Adipocyte fatty acid-binding protein exacerbates cerebral ischaemia injury by disrupting the blood–brain barrier

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Aims	Adipocyte fatty acid-binding protein (A-FABP) is an adipokine implicating in various metabolic diseases. Elevated circulating levels of A-FABP correlate positively with poor prognosis in ischaemic stroke (IS) patients. No information is available concerning the role of A-FABP in the pathogenesis of IS. Experiments were designed to determine whether or not A-FABP mediates blood–brain barrier (BBB) disruption, and if so, to explore the molecular mechanisms underlying this deleterious effects.
Methods and results	Circulating A-FABP and its cerebral expression were increased in mice after middle cerebral artery occlusion. Genetic deletion and pharmacological inhibition of A-FABP alleviated cerebral ischaemia injury with reduced infarction volume, cerebral oedema, neurological deficits, and neuronal apoptosis; BBB disruption was attenuated and accompanied by reduced degradation of tight junction proteins and induction of matrix metalloproteinases-9 (MMP-9). In patients with acute IS, elevated circulating A-FABP levels positively correlated with those of MMP-9 and cerebral infarct volume. Mechanistically, ischaemia-induced elevation of A-FABP selectively in peripheral blood monocyte-derived macrophages and cerebral resident microglia promoted MMP-9 transactivation by potentiating JNK/c-Jun signalling, enhancing degradation of tight junction proteins and BBB leakage. The detrimental effects of A-FABP were prevented by pharmacological inhibition of MMP-9.
Conclusion	A-FABP is a key mediator of cerebral ischaemia injury promoting MMP-9-mediated BBB disruption. Inhibition of A-FABP is a potential strategy to improve IS outcome.
Keywords	A-FABP • Blood–brain barrier • Ischaemic stroke • JNK/c-Jun signalling • MMP-9

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Translational perspective

Blood–brain barrier (BBB) disruption after ischaemic stroke (IS) is a key event that precedes various neurological disorders while the contributing factors that regulate the process remain elusive. In this study, adipocyte fatty acid-binding protein (A-FABP) is identified as a novel regulator of BBB disruption because it increases the expression and activity of matrix metalloproteinase-9 (MMP-9). Treatment with a selective A-FABP inhibitor alleviated cerebral ischaemic injury and improved functional outcome in mice. Since circulating A-FABP is increased and correlates positively with the MMP-9 level and infarct volume in IS patients, these findings imply that inhibition of A-FABP is a potential strategy for treating such patients.

Introduction

Stroke is a major worldwide cause of permanent disability and death, whereby ischaemic stroke (IS), accounts for more than 80% of its incidence.[1–3] Tissue plasminogen activator is the only current pharmacological treatment for acute IS approved by the USA Food and Drug Administration. However, its administration may lead to bleeding and the treatment window is short.[4] Hence, development of other effective pharmacological therapies is in need to improve outcome in patients with IS.

The blood–brain barrier (BBB) is a selective permeable membrane composed of endothelial cells, extracellular matrix components of the basement membrane, pericytes, and astrocyte end-feet.[5] The tight junctions between endothelial cells act as gatekeepers to restrict blood–borne substances from entering the brain, thus maintaining cerebral homeostasis.[5] Disruption of the BBB is a key event following IS worsening the outcome. Indeed, a compromised BBB integrity leads to increased paracellular permeability allowing the passage of toxins, various immune cells, and inflammatory factors into the brain. These result in increased cerebral vasogenic oedema, haemorrhagic transformation, and mortality.[6] In such BBB disruption, matrix metalloproteinases (MMPs; belonging to a family of zinc and calcium-dependent proteolytic enzymes), in particular the gelatinases MMP-2 and MMP-9 play a key role by degrading tight junction (TJ) and basal lamina proteins.[7] However, the principal mediators and risk factors that initiate and/or contribute to IS-mediated BBB breakdown remain to be defined.

Adipocyte fatty acid-binding protein (A-FABP, also known as FABP4 and ap2) is an adipokine expressed mainly in adipocytes, macrophages, and endothelial cells.[8] It has been originally described as a lipid chaperone transporting free fatty acids between cell compartments and in the circulation,[9] but later identified as a critical mediator of metabolic dysfunction due to its pro-inflammatory effects.[8,10,11] Several clinical studies suggest its involvement in the pathogenesis/progression of IS. Thus, the circulating A-FABP level is elevated in obese subjects and correlates positively with the incidence of type 2 diabetes, hypertension, and dyslipidaemia,[12] which all are risk factors for the occurrence of stroke.[13] Likewise, serum A-FABP levels are elevated in patients with carotid atherosclerosis which can cause cerebral embolization and symptoms of cerebral ischaemia thus causing transient ischaemic attacks and stroke.[14] Increased circulating A-FABP levels are also associated with higher levels of high-sensitivity C-reactive protein which are closely correlated with the severity of acute IS.[15] In addition, an elevated circulating A-FABP level is a potential biomarker of arterial stiffness which is associated with the onset, incidence and the clinical outcome of stroke.[16–18] Thus, not surprisingly, serum levels of A-FABP are actually increased in patients with IS and correlate positively with early death[19] and adverse functional outcome.[20] Thus, A-FABP can be regarded as an independent prognostic biomarker in patients with stroke. However, its exact pathophysiological role in the evolution of IS and the molecular mechanisms underlying its deleterious effects have not been explored so far.

In the present study, A-FABP knockout (A-FABP KO) mice and their wild-type (WT) littermates were subjected to middle cerebral artery occlusion (MCAO) to investigate whether or not A-FABP contributes actively, rather than only monitors (functioning as a biomarker only), at least in part, to the deleterious consequences of BBB disruption after IS and, to determine the plausible underlying mechanisms involved. The therapeutic potential of a selective A-FABP inhibitor, BMS309403 on the disease was determined also. Finally, the clinical implications of such contribution of A-FABP to the evolution of IS was evaluated in patients.

Methods

All experimental protocols were approved by the Committee on the Use of Live Animals in Teaching and Research at the University of Hong Kong. Ethics committee approval from the First Affiliated Hospital of Anhui Medical University and written informed consents from all patients and control subjects were obtained. For methods and materials of other experiments, please refer to Supplementary material online, *Materials and Methods*.

