Anti-ApoA-1 IgG serum levels predict worse post-stroke outcomes

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Abstract

Background: Autoantibodies to ApolipoproteinA-1 (anti-ApoA-1 IgG) were shown to predict major adverse cardiovascular events and promote atherogenesis. However, their potential relationship with clinical disability and ischemic lesion volume after acute ischemic stroke (AIS) remains unexplored.

Materials and methods: We included n=76 patients admitted for AIS and we investigated whether baseline serum anti-ApoA-1 IgG levels could predict i) AIS-induced clinical disability (assessed by the modified Rankin Scale [mRS]), and ii) AIS-related ischemic lesion volume (assessed by Computed Tomography [CT]). We also evaluated the possible pro-apoptotic and pro-necrotic effects of anti-ApoA-1 IgG on human astrocytoma cell line (U251) using flow cytometry.

Results: High levels of anti-ApoA-1 IgG were retrieved in 15.8% (12/76) of patients. Increased baseline levels of anti-ApoA-1 IgG were independently correlated with worse mRS (β=0.364; *p*=0.002; adjusted odds ratio [OR]: 1.05 [95% CI 1.01-1.09]; *p*=0.017) and CT-assessed ischemic lesion volume (β=0.333; p<0.001; adjusted OR: 1.06 [95% CI 1.01-1.12]; *p*=0.048) at 3 months. No difference in baseline clinical, biochemical and radiological characteristics was observed between patients with high versus low levels of anti-ApoA-1 IgG. Incubating human astrocytoma cells with anti-ApoA-1 IgG dose-dependently induced necrosis and apoptosis of U251 cells *in vitro*.

Conclusion: anti-ApoA-1 IgG serum levels at AIS onset are associated with poorer clinical recovery and worse brain lesion volume 3 months after AIS. These observations could be partly explained by the deleterious effect of anti-ApoA-1 IgG on human brain cell survival *in vitro* and may have clinical implication in the prediction of poor outcome in AIS.

Keywords: Acute ischemic stroke, anti-ApoA-1 IgG, computed tomography, modified Rankin Scale.

Introduction

A growing body of evidence indicates that humoral autoimmunity plays an active role in atherogenesis and its related complications, including acute coronary syndromes and acute ischemic stroke (AIS) [1, 2]. In AIS, different autoantibodies of various subclasses and directed against various antigens have been reported, some of them being associated with a worse cardiovascular (CV) risk and prognosis [3-7], while others appear as acting as protective antibodies [7-9]. Among possible pro-atherogenic auto-antibodies, we and others focused the interest on autoantibodies against ApolipoproteinA-1 (anti-ApoA-1 IgG), the major lipoprotein fraction of high-density lipoprotein cholesterol (HDL-c) [1, 10, 11]. Most of the studies have so far demonstrated that high serum level of anti-ApoA-1 IgG is an independent predictor of poor CV outcome associated with increased atherosclerotic plaque vulnerability in humans [12-15]. Furthermore, *in vitro* and animal studies pointed to toll-like receptor (TLR)-2, -4 and CD14 signalling as key receptors underlying the inflammatory and pro-atherogenic response of anti-ApoA-1 IgG [16-19]. However, despite the association with increased CV risk, their prognostic role in functional recovery and immune system activation after AIS remains largely unexplored. Therefore, in this study, we aimed at investigating the potential of anti-ApoA-1 IgG in predicting functional handicap (as determined by modified Rankin Scale [mRS] and National Institute of Health Stroke Scale [NIHSS]) [20] and ischemic lesion volume. [21] Finally, to explore the possible impact of anti-ApoA-1 IgG on human glioma cell survival, we evaluated their ability to promote necrosis and apoptosis on cells derived from U251 astrocytoma cell line using flow cytometry.

Methods

The Ethics Committee of Ferrara University Hospital approved this protocol, performed in accordance with the guidelines of the Declaration of Helsinki. Patients gave informed consent prior to entering the study.

