7.6	80.2 ± 5.4	76.4 ± 6.3
MMSE, score	25.5 ± 4.7*	22.3 ± 6.0	27.6 ± 1.8	27.0 ± 1.6	25.6 ± 2.0	25.6 ± 1.8	19.4 ± 5.1	17.4 ± 5.2
GDS, score	5.7 ± 3.7*	9.3 ± 4.1	5.1 ± 3.5 <sup>§</sup>	7.2 ± 3.8	4.9 ± 3.2*	10.9 ± 3.2	9.6 ± 2.6	10.7 ± 3.7
ADLs, score	76.8 ± 22.0	75.4 ± 18.1	80.6 ± 25.4	77.8 ± 16.7	80.0 ± 8.8	82.9 ± 10.1	66.0 ± 19.6	72.3 ± 19.6
IADL, score	4.7 ± 2.4	4.1 ± 2.5	6.0 ± 1.3	6.3 ± 0.6	5.3 ± 0.7*	6.4 ± 0.5	1.2 ± 1.5	2.0 ± 1.7
CIRS-T, score	32.8 ± 5.4	33.5 ± 3.8	29.8 ± 5.3 <sup>§</sup>	34.1 ± 4.1	34.7 ± 4.0*	30.7 ± 3.8	35.8 ± 4.2	33.7 ± 3.2
CIRS-MI	3.7 ± 0.7	3.6 ± 0.7	3.5 ± 0.6	3.6 ± 0.7	3.8 ± 0.4	3.2 ± 0.6	4.0 ± 0.8	3.8 ± 0.7
CIRS-SI	2.3 ± 0.4	2.3 ± 0.3	2.1 ± 0.4	2.4 ± 0.3	2.5 ± 0.3	2.1 ± 0.2	2.5 ± 0.4	2.3 ± 0.2
CIRS-CI	7.3 ± 2.2	7.6 ± 1.9	6.5 ± 2.3	7.9 ± 2.2	7.9 ± 2.0	6.3 ± 1.8	8.0 ± 1.8	7.7 ± 1.6
ORO score	1.3 ± 1.3*	2.0 ± 1.5	0.7 ± 0.8	0.7 ± 0.8	2.1 ± 1.2	1.9 ± 1.2	2.6 ± 1.4	3.2 ± 1.1
CF score	0.4 ± 0.7*	0.9 ± 0.9	0.1 ± 0.4 <sup>§</sup>	0.3 ± 0.5	0.7 ± 0.7	0.7 ± 0.7	1.0 ± 0.9*	1.6 ± 0.8*
% total cell area	9.1 ± 5.5*	11.6 ± 6.5	6.9 ± 3.8	7.5 ± 5.0	12.0 ± 4.5	12.4 ± 6.5	14.2 ± 7.6	15.1 ± 5.5

Results are shown as mean ± standard deviation (SD). Statistically significant differences are indicated in bold type  
t-test:

\* males vs. females: MMSE, **P=0.000**; GDS, **P=0.000**; ORO score, **P=0.001**; CF score, **P=0.000**, % of total cell area, **P=0.008**

<sup>§</sup> HC males vs. HC females: GDS, **P=0.007**; CIRS-T, **P=0.006**; CF score, **P=0.037**

\* males with MCI vs. females with MCI GDS, **P=0.000**; IADL, **P=0.000**; CIRS-T, **P=0.01**

\* Demented males vs. Demented females: **CF score, P=0.035**

**Abbreviations:** HC: Healthy Controls; MCI: Mild Cognitive Impairment; MMSE: Mini Mental State Examination; GDS: Geriatric Depression Scale; ADL: Activities of Daily Living; IADL: Instrumental Activities of Daily Living; CIRS: Cumulative Illness Rating Scale; CIRS MI: Maximum Impairment; CIRS-SI: Severity Index; CIRS-CI: Comorbidity Index; ORO score: Oil Red O Staining Intensity; CF score: Rate of Cluster Formation; % of total cell area: Percentage of Positively Stained Total Cell Area