Results

Acute ischaemic stroke up-regulates circulating and cerebral levels of A-FABP in mice

To explore the role of A-FABP in IS, dynamic changes in circulating A-FABP levels and its expression in the brain were determined in C57BL/6N mice after MCAO surgery. The serum level of A-FABP was elevated 2 h after MCAO, reached a peak after 48 h and gradually returned to baseline after 72 h (*Figure 1A*). The mRNA and protein levels of A-FABP were induced significantly in the ischaemic brain 2 h after stroke and remained high for at least 72 h (*Figure 1B* and *C*). To determine the cellular source of A-FABP contributing to its elevation in the circulation, various immune cells were sorted from whole blood by flow cytometry (Supplementary material online, *Figures S1* and *S2*). The mRNA levels of *A-FABP* were increased significantly in peripheral monocytes/macrophages (CD45^{high}CD11b$^+$F4/80$^+$) after MCAO while those in other sorted cells were not altered significantly (Supplementary material online, *Figure S3A*). Peripheral blood
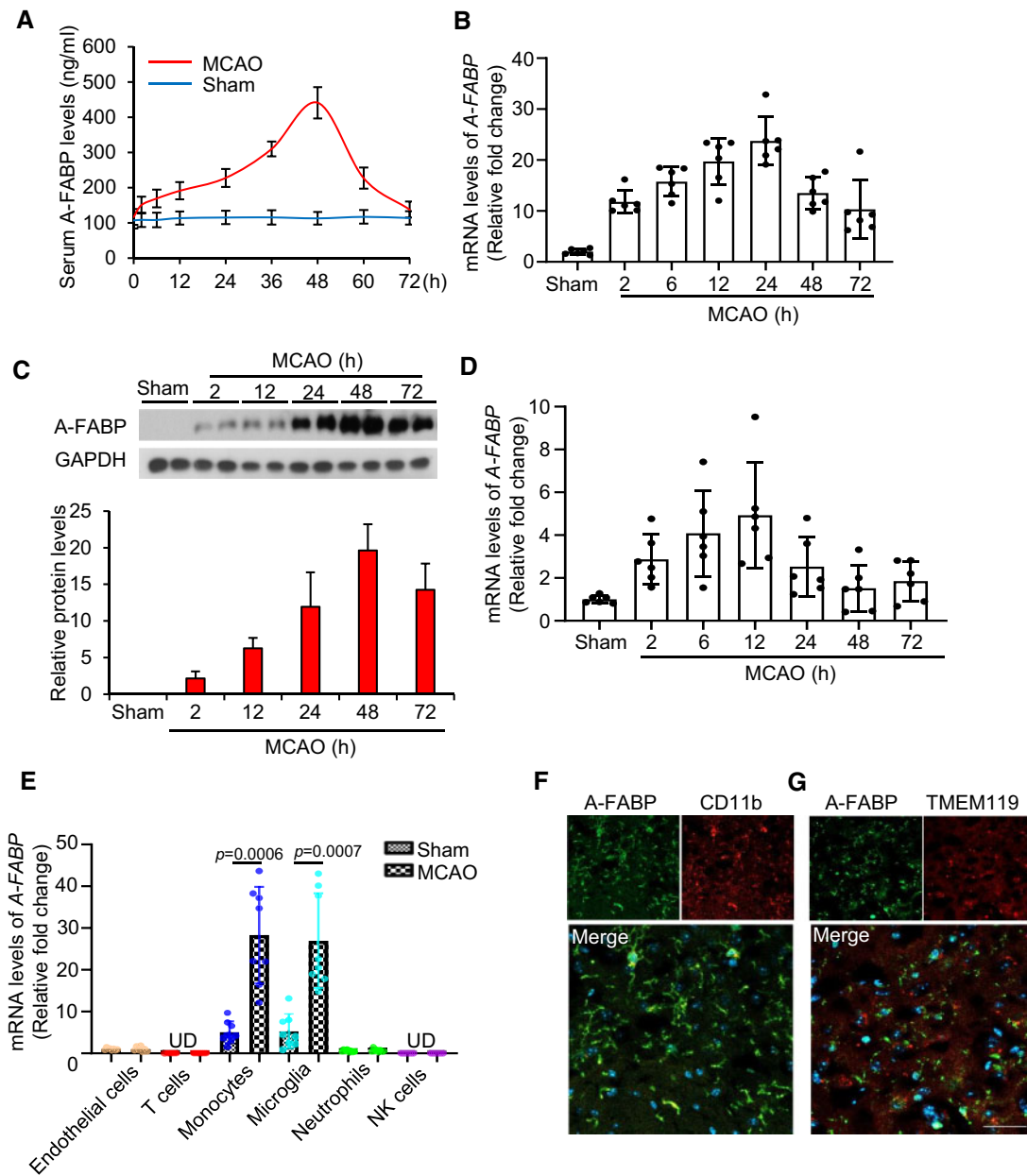
Figure 1 Circulating and cerebral A-FABP are increased in mice after acute ischaemic stroke. Eight-week-old male C57BL/6N mice were subjected to MCAO or sham operation for 1 h followed by 2-72 h reperfusion. (A) Serum A-FABP levels ($n = 9$). (B) The mRNA abundance of *A-FABP* in the ischaemic ipsilateral cortex of brain normalized to the *GAPDH* gene ($n = 9$). (C) Representative immunoblots for A-FABP of the ischaemic ipsilateral cortex of brain at various time points after MCAO and the same cortex region of the mice 24 h after sham operation and the band intensities relative to GAPDH ($n = 6$). (D and E) The mRNA abundance of *A-FABP* in peripheral blood monocytes ($n = 6$) (D) and in various inflammatory cells and endothelial cells isolated from the brain ($n = 8$) (E) of mice 24 h after MCAO normalized to the *GAPDH* gene. (F and G) Representative images of immunofluorescence co-staining of A-FABP with CD11b (F) or TMEM119 (G) in the ischaemic ipsilateral cortex of brain 24 h after MCAO. Scale bar: 50 μm. Data are presented as means ± SD. UD, undetectable.