Patients and clinical assessment

This pilot observational prospective study is a sub-analysis of a larger single center cohort (Neurology Department, Ferrara University Hospital, Italy) enrolling 90 patients with first AIS from April 2009 to December 2011 [22]. Serum samples were available for 76 of 90 patients admitted for first AIS. Acute ischemic stroke (AIS) was defined as acute-onset focal neurological deficit characterized by neuroimaging evidence of cerebral infarction [23]. Patients admitted within 6 hours from AIS onset were recruited, excepted those with haemorrhagic stroke, intracranial abscess, acute infection, recent (<30 days) acute CV event, malignancy, and renal/hepatic failure. All patients were then treated according to the current guidelines of the American Heart Association [24]. Thrombolysis exclusion criteria included patients with minor or rapidly improving stroke symptoms, history of previous intracranial haemorrhage, significant head trauma in previous 3 months, elevated blood pressure (systolic >185 mmHg or diastolic >110 mmHg), active internal bleeding, major surgery within previous 14 days, and those admitted after 4.5 hours from onset of symptoms in presence of an hypodensity >1/3 cerebral hemisphere on non-contrast cranial computed tomography (NCCT) [22]. Finally, the clinical assessment also included a categorization of stroke aetiology according to the TOAST (Trial of ORG 10172 in Acute Stroke Treatment) criteria: i) large-artery atherosclerosis, ii) cardio-embolism, iii) small-vessel occlusion, iv) stroke of other determined aetiology, or v) stroke of undetermined aetiology [25].

Patient follow-up and study endpoint adjudication

The primary end-point of this study was to test the ability of serum anti-ApoA-1 IgG to predict 90-day disability according to the mRS. As previously validated, [20] mRS ≤2 at day 90 after AIS onset identified patients with recovered autonomy in carrying out usual activities, whereas persistent long-term disability was defined by mRS >2. The secondary endpoint of this study was to explore the relationship of circulating anti-ApoA-1 IgG with stroke severity at day 1 by NIHSS. In this regard, NIHSS ≥5 was used as cut-off to determine neurological severity after AIS onset [26]. A further endpoint was to assess the relationship between anti-ApoA-1 IgG and ischemic lesion volume at day 1 and 90 by non-contrast computerised tomography (NCCT). Finally, we also explored the potential association of anti-ApoA-1 IgG positivity with the haemorrhagic transformation during the first day after AIS. As previously described, both study endpoints were independently adjudicated by two study investigators (neurologists) at Ferrara University Hospital, who were blinded to the results of biochemical analyses [22].

Neuroimaging

NCCT was performed by a 64-slice Lightspeed VCT (GE Medical System, Milwaukee, WI; USA) from the skull base to the vertex by using an axial technique, as previously reported. [22]. All NCCT images were acquired along the orbito-meteal plane with 2.5-mm (8 images/rotation) and 5-mm (4-images/rotation) slice thickness reconstruction for posterior fossa and supratentorial region, respectively. As reported elsewhere, ischemic volume was calculated on NCCT at day 1, 7 and 90 after symptom onset with a multi-slice planimetric method by summation of the hypodense areas, manually traced on each slice in which they were detectable, multiplied by slice thickness [27]. The lesion volume obtained at 90 days was considered as the final infarct size. Finally, also the occurrence of haemorrhagic transformation (HT) was recognized by NCCT at day 1 and 7 after stroke onset.

Blood collection and quantification

For all patients, blood samples were collected by using a butterfly to reduce membrane shear stress and then drawn in tubes to obtain serum. The first sample was collected within 1 hour from admission and within 6 hours of symptom onset. In addition to anti-ApoA-1 IgG assessment, haematological parameters and blood chemistry including plasma glucose, triglycerides, total cholesterol, high-density lipoprotein, and low-density lipoprotein cholesterol were measured by routine auto-analyser.