**Table 2:** Base statistical gender distribution in our study population.

A number of studies have linked cardiovascular disease to cognitive impairment and dementia [21,22]. To exclude a possible confounding effect of cardiovascular disease on ORO staining and CF scores, we also estimated the impact of the most common cardiovascular risk factors (hypertension, atherosclerosis, dyslipidaemia, diabetes) on these parameters; as shown in Table 3, no association was found between ORO staining and CF variables and cardiovascular risk factors, thus indicating low probability that these risk factors may influence the levels of these variables.

To maximize the statistical power of the study, we merged data collected from the entire population (n=192) and performed Spearman correlation analysis between age and MMSE, age and ORO staining and between MMSE and ORO staining. Spearman's coefficient showed a moderate but significant correlation coefficient between MMSE and age (r=0.150, P=0.037). No significant correlation was observed between ORO score and % of total cell area and age (r=-0.078, P=0.284; r=-0.087, P=0.228, respectively). A significant and negative correlation is evident between the MMSE and ORO score (r=-0.699, P<0.001) and MMSE and % of total cell area (r=-0.586, P<0.001). In general, these results suggest that normal aging has little effect on the changes in ORO staining found in PBMCs from AD and MCI subjects.

Cataract, glaucoma, diabetic retinopathy and age-related macular degeneration are the four most common eye conditions among people over age 65 and share a number of striking similarities with AD, MD and VD [23,24]. Consequently, as a final point, we conducted separate analyses to examine whether and how these related eye problems may influence ORO staining and CF score.

In our study population, eye diseases were detected in 52 of the 192 enrolled subjects, giving prevalence of 27.1%; of these, 24 subjects suffered from cataract, 8 glaucoma, 8 age-related macular degeneration and 12 diabetic retinopathy. Eye disorders in relation to all types of cognitive impairment are summarized in Table 4. The small number of participants for each type of disease made it difficult to achieve statistical comparisons between subgroups. Participants were divided

into two groups according to the presence or absence of eye disorders and subdivided in subgroups according to the absence of cognitive impairment or the presence of dementia or MCI (HC with or without eye disorders; MCI with or without eye disorders; demented patients with or without eye disorders). These groups were compared to CGA parameters and ORO staining and CF rate (Table 5). The t-test showed that, in the group of all patients with at least an eye disorder, GDS, CIRS-T and CIRS-CI scores were significantly higher than all subjects without eye disorders. In the group of cognitively unimpaired subjects with at least an eye disorder, GDS, CIRS-T, CIRS-SI and CIRS-CI scores were significantly higher and MMSE and IADL scores were significantly lower compared to cognitively unimpaired subjects without eye disorders. Demented patients with at least an eye disorder had GDS, CIRS-T, ORO and CF scores significantly higher compared to demented patients without eye disorders. Although failing to reach significance, the percentage of ORO staining of total cell area was lower in demented patients without eye disorders compared with demented patients with at least an eye disorder. T-test was not performed between MCI subjects with or without eye disorders given the small number of participants of the second group.

## Discussion

In this study, we found that freshly isolated (naive) PBMCs from subjects with dementia accumulate NLs, as determined in ORO staining, irrespective of dementia type: ORO score in PBMCs from demented patients and MCI was significantly higher than in HC, and significantly lower in MCI compared to demented patients. Moreover, while no significant differences in ORO score and CF rate were found between AD, MD and VD groups, the percentage of ORO staining intensity in total cell area was significantly higher in PBMCs from MD than in those from VD. It has also been shown that ORO staining in all enrolled subjects was not age-correlated, suggesting that, in an elderly population, NL levels in PBMCs increase with cognitive impairment severity and are not influenced by aging. We also observed, in demented females, a tendency to reach greater ORO staining intensity (ORO score and % of total cell area) and CF rate, although only in CF