monocytes were further isolated after MCAO using monocyte isolation kit. The *A-FABP* mRNA levels in peripheral blood monocytes showed a similar induction pattern as the serum A-FABP level (*Figure 1D*). No significant change was observed in the *A-FABP* mRNA levels in subcutaneous and epididymal fat (Supplementary material online, *Figure S3B* and *C*). Next, various cells were sorted from the

brain of mice after MCAO to elucidate the cellular source of cerebral A-FABP (Supplementary material online, *Figures S1*, *S4*, and *S5*). The mRNA levels of *A-FABP* were increased significantly in microglia (CD45^{int}CD11b$^+$Ly6G$^-$) and infiltrated monocytes and monocyte-derived macrophages (CD45^{high}CD11b$^+$F4/80$^+$) but not in the other sorted cells (*Figure 1E*). Immunofluorescence co-staining

studies and co-localization analysis showed the co-localization of A-FABP with CD11b$^+$ (marker for monocytes/macrophages and microglia) cells and TMEM119$^+$ (specific marker for microglia) cells in the ischaemic cores of brains with Pearson correlation coefficient[21] 0.868 and 0.427, respectively (*Figure 1F* and *G* and Supplementary material online, *Figure S6*) suggesting that the infiltrated monocytes and monocyte-derived macrophages and resident microglia were the major cellular sources of cerebral A-FABP in response to IS. These results indicated that under ischaemia-induced cerebral hypoxia condition, A-FABP was expressed mainly in peripheral blood monocytes and microglia, contributing to the elevated levels in the circulation and brain.

Genetic ablation of A-FABP ameliorates cerebral ischaemic injury

To determine the impact of A-FABP on cerebral ischaemic damage, 1 h of MCAO was imposed in male A-FABP KO mice and their WT littermates followed by reperfusion for 23 h or 7 days. The infarct areas of the brain of the A-FABP KO mice on Day 1 after MCAO were significantly smaller than those of the WT mice (*Figure 2A*) with the percentage of cortical infarct volume of WT vs. KO as 25.14 ± 4.01% vs. 17.60 ± 2.20% (relative to contralateral hemisphere; $P < 0.0001$) and subcortical infarct volume of WT vs. KO as 17.73 ± 1.64% vs. 9.92 ± 2.48% (relative to contralateral hemisphere; $P < 0.0001$) (data not shown). Similar trend of infarct volume was observed on Day 7 after MCAO (Supplementary material online, *Figure S7A*). Consistently, A-FABP KO mice exhibited a significant decrease in ischaemia-induced brain oedema when compared with their WT littermates on Day 1 and Day 7 after MCAO (*Figure 2B* and Supplementary material online, *Figure S7B*). Long-term neurological deficits during 7 days after MCAO were improved significantly (*Figure 2C* and *D*) and were associated with an increased survival rate (*Figure 2E*) in the A-FABP KO mice when compared with the WT mice. Neuronal apoptosis in the infarct core in the A-FABP KO mice was attenuated significantly (*Figure 2F*). Furthermore, MCAO-induced immune cell infiltration in the brain was reduced significantly in the A-FABP KO mice (Supplementary material online, *Figure S8A–E*) which was associated with decreased expression of pro-inflammatory cytokines (Supplementary material online, *Figure S8F*) when compared with the WT mice. The protective effects of A-FABP deficiency on acute IS-induced infarct area, cerebral oedema, neurological dysfunction, and survival rate were also observed in female mice (Supplementary material online, *Figure S9*) with findings comparable to those in male animals. These data suggest that elevation of A-FABP induced by IS promotes cerebral injury and poor functional outcome.

Monocytes and microglia-derived A-FABP exerts a detrimental effect on ischaemic stroke

Next, the detrimental effect of A-FABP derived from peripheral monocytes and microglia (macrophages) in stroke outcome were elucidated by bone marrow transplantation (BMT) experiments between WT and A-FABP KO mice. Bone marrow (BM) from either A-FABP KO or WT donor mice was transplanted into 6-week-old recipient WT or A-FABP KO mice (Supplementary material online, *Figure S10A*). The four groups of recipient mice (Supplementary material online, *Figure S10B*) were subjected to MCAO followed by 7 days of reperfusion. WT mice receiving A-FABP deficient BM exhibited reduced expression of cerebral and circulating A-FABP and an alleviated stroke outcome when compared with the WT mice receiving WT-BM (Supplementary material online, *Figure S10C–I*). In contrast, A-FABP KO mice receiving WT-BM exhibited elevated cerebral and circulating A-FABP and a worse stroke outcome when compared with the A-FABP KO mice receiving A-FABP KO-BM (Supplementary material online, *Figure S10C–I*). These findings suggest that bone marrow-derived macrophages (BMDMs) expressing A-FABP play essential role in mediating stroke outcome. Furthermore, WT mice receiving A-FABP KO-BM exhibited similar stroke outcome as that of A-FABP KO mice receiving WT-BM indicating that A-FABP expressing-microglia and -BMDMs contribute similarly to the IS outcome (Supplementary material online, *Figure S10E–I*). Thus, these data demonstrate that the detrimental effects of A-FABP on IS injury and the resulting neurological deficits occur largely through its effects on microglia and BMDMs.

Pharmacological inhibition of A-FABP attenuates cerebral ischaemic injury in mice

Next, the effect of pharmacological inhibition of A-FABP on IS outcome was investigated. C57BL/6N mice were subjected to MCAO followed by treatment with the selective A-FABP inhibitor, BMS309403 (BMS)[22] or vehicle (Veh). BMS significantly decreased ischaemia-induced infarct areas on Day 1 after reperfusion (Supplementary material online, *Figure S11A*) with percentage of cortical infarct volume of Veh vs. BMS as 22.18 ± 2.70% vs. 14.3 ± 1.66% (relative to contralateral hemisphere; $p < 0.0001$) and subcortical infarct volume of Veh vs. BMS as 18.30 ± 2.22% vs. 10.11 ± 2.83% (relative to contralateral hemisphere; $P < 0.0001$) (data not shown). Similar trend in infract area was observed on Day 7 (Supplementary material online, *Figure S12A*). BMS also significantly reduced brain oedema, neurological deficits, and numbers of apoptotic neurons, but increased the survival rate of mice subjected to MCAO followed by 1-day or 7-day reperfusion when compared with those treated with vehicle (Supplementary material online, *Figures S11B–F* and *S12B*), suggesting that pharmacological inhibition of A-FABP possesses a long-term protective effect on IS outcome. Furthermore, A-FABP deficiency and its pharmacological inhibition attenuated ischaemia-induced intracerebral haemorrhage in mice (Supplementary material online, *Figure S13*).