Biomarker measurements in serum

Anti-ApoA-1 IgG serum levels were measured as previously described [12-16]. Briefly, Maxi-Sorb plates (Nunc™ from Thermo Scientific, Waltham, MA) were coated with purified human-derived delipidated ApoA-1 (20 μg/ml; 50 μl/well) for 1 hour at 37°C. After 3 washes with phosphate buffered saline (PBS)-2% bovine serum albumin (BSA; 100 μl/well), all wells were blocked for 1 hour with 2% BSA at 37°C. Samples were diluted 1:50 in PBS-2% BSA and incubated for 60 minutes. Additional patient samples at the same dilution were also added to an uncoated well to assess individual nonspecific binding. After 6 further washes, 50 μl/well of signal antibody (alkaline phosphatase conjugated anti-human IgG; Sigma-Aldrich, Saint Louis MO) diluted 1:1000 in PBS-2% BSA solution was incubated for 1 hour at 37°C. After 6 more washes (150 μl/well) with PBS-2% BSA solution, the phosphatase substrate p-nitrophenyl phosphate disodium (50 μl/well; Sigma-Aldrich) dissolved in diethanolamine buffer (pH 9.8) was added. Each sample was tested in duplicate, and absorbance, determined as the optical density at 405 nm (OD 405 nm), was determined after 20 minutes of incubation at 37°C (VersaMax, Molecular Devices, Sunnyvale, CA). The corresponding nonspecific binding value was subtracted from the mean absorbance value for each sample. The positivity

cut-off point is set at an arbitrary unit (AU; optical density: 405 nm) >0.64 AU, and an index (ratio between sample net AU and the positive control net AU x 100) > 37%, corresponding to the 97.5th percentile of a reference population of 140 healthy blood donors [28]. This cut-off has been subsequently validated in numerous different prospective studies [13-16], the reason why the same cut-off was used presently.

Binding of anti-ApoA-1 IgG on U251 cells in vitro

5x10^5 cells were resuspended in 100 µl of PBS + 2% BSA and 20 µg/ml of polyclonal goat anti-human apoA-1 IgG (Academy Bio-Medical Company, Inc., Houston, TX,) or polyclonal goat isotype control (CTL) IgG (Meridian Life Science, Saco, ME.) were added for 30 min on ice. After washed with PBS + 2% BSA, cells were incubated with 10 µg/ml of FITC-donkey anti-goat IgG (Invitrogen™, ThermoFisher Scientific, Waltham, MA) for 30 min then washed. Fluorescence quantification was performed using an ACCURI C6 flow cytometry (BD Biosciences, San Jose, CA), and analysed using FlowJo data analysis software (version 10.0.7) (FlowJo LLC, Ashland, OR).

In vitro detection of necrosis and apoptosis of U251 cells

Astrocytoma cell line U251 (Sigma-Aldrich) were grown in RPMI-1640-GlutaMax culture medium with supplementation of 25 mM HEPES and 10 % Foetal Bovine Serum (Gibco BRL-Life Technology, Rockville, MD). Cells were seeded at 1x10^5 cell/well in a 12 wells plate (Nunc™) in complete medium for 24 hours. Then, cells underwent 24 hours starvation with RPMI + 1% FBS. Polyclonal goat anti-human ApoA-1 IgG (Academy) or polyclonal goat CTL IgG (Meridian Life Science) were added to the cell media at the concentration of 20, 40, 80 or 160 µg/ml for 48h. Cells were finally washed with D-PBS (Gibco) and detached using Accutase (StemCell Technologies SARL, Köln, Germany). Necrotic/apoptotic cells

were detected using the PE active caspase-3 apoptosis kit from BD-Pharmigen and Zombie Red Fixable Viability Kit (BioLegend, San Diego, CA). Briefly, the detached cells were washed in PBS and resuspended in 100 µl of PBS. 1 µl of Zombie red dye was added to the cells for 15 min in the dark. The cells were washed with 2 ml of PBS + 1% BSA then re-suspended in 0.5 ml of cytofix/cytoperm buffer for 15 min on ice. After 2 washes in Perm/Wash buffer, the cells were incubated with 20 µl of PE anti-activate caspase 3 antibody in 100 µl buffer for 30 min. After 1 wash, the cells were resuspended in 300 µl of buffer and necrosis/apoptosis were quantified using an Accuri C6 Flow cytometer (BD Biosciences). The data were analysed with FlowJo V10.0.7 software. Data are expressed as a percentage of cells positive for Zombie dye labelling (necrotic cells) and/or positive for active caspase labelling (apoptotic cells) for 4 independent experiments.