Participants (numbers)	Dyslipidaemia	Atherosclerosis	Diabetes	Hypertension
	No Yes	No Yes	No Yes	No Yes
<b>% cell area</b>				
High	65 45	40 70	71 39	28 82
Low	53 29	30 52	53 29	16 66
$\chi^2$	0.609	0.001	0.000	0.939
P-value	0.435	0.975	0.990	0.333
<b>ORO score</b>				
High	60 46	39 67	70 36	25 87
Low	58 28	31 55	54 32	19 67
$\chi^2$	2.354	0.011	0.219	0.060
P-value	0.125	0.915	0.640	0.807
<b>CF score</b>				
High	63 44	40 67	72 35	27 80
Low	55 30	30 55	52 33	17 68
$\chi^2$	0.679	0.089	0.774	0.735
P-value	0.410	0.765	0.379	0.391

**Abbreviations:** ORO score: Oil Red O Staining Intensity; CF score: Rate of Cluster Formation; % of total cell area: Percentage of Positively Stained Total Cell Area

**Table 3:** Relationship between cardiovascular risk factors and ORO staining and CF score.

	Tot	HC	MCI	Dementia	AD	DV	DM
No Eye Disorders	140	65	25	50	16	24	10
All Eye Disorders	52	30	4	18	4	10	4
Cataract	24	10	2	12	2	8	2
Age-related macular degeneration	8	6	0	2	2	0	0
Glaucoma	8	8	0	0	0	0	0
Diabetic retinopathy	12	6	2	4	0	2	2

**Abbreviations:** HC: Healthy Controls; MCI: Mild Cognitive Impairment; AD: Alzheimer's Disease; MD: Mixed Dementia; VD: Vascular Dementia

$\chi^2$ : No significant differences ( $P=0.167$ ) were observed between HC, patients with Dementia, patients with MCI and patients with or without eye disorders

**Table 4:** Eye disorders in relation to all types of cognitive impairment.

	No Eye Disorders (140) M ± SD	All Eye Disorders (52) M ± SD	HC without Eye disorders (65) M ± SD	HC with an Eye disorder (30) M ± SD	MCI without Eye disorders (25) M ± SD	MCI with an Eye disorder (4) M ± SD	Dementia without Eye Disorders (50) M ± SD	Dementia with Eye Disorders (18) M ± SD
Age, y	77.9 ± 7.1	77.4 ± 6.7	78.0 ± 6.4	79.1 ± 7.5	78.8 ± 8.2	68 ± 4.1	77.2 ± 7.2	76.7 ± 2.7
MMSE, score	23.7 ± 5.8	23.4 ± 4.9	27.5 ± 1.7	26.7 ± 1.6**	25.9 ± 1.9	24.0 ± 0	17.7 ± 5.6	17.7.0 ± 3.9
GDS, score	7.0 ± 4.1	10.7 ± 3.3*	4.9 ± 3.5	9.2 ± 2.3**	7.7 ± 4.4	8.5 ± 4.6	9.4 ± 3.3	13.7 ± 2.0 <sup>#</sup>
ADLs, score	75.9 ± 19.7	75.8 ± 19.0	79.5 ± 23.3	78.1 ± 15.5	79.9 ± 8.6	94.0 ± 4.0	71.3 ± 18.8	70.7 ± 21.8
IADL, score	4.3 ± 2.5	4.3 ± 2.3	6.5 ± 1.1	5.8 ± 0.4**	5.8 ± 0.8	6.0 ± 1.1	1.8 ± 1.5	2.0 ± 1.9
CIRS-T, score	32.3 ± 4.6	35.7 ± 0.7*	30.8 ± 5.4	35.7 ± 1.7**	32.9 ± 4.6	31.0 ± 1.1	33.2 ± 3.5	36.3 ± 2.4 <sup>#</sup>
CIRS-MI	3.6 ± 0.7	3.7 ± 0.4	3.5 ± 0.6	3.7 ± 0.7	3.6 ± 0.6	3.0 ± 0.6	3.8 ± 0.8	3.9 ± 0.6
CIRS-SI	2.3 ± 0.3	2.5 ± 0.2	2.2 ± 0.4	2.5 ± 0.2**	2.3 ± 0.3	2.1 ± 0.1	2.3 ± 0.3	2.5 ± 0.2
CIRS-CI	7.1 ± 2.0	8.4 ± 1.7*	6.6 ± 2.4	8.7 ± 1.7**	7.1 ± 2.1	7.5 ± 0.7	7.6 ± 1.6	8.1 ± 1.8
ORO score	1.8 ± 1.5	2.1 ± 1.6	0.7 ± 1.0	1.0 ± 1.2	2.3 ± 1.3	3.0 ± 1.3	2.9 ± 1.2	3.7 ± 0.5 <sup>#</sup>
CF score	1.0 ± 0.8	1.0 ± 0.9	0.3 ± 0.4	0.5 ± 0.6	1.0 ± 0.8	1.2 ± 0.8	1.3 ± 0.7	1.9 ± 0.3 <sup>#</sup>
% of total cell area	10.5 ± 6.0	11.2 ± 6.8	7.0 ± 4.0	7.7 ± 5.4	11.6 ± 4.5	15.7 ± 9.6	14.5 ± 6.1	16.1 ± 4.8