A-FABP promotes ischaemia-induced blood–brain barrier disruption

The BBB disruption after IS is a key event that precedes various neurological disorders.[23] Ischaemia-induced BBB leakage decreased significantly in A-FABP KO mice as indicated by the reduced permeability to Evans blue dye and attenuated IgG extravasation when compared with WT controls (*Figure 3A* and *B*). Ischaemia also led to a significantly decreased expression of the major TJ membrane
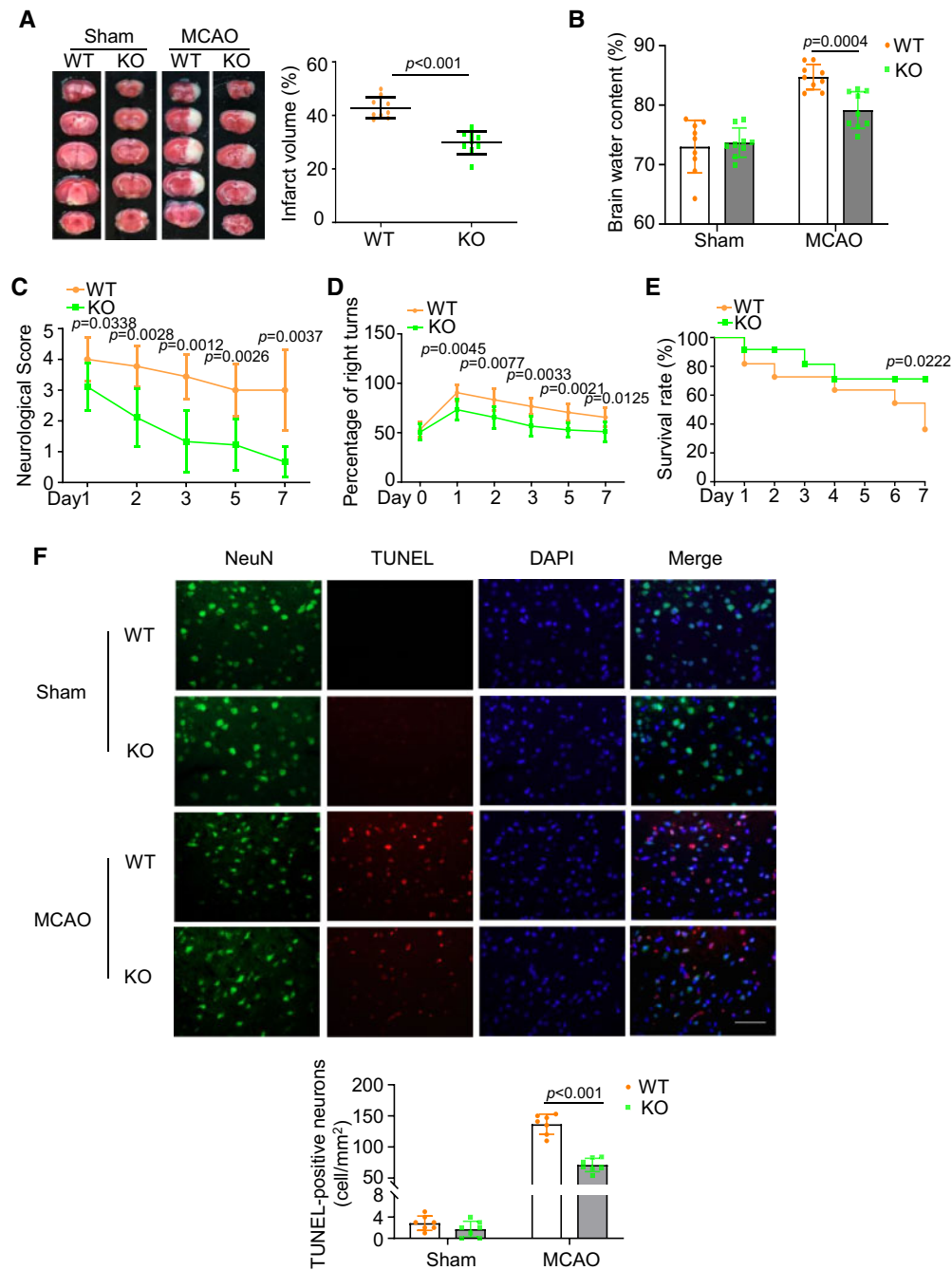
Figure 2 A-FABP deficiency protects mice against cerebral ischaemia injury. Eight-week-old male A-FABP KO mice and their WT littermates were subjected to MCAO or sham operation for 1 h, followed by reperfusion for 23 h to 7 days. (A) Representative photographs of coronal brain sections of mice stained with TTC 24 h after MCAO or sham operation and the relative infarct volume (n = 9). (B) Percentage of brain water content (n = 9). (C–E) Neurological score (C), percentage of right turns in the Corner turning test (D), and survival rate (E) of mice during the 7 days after MCAO (n = 9). (F) Representative images of TUNEL staining with immuno-costaining with the neuronal marker NeuN in the ipsilateral cortex of brain of mice 24 h after MCAO or sham operation and the quantification of TUNEL positive neurons (n = 7). Scale bar: 50 μm. Data are presented as means ± SD.