Expression of toll like receptor-2,-4 CD 14, and GFAP on U251 cells

$5x10^5$ cells/condition were re-suspended in 100 µl of PBS + 2% BSA. Then 2.5 µl of the following antibodies were added: FITC-mouse anti-human CD14 (clone HCD14), AF488-mouse anti-human TLR2 (clone TL2.5), APC-mouse anti-human TLR4 (clone HTA125) or isotype-matched control mouse IgG (Biolegend). The cells were incubated 30 min on ice followed by two washes in PBS + 2% BSA. For the astrocyte marker glial fibrillary acidic protein (GFAP) labelling, the cells were first fixed and permeabilized with a solution of 4%PAF/0.1% saponin in PBS buffer for 20 min on ice. After one wash in PBS, the cells were resuspended in 100 µl of PBS + 2% BSA and 2 µl of GFAP rabbit whole antisera (ab7260 by Abcam Cambridge, UK) were added for 30 min followed by secondary labelling with FITC goat-anti-rabbit IgG (Invitrogen) for 30 min. After 2 washes with PBS + 2% BSA, cells were resuspended in 300 µl of PBS + 2% BSA. Fluorescence quantification was performed using

an ACCURI C6 flow cytometer (BD Biosciences) and analysed using FlowJo data analysis software.

Statistical analysis

Analyses were performed with IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY). Categorical data were presented as relative and absolute frequencies, whereas continuous variables were expressed as median and interquartile range (IQR), due to the fact that data do not follow a normal distribution. Comparisons between groups were drawn by Fisher's exact test or non-parametric Mann-Whitney *U* test when appropriate. Spearman test was used to test the correlations between continuous variables. Then, the independence of the correlation was estimated by a linear regression model adjusted for potential confounders. In addition, age, gender, and thrombolysis were set as covariates, according to with previous studies from this cohort [29, 30] and other published data [31-33]. Multivariate risk analysis was finally performed by logistic regression models, presented as odds ratio (OR) and corresponding 95% confidence intervals (95% CI). In these models, categorized NIHSS and mRS, the occurrence of haemorrhagic transformation and tertiles of NCCT-assessed ischaemic lesion volume were set as dependent variables, whereas anti-apoA-1 IgG age, gender and thrombolysis were set as covariates. For in vitro experiments, the data were presented as mean ± standard deviation (SD). Statistical analyses were performed using one-way ANOVA (corrected with Bonferroni posthoc test), when appropriate. A 2-sided p-value <0.05 was considered as statistically significant.

Results

Patients' characteristics

Baseline demographic, biochemical and clinical characteristics, as well as medications for AIS patients, are shown in Table 1. In the overall cohort, median age was 67 years and 44 patients (57.9%) were male. Considering traditional risk factors for AIS, 59.2% of subjects had hypertension, 34.7% were active smokers, 25.0% suffered from dyslipidaemia, and 14.4% displayed a body mass index ≥30 (Table 1). High levels of anti-ApoA-1 IgG was retrieved in 15.8% (12/76) of the patients. At the exception of angiotensin receptor blockers and diuretics use that were more frequent in anti-ApoA-1 IgG positive patients, no other differences were observed for the remaining clinical and biochemical parameters (Table 1). The aetiology of AIS and the administration of thrombolytic treatment did not differ between these two groups of patients. Similarly, no significant differences in clinical NIHSS score and NCCT-assessed ischemic lesion volume were found among both study groups at onset and at day 1 after AIS (Table 1).