Values are mean ± standard deviation (M ± SD)

t test:

\*No Eye Disorders vs. All Eye Disorders: GDS  $P<0.001$ ; CIRS-T  $P<0.001$ ; CIRS-CI  $P=0.003$

\*\*HC without Eye disorders vs. HC with an Eye disorder: MMSE  $P=0.032$ ; GDS  $P<0.001$ ; IADL  $P=0.001$ ; CIRS-T  $P<0.001$ ; CIRS-SI  $P=0.009$ ; CIRS-CI  $P=0.005$

MCI without Eye disorders vs. MCI with an Eye disorder: comparison not evaluated because of small number of Eye Disorders among MCI subjects

<sup>#</sup>Dementia without Eye Disorders vs. Dementia with Eye Disorders: GDS  $P<0.001$ ; CIRS-T  $P=0.005$ ; ORO score  $P=0.008$ ; CF score  $P=0.002$

**Abbreviations:** MMSE: Mini Mental State Examination; GDS: Geriatric Depression Scale; ADL: Activities of Daily Living; IADL: Instrumental Activities of Daily Living; CIRS: Cumulative Illness Rating Scale; CIRS MI: Maximum Impairment; CIRS-SI: Severity Index; CIRS-CI: Comorbidity Index; ORO score: Oil Red O Staining Intensity; CF score: Rate of Cluster Formation; % of total cell area: Percentage of Positively Stained Total Cell Area

**Table 5:** CGA parameters, ORO score, CF score and % of total cell area in relation to cognitive impairment and eye disorders.

rate it resulted statistically significant. This is probably a consequence of the imbalance in the number of dementia affected between genders, with a greater prevalence of dementia in the female group rather than the male one (46.8% vs. 14.7%). Interestingly, ORO score, % of total cell area and CF rate do not differ in MCI males and females, suggesting that changes in these three parameters are present not only in full-blown dementia, but probably also in the early stages of disease.