proteins occludin and Zona occludens-1 (ZO-1), which interact with each other to maintain BBB integrity,[24] in both mouse genotypes, whereby, however, WT controls exhibited a more severe reduction when compared with A-FABP KO mice (*Figure 3C*). Similarly, post-surgery treatment with BMS alleviated MCAO-induced BBB leakage (*Figure 3D–F*). These results suggest that genetic ablation or pharmacological inhibition of A-FABP attenuates ischaemia-induced BBB disruption.
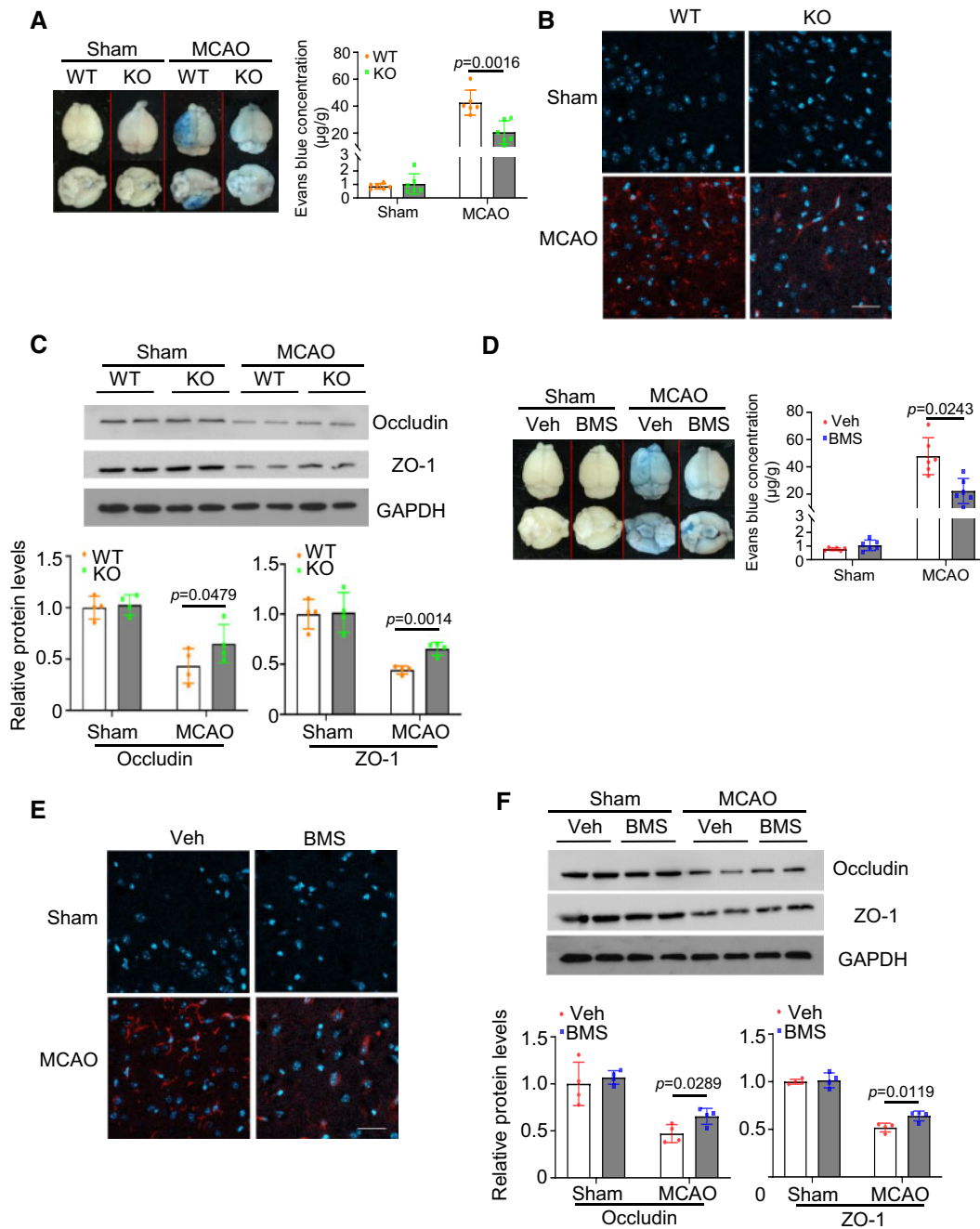
Figure 3 A-FABP deficiency alleviates ischaemia-induced blood–brain barrier disruption. Eight-week-old male A-FABP KO mice and their WT littermates were subjected to MCAO for 1 h, followed by 23 h of reperfusion. Another group of 8-week-old male C57BL/6N mice subjected to MCAO were treated with vehicle or BMS309403 (BMS) 1- or 12-h post-surgery and then every 24 h for the following 6 days. (*A*) Representative photographs of the brain of mice stained with Evans Blue 24 h after sham operation or MCAO (*n* = 6). (*B*) Representative immune-microscopic images of IgG extravasation in the cortex of mice [red: IgG; blue: 4′,6-diamidino-2-phenylindole (DAPI); scale bar = 50 μm]. (*C*) Representative immunoblots of TJ proteins in the brains of mice and the band intensity of each protein relative to GAPDH. (*D*) Representative photographs of Evans Blue-stained brains of MCAO-subjected C57BL/6N mice treated with BMS or vehicle (*n* = 6). (*E*) Representative immuno-microscopic images of IgG extravasation in the cortex of vehicle- or BMS-treated mice (red: IgG; blue: DAPI; scale bar = 50 μm). (*F*) Representative immunoblots of TJ proteins in the brains of mice treated with vehicle or BMS after sham operation or MCAO and the band intensity of each protein relative to GAPDH. Data are presented as means ± SD.

A-FABP deficiency abolishes MCAO-induced up-regulation of MMP-9 in mice

MMPs play key roles in the disruption of BBB through degradation of extracellular matrix and TJ proteins.[7] MCAO induced a significant elevation of cerebral *MMP-2*, *MMP-3*, and *MMP-12* mRNA expressions, to a similar extent (~four-fold increase), in both WT and A-FABP KO mice (*Figure 4A*). In contrast, MCAO significantly increased (by ~nine-fold) the cerebral expression of *MMP-9* in WT mice, whereas in A-FABP KO mice such increase in expression was suppressed (*Figure 4A*), indicating that A-FABP is an important modulator of cerebral *MMP-9* expression. MCAO-induced protein presence and enzymatic activity of cerebral MMP-9 were also decreased significantly in A-FABP KO mice (*Figure 4B–C*) when compared with WT littermates. An increase of total serum MMP-9 levels was observed in both mouse genotypes after MCAO but that in A-FABP KO mice was significantly lower (*Figure 4D*). Consistently, treatment with BMS suppressed MCAO-induced MMP-9 expression and its enzymatic activity (Supplementary material online, *Figure S14*). These data suggest that ischaemia-induced A-FABP elevations are associated with increased presence of MMP-9 in the brain and the circulation.