Baseline anti-ApoA-1 IgG levels do not correlate with neurological disability score (NIHSS) or stroke lesion volume in the acute phase

The levels of anti-ApoA-1 antibodies did not correlate with the severity of neurological injury in the acute phase of AIS, as assessed by NIHSS at day 1 (r=0.186; p=0.108 according to with the Spearman test).

Likewise, univariate linear regression analysis failed to show a significant relationship between anti-ApoA-1 antibodies and the severity of neurological injury in the acute phase of AIS, as assessed by NIHSS at day 1 (β=0.169; p=0.145) (Table 2). Similarly, no correlation was observed between anti-ApoA-1 IgG and ischemic lesion volume (assessed by NCCT) at day 1 (r=0.107; p=0.358 according to with the Spearman test). These results were confirmed by univariate linear regression analysis (Table 2). However, after adjustment for potential confounders, an independent association between anti-ApoA-1 antibodies and NIHSS at day 1

was shown (adjusted β=0.246; *p*=0.034), whereas no association was demonstrated with NCCT-assessed lesion volume (Table 2).

Concerning thrombolytic treatment, linear regression analysis showed an independent relationship with increased NIHSS in both univariate and adjusted analysis at days 1 (adjusted β=0.279; *p*=0.013) (Table 2). Male gender was not associated with NCCT-assessed lesion volume on day 1 (Table 2). The relationship between male gender and NIHSS became non-significant after adjustment for confounding factors (Table 2). Finally, age was negatively correlated with ischemic lesion volume at the same time point (Table 2).

Logistic regression did not indicate anti-ApoA-1 IgG status as predictor of poor neurological outcome at day 1 after AIS (Table 3). Conversely, risk analysis further confirmed the prognostic impact of thrombolysis in the early phase of ischemic stroke. Thrombolysis was able to predict a severe neurological injury according to NIHSS at day 1 (adjusted OR 7.35 [95% CI 2.06-3.28]; *p*=0.002; Table 3). Similarly, thrombolytic treatment was associated with the risk of increased ischemic lesion volume at days 1 (adjusted OR 7.18 [95% CI 1.62-31.71]; *p*=0.009; Table 3). Taken together, these results indicate that anti-ApoA-1 IgG does not correlate with neurological disability score (NIHSS) or stroke lesion volume in the acute phase of ischemic stroke.

Baseline anti-ApoA-1 IgG levels predict neurological disability (mRS) and stroke lesion volume at 90-days after AIS.

Significant correlations of anti-ApoA-1 IgG values with both the marker of poor neurological outcome mRS (*r*=0.293; *p*=0.010) and NCCT-assessed lesion volume (*r*=0.312; *p*=0.006) were retrieved at day 90 after AIS according to with the Spearman test.

Furthermore, linear regression analysis showed that anti-ApoA-1 IgG serum levels were independently correlated with worse functional recovery according to mRS (β=0.303;

p=0.008) and final infarct volume (β=0.286; *p*=0.012) at day 90 after AIS onset (Table 2). Also after the adjustment for confounding factors (age, male gender and thrombolysis), anti-ApoA-1 IgG serum levels remained independently correlated with both worse neurological outcome (adjusted β=0.364; *p*=0.002) and final infarct volume (adjusted β=0.333; *p*<0.001) (Table 2). Similar results were observed also for thrombolysis (Table 2).

The clinical characteristics of patients according to with mRS status at day 90 were also shown. Patients with a good neurological recovery after AIS (mRS ≤2) had higher circulating levels of HDL-c and lower serum glucose at admission as compared to mRS >2 (Table 4). Conversely, patients with a worse functional recovery after AIS (mRS >2) had higher proportions of atherothrombotic stroke and thrombolytic treatment as well as lower lacunar stroke (Table 4).