These results are in agreement with our earlier observations which showed that skin fibroblasts and PBMCs from patients with diagnosis of probable sporadic AD display an imbalance between free cholesterol and CE pools due to cytoplasmic NL accumulation, mainly CEs [3,4]. In addition, they seem to be consistent with other research, which either found altered lipid composition-cholesterol, in particular-in the brains of patients affected by different neurodegenerative diseases [1,25] or a potential link between diet-induced alterations in brain cholesterol metabolism and  $\beta$ -site A $\beta$ PP-cleaving enzyme 1 (BACE1) overexpression, which represents an early event in AD development [26]. Dysregulation of cholesterol homeostasis in the brain has, in fact, been linked to chronic neurodegenerative disorders, including AD, Huntington's disease, Parkinson's disease, Niemann-Pick type C disease and Smith-Lemli Opitz syndrome, as well as to acute neuronal injuries such as stroke and brain trauma [1,25]. The first clear proof for a critical role of CEs in the pathogenesis of neurological disorders derives from observations by Huttunen and Kovacs [27], who focused their studies on acyl-coenzyme A:cholesterol acyltransferase (ACAT), an endoplasmic reticulum membrane-bound enzyme that catalyses the conversion of free cholesterol to CEs, which coalesce to form lipid droplets in the cytoplasm. They found that cells with elevated CE levels produced more amyloid- $\beta$  (A $\beta$ ) peptides, which are crucially involved in AD as the main component of the amyloid plaques [28,29]. By contrast, cells that lacked CEs, but had elevated free cholesterol, produced almost no A $\beta$ . The researchers also found that ACAT inhibitors CP-113,818 and CI 1011 administered to AD-affected mice significantly reduced amyloid plaques and rescued cognitive deficits.

Following that, Bryleva et al. [30], using a combined mouse-genetic and biochemical approach to evaluate the potential role of ACAT in AD, showed that ACAT1 gene ablation in triple AD transgenic mice leads to more than 60% reduction in full-length human A $\beta$  precursor protein (A $\beta$ PP), as well as in its proteolytic fragments, and ameliorates cognitive deficits. These exciting findings led them to hypothesize that ACAT inhibitors might be of therapeutic value in the treatment of cognitive impairment. Overall, these data clearly indicate a direct association between impaired cholesterol metabolisms in the brain during neurodegeneration; however, whether a common molecular mechanism links the neurodegenerative phenotype to altered brain cholesterol metabolism remains to be established. This is an important point, because if this link existed, it would mean that a common therapeutic approach for these diseases might be possible. Awareness of these problems and possible solutions to them is severely limited by the inaccessibility of direct examination of the living human brain; for this reason, potential surrogate tissues must be utilized. In this context, PBMCs appear particularly attractive because they seem to participate directly in neurodegenerative processes. PBMCs have been shown to share much of the non-synaptic biochemical environment of neurons, and contain the full complement of epigenetic enzymes and machinery that are found in both neurons and peripheral nucleated cells, as in most other tissues, as suggested by Arosio et al. [31]. The present results show that NL accumulation in PBMC cytoplasm is not restricted to AD, but may also occur in other diseases characterized by the deterioration of cognitive functions. These changes have also been observed in MCI, but with less magnitude and extent. The control subjects, cognitively healthy and free of any neurological or psychiatric illnesses, had minimal NL content changes in their PBMCs, as previously reported by us [2-4,32]. Interestingly, NL content was significantly correlated to clinical severity, as assessed by MMSE [2-4,32]. These results led us to hypothesize that NL changes in PBMCs might reflect dynamic modifications in cholesterol homeostasis, which occur in the brain during early development of cognitive deficit [2-4,32]. Another interesting finding of this study was that PBMCs from dementia and MCI groups, freshly isolated from buffy coat and plated in 6-well tissue culture dishes, merged together to form large aggregates or clusters, which are visible under light microscope. By rating cell-clustering using a semi-quantitative scale ranging from 0 to 2 we found that it follows a pattern similar to ORO staining, with CF score being significantly lower in HC than in the other four groups (MCI, AD, MD and VD) and higher in AD, MD and VD than in MCI. The mechanism by which PBMCs from dementia and MCI subjects form clusters remains to be determined. However, since clusters observed in dementia and MCI cells resemble those seen in PHA-stimulated PBMCs [2,3], we assumed that cluster formation in the unstimulated cultures of PBMCs may reflect the fact that they pre-exist in *in vivo*-activated state, secondary to the release of inflammatory mediators, such as cytokines, by brain cells [2-4,31]. Studies have consistently documented the presence of sustained inflammatory responses, involving glial cells in the early stages of neurodegeneration [33]. It is generally agreed that brain injury results in the immediate recruitment of glial cells to the damaged area, where they become activated and secrete cytokines and chemokines [34-36]. It should be stressed that the term 'glial cell activation' is not specific, and may reflect different pathological states. Inflammatory plasma-protein levels (i.e., interleukin-1, interleukin-6 and a-1-antichymotrypsin) have been found to increase before clinical onset of dementia in patients with AD and VD. Detection of circulating cytokines has also been proposed as a potential biomarker for screening, diagnosis and monitoring of neurodegenerative diseases and other neurological pathologies [37,38]