Circulating A-FABP is positively correlated with MMP-9 levels and infarct volume in patients with ischaemic stroke

To explore the clinical relevance of the relationship between A-FABP and MMP-9 in acute IS, serum concentrations of MMP-9's active form and A-FABP were determined in patients within 24 h of stroke onset. Serum active MMP-9 and A-FABP levels were elevated significantly in patients after acute stroke when compared with control subjects (*Figure 4E* and *F*). Moreover, the serum levels of active MMP-9 and A-FABP were positively correlated with each other, further supporting that A-FABP is a positive regulator of MMP-9 expression in patients with IS (*Figure 4G*). The serum A-FABP level was also positively correlated with cerebral infarct volume of stroke patient which suggested that the adipokine modulates the outcome of IS (*Figure 4H*).

MMP-9 confers the deleterious effects of A-FABP on cerebral ischaemia injury

To determine if the detrimental effects of A-FABP on IS are mediated by induction of MMP-9, A-FABP KO mice were subjected to cerebral injection of adenovirus over-expressing A-FABP (Ad-A-FABP) or luciferase (Ad-Luci), followed by MCAO and subsequent treatment with the MMP-9 inhibitor SB-3CT or vehicle. The adenovirus-mediated A-FABP overexpression in the brain of A-FABP KO mice was confirmed and the expression level of A-FABP was comparable to that observed in MCAO-subjected WT mice injected with Ad-Luci (Supplementary material online, *Figure S15*). The significantly enhanced MCAO-induced MMP-9 activity in the A-FABP KO mice with adenovirus-mediated A-FABP overexpression, was suppressed by treatment with SB-3CT (*Figure 5A*). Consistently, A-FABP-mediated induction of MMP-9 activity was accompanied by increased infarct volume, cerebral oedema, neurological deficits, reduced survival rate, and increased BBB permeability in vehicle-treated KO mice and these deleterious effects were alleviated significantly by SB-3CT (*Figure 5B–G*). These data indicating the adverse effects of A-FABP on

IS are mediated by induction of MMP-9, supporting the notion that the protective effect of A-FABP deficiency on BBB disruption is largely attributable to the attenuated MMP-9 expression.

A-FABP promotes MMP-9 expression through JNK/c-Jun signalling

The molecular mechanism whereby A-FABP promotes the expression of MMP-9 was explored next. A-FABP is part of a finely tuned positive feedback loop with c-Jun NH2-terminal Kinase (JNK) and activator protein-1 (AP-1) to exacerbate lipopolysaccharide (LPS)-induced inflammatory responses in macrophages.[25] In addition, AP-1 regulates the transactivation of murine *MMP-9*.[26] Thus, the possibility was explored that A-FABP induces MMP-9 expression during IS by activating the JNK/c-Jun signalling pathway. MCAO-induced expression of A-FABP and MMP-9 in the brain (*Figure 1C* and *4B*) was associated with a significant enhanced phosphorylation of JNK (Thr183/Tyr185) and c-Jun (Ser63/73) in both WT and A-FABP KO mice although the magnitude of phosphorylation was significantly lower in the latter (Supplementary material online, *Figure S16A*). Furthermore, BMDMs isolated from WT and A-FABP KO mice were subjected to 1 h of oxygen and glucose deprivation (OGD), a condition mimicking IS. OGD significantly induced the expression of A-FABP and MMP-9 in WT-BMDMs (Supplementary material online, *Figure S16B* and *C*). Such induction was accompanied by robust activation of JNK/c-Jun signalling (Supplementary material online, *Figure S16B*). However, the OGD-induced expression of MMP-9 and activation of JNK/c-Jun were attenuated significantly in A-FABP-KO BMDMs (Supplementary material online, *Figure S16B*). To elucidate if A-FABP enhances the expression of MMP-9 by potentiating JNK/c-Jun signalling, MMP-9 expression and JNK/c-Jun activation were examined in A-FABP-KO BMDMs infected with Ad-A-FABP or Ad-Luci for 3 days, followed by pre-treatment with either vehicle or the JNK inhibitor SP600125 (40 μM)[27] before subjecting the cells to OGD. The inhibition of JNK signalling significantly impaired the A-FABP-induced MMP-9 expression (Supplementary material online, *Figure S16D* and *E*). Previous studies identified two transcriptional cis-acting elements of AP-1 (-31/-24 bp and -467/-460 bp) within the promoter region of the murine *MMP-9* gene.[26] A chromatin immunoprecipitation (ChIP) assay, using specific primers spanning these two AP-1 sites, showed that A-FABP deficiency significantly decreased the binding between p-c-Jun and AP-1 sites during ischaemia (Supplementary material online, *Figure S16F*).