On this basis, logistic regression analysis indicated a significant risk of poor neurological recovery at day 90 after AIS in patients with high serum levels of anti-ApoA-1 IgG (OR for mRS >2 1.03 [95% CI 1.01-1.06]; *p*=0.036; Table 3). This association remained statistically significant also after adjustment for age, male gender and thrombolysis (adjusted OR for mRS >2 1.05 [95% CI 1.01-1.09]; *p*=0.017; Table 3). In addition, anti-ApoA-1 IgG positivity slightly increased the risk of large infarct volume at day 90 after AIS (OR for tertiles of NCCT-assessed ischemic lesion volume 1.04 [95% CI 0.99-1.09]; *p*=0.074; Table 3). However, anti-ApoA-1 IgG positivity significantly predicted an increased final infarct volume in multivariate analysis (adjusted OR 1.06 [95% CI 1.01-1.12]; *p*=0.048; Table 3).

Anti-ApoA-1 IgGs induce a dose-dependent necrosis and apoptosis of human astrocytoma cell line U251

In an attempt to provide a biological substrate to better understand the aforementioned clinical associations, we evaluated the possible direct effect of anti-ApoA-1 IgG exposure on the

human astrocytoma cell line (U251) survival by flow cytometry. As first, it was observed that anti-ApoA-1 antibodies but not CTL IgG bind on the U251 cell surface (Figure 1). Therefore, incubating U251 cells with anti-ApoA-1 IgG was shown to induce a dose-dependent necrosis and apoptosis, whereas no effect of CTL IgG was observed, even at the highest tested dose of 160 µg/ml (Figures 2A and 2B). As the anti-ApoA-1 IgG cellular signalling was shown to involve TLR2, TLR4, and CD14 complex [17-19], we first evaluated whether U251 cells expressed any of these receptors. As expected, U251 cells expressed the specific marker GFAP (Fig. 3A), but not TLR2, TLR4 or CD14 (Figs 3B-D), thus indicating that the direct pro-apoptotic and pro-necrotic effects of anti-ApoA-1 IgG effects do not involve TLR2, TL4 or CD14 signalling. The exact mechanisms underlying these observations are currently under investigation.

Discussion

In this pilot study, we demonstrated that high baseline levels of anti-ApoA-1 IgG predict disability and brain lesion volume at day 90 after AIS. In this regard, the detrimental effect of anti-ApoA-1 status has been shown to be independent of other well-known determinants of neurological outcome. Specifically, we considered age, gender and thrombolysis, previously demonstrated as major determinants of post-stroke outcome in large clinical studies [31-33]. Furthermore, our study suggested a detrimental role of anti-ApoA-1 IgG on human glioma cells *in vitro*. Although preliminary, these results raise several questions that may have a practical clinical implication in the future. Indeed, from these results, one could extrapolate that patient with AIS and high levels of anti-apoA-1 IgG (15.8% of AIS patients) could represent a high-risk subgroup particularly prone to benefit from earlier and/or more intensive neuro-rehabilitation than patients tested negative for these antibodies. Whether such patients would benefit, on top of the standard of care, from reconstituted HDL-c or ApoA-1 mimetic

peptide-mediated neuroprotection remains to be explored [34, 35]. The *in vitro* demonstration of the neurotoxic and pro-necrotic effects of anti-ApoA-1 IgG on human glioma cells broadens the scope of the currently known biological functions ascribed to these antibodies. Even if the exact molecular mechanisms leading to the anti-ApoA-1-IgG induced apoptosis and necrosis are currently unknown, a role of TLR2, TLR4 and CD14 cannot rule out, considering their expression by glial cells and their involvement in post-ischemic brain inflammation [36],

Another remaining issue is to know whether and how plasmatic anti-ApoA-1 IgG could migrate through the blood-brain barrier (BBB) into the central nervous system (CNS). A direct effect of anti-ApoA-1 IgG on BBB integrity through decreasing astrocyte survival cannot be excluded considering that U251 cells are derived from astrocytes, a key determinant of BBB [37-39]. In this regard, it is very interesting to note that the BBB integrity appears to be a major determinant of the autoantibody's protective, neutral or deleterious effect on brain tissue [3, 7]. Whether this observation could also apply to anti-ApoA-1 IgG remains to be clarified.