as well as a tool to evaluate the progression of brain inflammation associated with cerebrovascular disease. Taken together, these data indicate that profound alterations in cholesterol metabolism, associated with signals of enhanced inflammatory response, occur both in the periphery and in the CNS, in the early stages of many neurological disorders, but whether these changes are a cause or a consequence of neurodegeneration remains uncertain. In addition, no clear molecular mechanism has yet emerged that would directly relate alterations in cholesterol homeostasis and neuroinflammation in the brain to molecular changes occurring in peripheral cells. Based on the information currently available and that of the present study, we propose a plausible mechanism linking changes in cholesterol metabolism and brain neurodegenerative phenotype inflammation, and explaining how changes in NLs and in PBMC cluster formation may reflect these effects. There is ample evidence that neuronal function and survival is compromised by neuroinflammation, which may be initiated as the result of a variety of causes, including infection, traumatic brain injury, toxic metabolites and autoimmunity [33,36] and also by perturbed cholesterol homeostasis, which may occur as a consequence of genetic and/or environmental factors [2]. So, it may be argued that these two processes may be mutually dependent upon each other, indicating that altered cholesterol homeostasis may induce neuroinflammation and vice versa. In any case, this would lead to neuronal damage. Response to neuronal damage includes activation of resident immune cells (microglia), resulting in a phagocytic phenotype and in the release of inflammatory mediators, such as cytokines and chemokines, which could pass into the systemic circulation, leading to PBMC activation [36]. This activation may be responsible for the observed PBMC cluster formation *in vitro*. On the other hand, following brain injury, damaged cells may release free cholesterol, which is taken up by neighbouring neurons, where, at first, it is presumably esterified by ACAT1 and stored within cytoplasmic lipid droplets. If in excess, free cholesterol, which cannot pass the blood brain barrier (BBB), is hydroxylated in neurons to 24(S)-hydroxycholesterol (24-OHC) and transferred into the blood circulation. At this point it should be mentioned that 24-OHC is an oxygenated cholesterol derivative and, consequently, a potential inducer of LDL oxidation. Consistent with this, increased levels of circulating oxidized low-density lipoprotein (LDLox) were found in patients affected by neurological disorders [2,32]. Through a mechanism similar to that described for atherosclerosis, these LDLox, which can be recognized by receptors on the surface of PBMCs, release CEs, once internalized, transforming PBMCs into foam cells. Such a scenario may explain why subjects with neurological disorders are characterized by an accumulation of CEs in the cytoplasm of their PBMCs [2,32]. 24-OHC can be responsible for amplifying neuronal damage in AD by inducing oxidative stress, as well as net synthesis of A $\beta$ <sub>1-42</sub> by up-regulating expression levels of A $\beta$ PP and b-secretase and its activity [39,40].