To further assess if *MMP-9* expression is regulated directly by A-FABP/JNK/c-Jun signalling, luciferase-reporter constructs containing a murine *MMP-9* promoter fragment spanning from -1614 to +38 (WT), or promoters in which either one (Ap-1a or Ap-1b) or both (AP-1a and 1b) core motives of AP-1 sites were mutated (TC to AA)[26] (Supplementary material online, *Figure S17A*) were transfected into HEK293 cells with or without adenovirus-mediated A-FABP overexpression. The reporter activity of the WT-construct increased approximately five-fold by overexpression of A-FABP. However, such induction was prevented by treatment with SP600125 (Supplementary material online, *Figure S17B*). Furthermore, mutation at either one of the AP-1 site (Ap-1a or Ap-1b) resulted in a significant loss of the response of the HEK293 cells to A-FABP over-expression when compared with that of the WT construct.
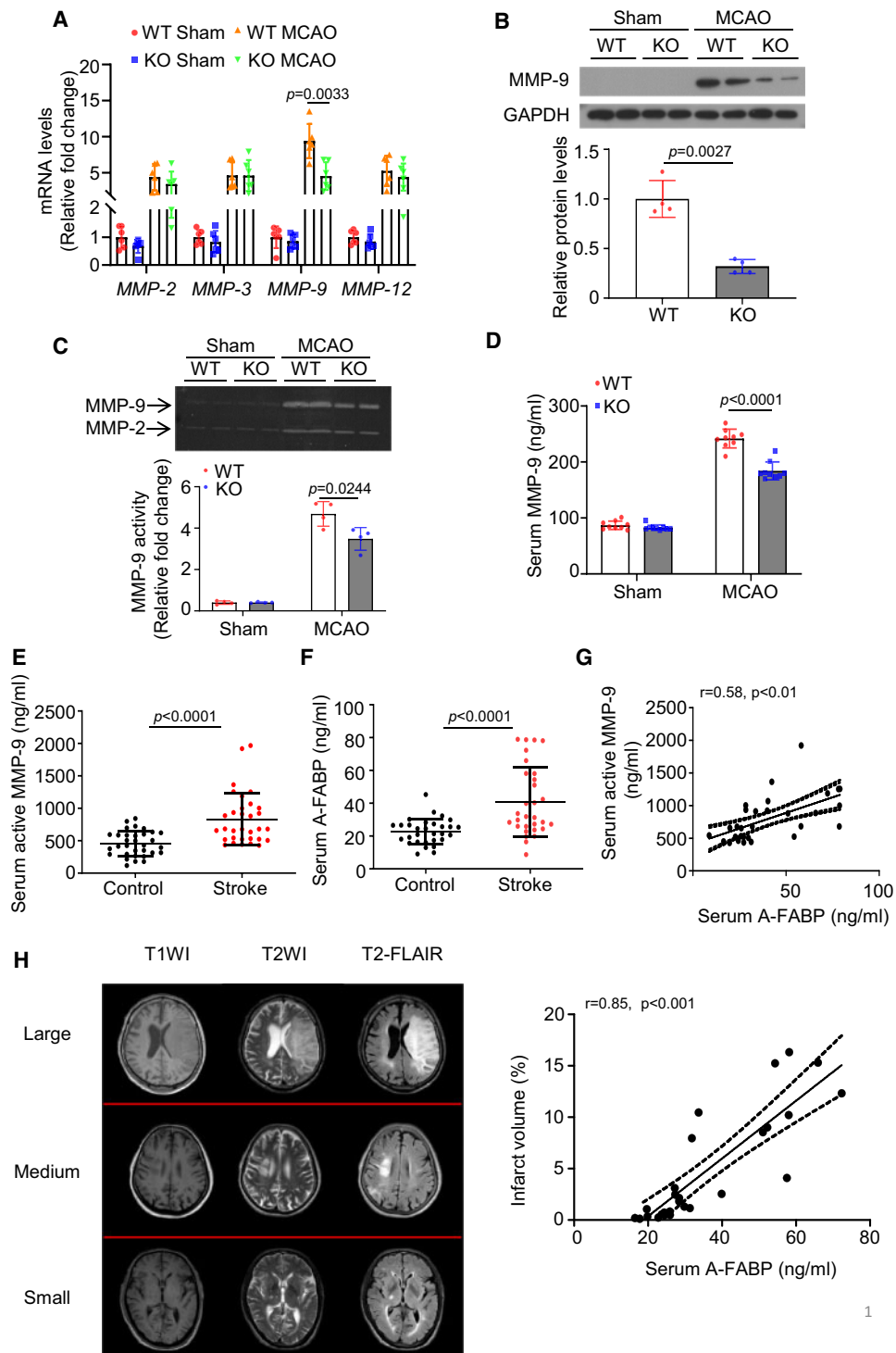
Figure 4 A-FABP modulates the expression of MMP-9 in response to ischaemic stroke. Eight-week-old male A-FABP KO mice and their WT littermates were subjected to sham operation or MCAO for 1 h, followed by 23 h of reperfusion. In the clinical study, sera of IS patients were collected within 24 h of stroke onset and compared with those of the control subjects. (*A*) The mRNA abundance of *MMPs* related to BBB disruption in brain of mice after MCAO normalized to the *GAPDH* gene. (*B*) Representative immunoblots of MMP-9 in the infarct cortex of mice after MCAO and the band intensity of MMP-9 relative to GAPDH. (*C*) MMP-9 activity in brain of mice after MCAO and the relative fold changes in MMP-9 activity accepting the control value as 1. (*D*) Serum levels of MMP-9 of mice. (*E* and *F*) Serum levels of the active form of MMP-9 (*E*) and A-FABP (*F*) in either patients or control subjects; *n* = 30 per group. (*G*) Correlation between serum A-FABP and the active form of MMP-9 in patients analysed by Pearson's correlation test; *n* = 30. (*H*) Classification of brain MRI images in IS patients based on the diameter of the infarct zone and the correlation between serum A-FABP levels and percentages of infarct volume; *n* = 30. T1WI, T1-weighted imaging; T2WI, T2-weighted imaging; T2-FLAIR, T2-weighted-fluid-attenuated inversion recovery. Data are presented as means ± SD.
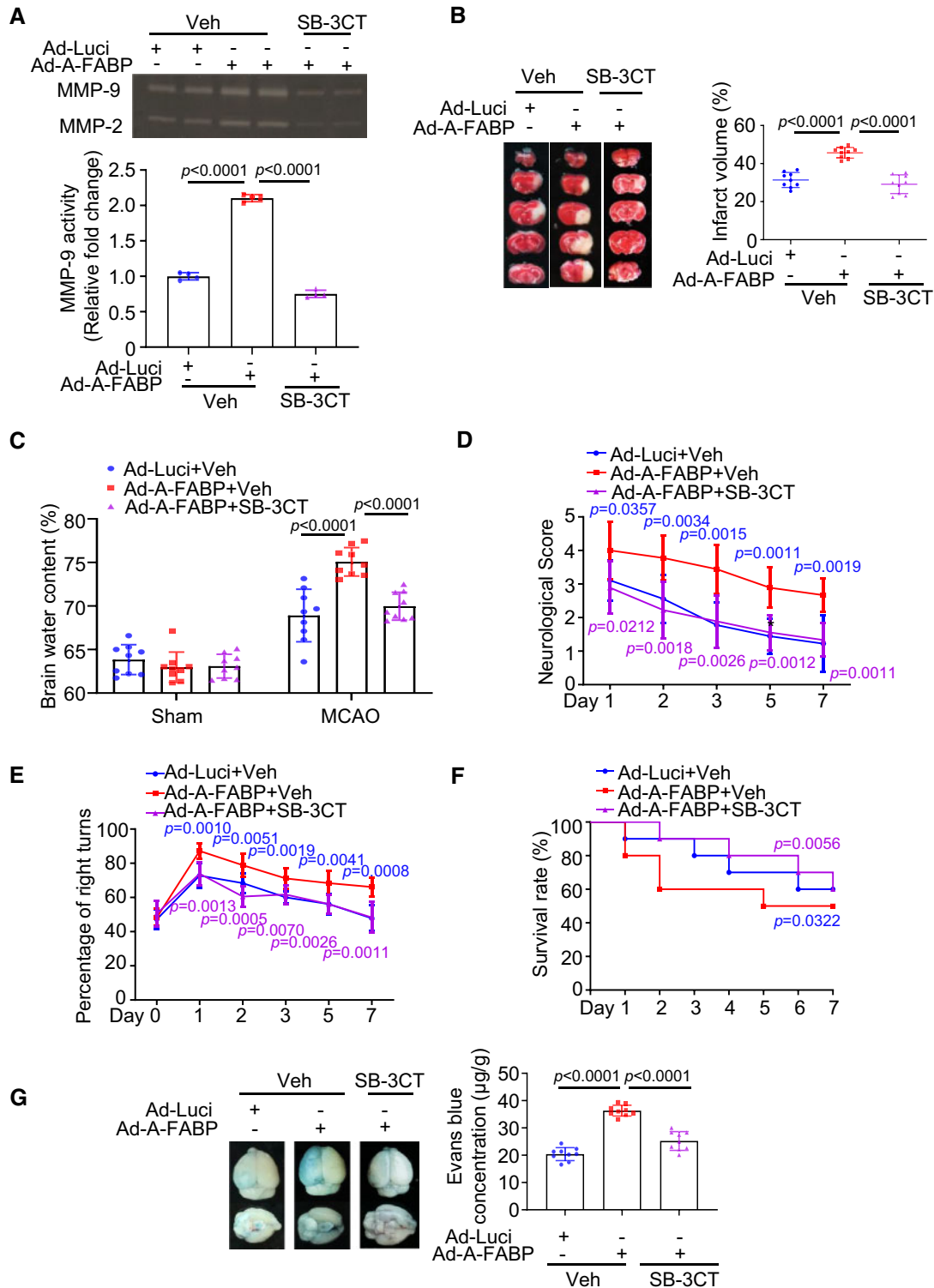
Figure 5 MMP-9 mediates the detrimental effect of A-FABP in cerebral ischaemia injury. Eight-week-old male A-FABP KO mice were injected with Ad-A-FABP or Ad-Luci in the lateral ventricles and caudate nucleus 3 days before MCAO. Mice were injected with SB-3CT or vehicle (Veh; 10% DMSO in Saline) after MCAO. (A) Activity of MMP-9 in the brain of the mice 24 h after MCAO and the relative fold changes in MMP-9 activity accepting the control value as 1. (B) Representative photographs of coronal brain sections stained with TTC 24 h after MCAO and the percentages of infarct volume (n = 9). (C) Percentage of brain water content of mice (n = 9). (D–F) Neurological score (D), percentage of right turns in the Corner turning test (E) and survival rate (F) of KO mice treated with Ad-Luci+Veh, Ad-A-FABP+Veh, and Ad-A-FABP+SB-3CT after MCAO (n = 9). (G) Representative photographs of, and quantification in brains stained with Evans Blue of mice treated with Ad-luci+Veh, Ad-A-FABP+Veh, and Ad-A-FABP+SB-3CT after MCAO. Data are presented as means ± SD.