This study has different limitations. Firstly, due to the very limited number of patients enrolled in the present pilot study, the results needs to be reproduced at a larger scale to confirm the association between circulating levels of anti-ApoA-1 IgG and the post-AIS functional and radiological outcomes. This power issue may also potentially explain the lack of association between anti-ApoA-1 IgG and both stroke subtypes and the risk of haemorrhagic transformation. Further larger studies are requested to provide a defined answer to this important question. Secondly, it can be argued that the use of a glioma cell line instead of human neurons in primary culture may be too far away for human pathophysiology. As we were able to reproduce the same results in other cell lines (oligodendrocytes and neurons, data not shown), we consider that neurotoxic effects reported in U251 cells are likely to be a

generic property of anti-ApoA-1 IG on neurons. Nevertheless, the replication of these *in vitro* results on human neurons in primary culture will be of utmost importance not only to draw definite conclusions regarding the possible anti-ApoA-1 neurotoxic effect but also to determine the importance of cellular replication rate in this process. Lastly, the present demonstration that anti-ApoA-1 IgG could negatively affect glioma cells survival in terms of both necrosis and apoptosis in the absence of TLR2, TLR4 and CD14 was rather surprising and represents an important mechanistic limitation. Other pathways independent of innate immune receptors currently under investigation are likely to be involved and are currently under investigation.

In conclusion, this pilot study suggests that high serum levels of anti-ApoA-1 IgG detected at the onset of AIS are associated with poorer clinical recovery and larger infarcted brain area 3 months after AIS. The possible causal nature of this association is supported by the deleterious effects of anti-ApoA-1 IgG on human glial cells apoptosis and necrosis *in vitro*. Further studies are needed to: i) validate these preliminary results at a larger scale, ii) to determine the potential impact of anti-ApoA-1 IgG on the clinical follow-up, and iii) to clarify the pathophysiological mechanism underlying the neurotoxic properties of anti-ApoA-1 IgG on glial and neuronal cells.

Conflict of interest statement: none to be declared

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Author contributions

E. Fainardi, G. Roversi, C. Tamborino, I. Casetta, S. Seraceni, A. Trentini, and M. Padroni recruited the patients and collected clinical and biochemical data. F. Montecucco, N. Satta, J Virzi, F. Burger and A. Roth performed biomarker measurements in serum, including anti-ApoliproteinA-1 assay, and *in vitro* experiments. F. Carbone performed statistical analysis and wrote the paper. F. Montecucco, N. Vuilleumier, P.H. Lalive, F. Mach, and F. Dallegri revised the manuscript.

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Figure Legend

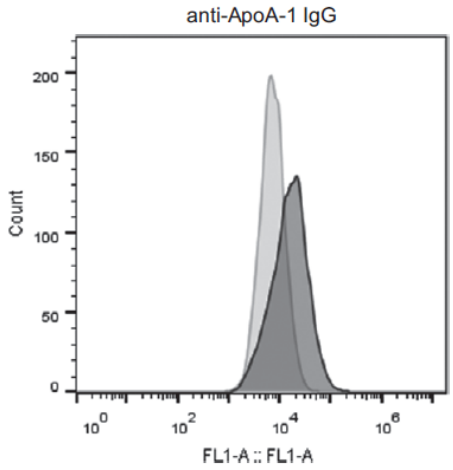

Figure 1. Detection of anti-ApoA-1 IgG binding on the U251 cell surface. Histogram representation of anti-ApoA-1 (dark grey peak) and control (CTL) IgG (light grey peak) fluorescence labelling on the U251 cell surface. Data representative of 2 different experiments.