The retina contains a specialized type of glia, the Müller glia, not found anywhere else in the CNS. Like other glial cells of the CNS, Müller cells undergo reactive gliosis following acute retinal injury or chronic neuronal stress [41]. Growing evidence in clinical and experimental studies strongly suggests involvement of Müller glia activation in the pathogenesis of the most common age-related eye disorders, including cataract, glaucoma, diabetic retinopathy and age-related macular degeneration. In addition, high CE levels, which tend to become oxidized into oxysterols, have also been reported in several eye lesions in particular, drusen and in ageing Bruch's membrane, which are all hallmark features of age-related macular degeneration [41-43].

We thus sought to determine whether unstimulated PBMCs derived from subjects with various age-related eye diseases have the capacity to self-assemble into clusters and to accumulate NLs. To do this, participants were divided into two groups, according to the presence or absence of eye disorders. Each group was further divided according to the absence of cognitive impairment or the presence of dementia or MCI (HC with or without eye disorders; MCI with or without eye disorders; demented patients with or without eye disorders). No significant difference in ORO and CF scores and percentage of cell area red intensity was observed between subjects with ocular disorders and those without ocular disorders. However, ORO and CF scores were significantly higher in demented patients with at least an eye disorder than demented patients without eye disorders, and, although failing to reach significance, the percentage of ORO staining of total cell area was lower in this second group compared to the first. These results indicate that eye damage may exacerbate NL accumulation and the rate of cell clustering observed in PBMCs of demented patients.

It has been previously established that MMSE is not well suited for identifying MCI, which raises the question whether it is an adequate "standard" to be used to assess cognitive performance. The aim of the present work was to characterize potential biological marker(s), which could be used either in early diagnosis or for prediction of the risk of severe neurological symptom development. Unlike other organ-based diseases, where diagnosis can be done rapidly by using biomarkers-usually involving blood tests-there are no rapid, definitive biochemical markers to detect potential brain injury early, nor is there a gold standard for subject stratification by neurological injury severity, or for monitoring injury progression. In this context, it appears imperative to develop tools that might facilitate disease state assessment and new drug efficacy, in particular, tools that may help to predict the early stages of the disease and the long period of time that precedes the onset of manifest signs. In fact, an effective therapeutic approach is most likely to be successful in pre-manifest subjects who have not yet developed irreversible neurodegeneration. Here, we present a novel, non-invasive approach for early neuronal injury detection based on the presence of NLs in the cytoplasm of unstimulated-PBMCs, combined with their potential tendency to form clusters. This approach is the result of two independent lines of investigation: the first derives from the general problem of cholesterol metabolism in the CNS: a constant equilibrium between synthesis and degradation is maintained through cholesterol-to-24-OHC oxidation-a metabolite able to cross BBB-the blood circulation levels of which are taken as an index of brain cholesterol elimination [2,32]; the second, from the connection between inflammation and neurodegeneration: the immune response of the CNS is orchestrated by microglial cells, which, on activation, become phagocytes and secrete a wide range of inflammatory mediators, including cytokines and chemokines, growth factors, complement molecules and adhesion molecules [2,32]. Inflammatory response detectable in the periphery is considered an index of neurological disease progression [44].

## Conclusion

In summary, our data demonstrate that: (i) NLs measurable in naïve PBMCs are shown to parallel a critical phase of neuronal loss in disease characterized by progressive brain damage; (ii) NLs and CF in PBMCs appear to be a valuable tool to discriminate pre-manifest subjects from neurological patients and might, therefore, be helpful to investigate subjects before the onset of neural symptoms. To our knowledge, this is the first evidence indicating that neurodegenerative processes could be monitored by testing the effects of compounds generated by altered

CNS metabolism on PBMCs. To further validate these data and their clinical implications, larger cross-sectional and longitudinal studies are essential. Furthermore, discovering if ORO score and CF remain low in MCI patients who do not develop the overt disease could be very important. Further longitudinal studies in this matter will be necessary.

## Acknowledgement

This paper is dedicated to the memory of Prof. Sandra Dessi.

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