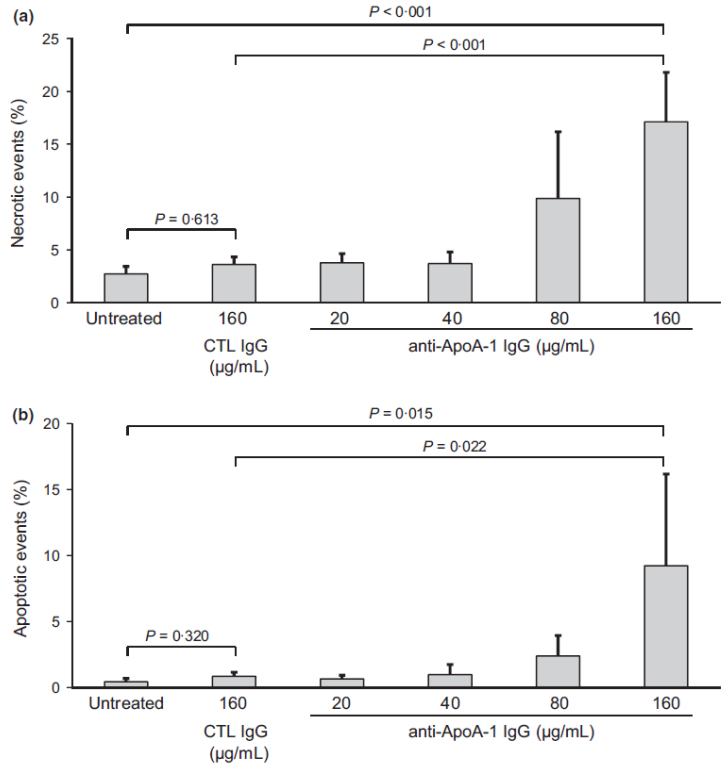

Figure 2. Treatment with anti-ApoA-1 IgG increases necrosis and apoptosis of human astrocytoma cell line U251. A. Necrotic events (expressed as a percentage [%] of the events in untreated cells) in the presence or absence of control (CTL) IgG or different concentrations of anti-ApoA-1 IgG. Data are expressed as mean±SD, n=4 per group. **B.** Apoptotic events (expressed as percentage [%] of the events in untreated cells [Medium]) in the presence or absence of CTL IgG or different concentrations of anti-ApoA-1 IgG. Data are expressed as mean ± SD, n=4 per group.

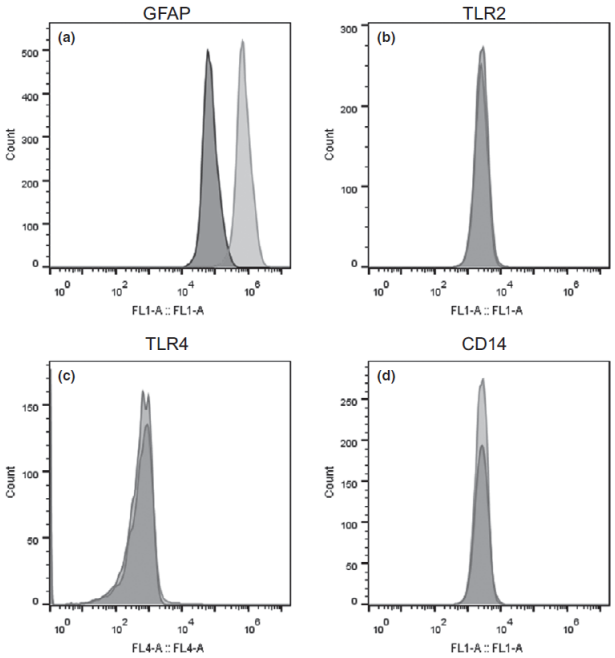

Figure 3. Detection of TLR2, TLR4, CD14 and GFAP expression on human astrocytoma cell line U251. Histogram representation of glial fibrillary acidic protein (GFAP) (**A**), TLR2 (**B**) TLR4 (**C**) and CD14 (**D**) and specific fluorescence labelling (light gray peak) in comparison to the isotype-matched control (CTL) IgG (dark gray peak). Only GFAP was identified in the U251. Data are representative of 2 independent experiments.