Furthermore, mutation of both AP-1 sites (AP-1a and 1b) abolished A-FABP-mediated activation of the *MMP-9* promoters (Supplementary material online, *Figure S17C*), suggesting that the two AP-1 sites are essential for the induction of *MMP-9* by A-FABP. These data indicate that A-FABP enhances JNK/c-Jun activity and promotes the binding between p-c-Jun and AP-1 sites within the promoter of *MMP-9*, triggering the transactivation of *MMP-9* during hypoxia.

Discussion

Previous studies relating A-FABP and IS were clinical investigations showing a close association between circulating A-FABP with severity, poor functional outcome, and mortality of cerebral IS, but permitted no conclusion as to the pathological participation of the adipokine in the deleterious consequences of brain hypoxia.[19,20,28] In the present experiments, evidence from both genetic ablation and pharmacological inhibition in animals together with the support from further studies in humans demonstrate, for the first time, that A-FABP is actually pathogenically involved in the worsening of IS outcome.

Indeed, the present study reveals a rapid and significant elevation of A-FABP in the circulation and in the brain of mice exposed to acute IS. Microglia and blood monocytes were identified as the major cellular source of A-FABP and demonstrated to induce MMP-9 expression contributing to a poor stroke outcome. IS leading to insufficient supply of oxygen to the brain induces the expression of hypoxia-inducible factor 1 alpha (HIF-1α).[29] Although the present experiments were not designed to unravel the mechanism(s) underlying the increased presence/expression of A-FABP after IS, it seems logical to attribute it to such HIF-1α induction. Indeed, the expression of A-FABP in ischaemia/reperfusion (I/R)-induced hepatic injury is upregulated by HIF-1α.[30] Rapidly increased levels of A-FABP have been observed in the circulation, heart or liver of mice subjected to either cardiac or hepatic I/R injury,[30,31] respectively. Overexpression of A-FABP sensitizes mice to hepatic I/R injury,[30] while A-FABP deficiency protects them against cardiac I/R injury.[31] These previous and the present data indicate that A-FABP is a stress-induced protein immediately responding to ischaemia and mediating the detrimental effects of the latter.
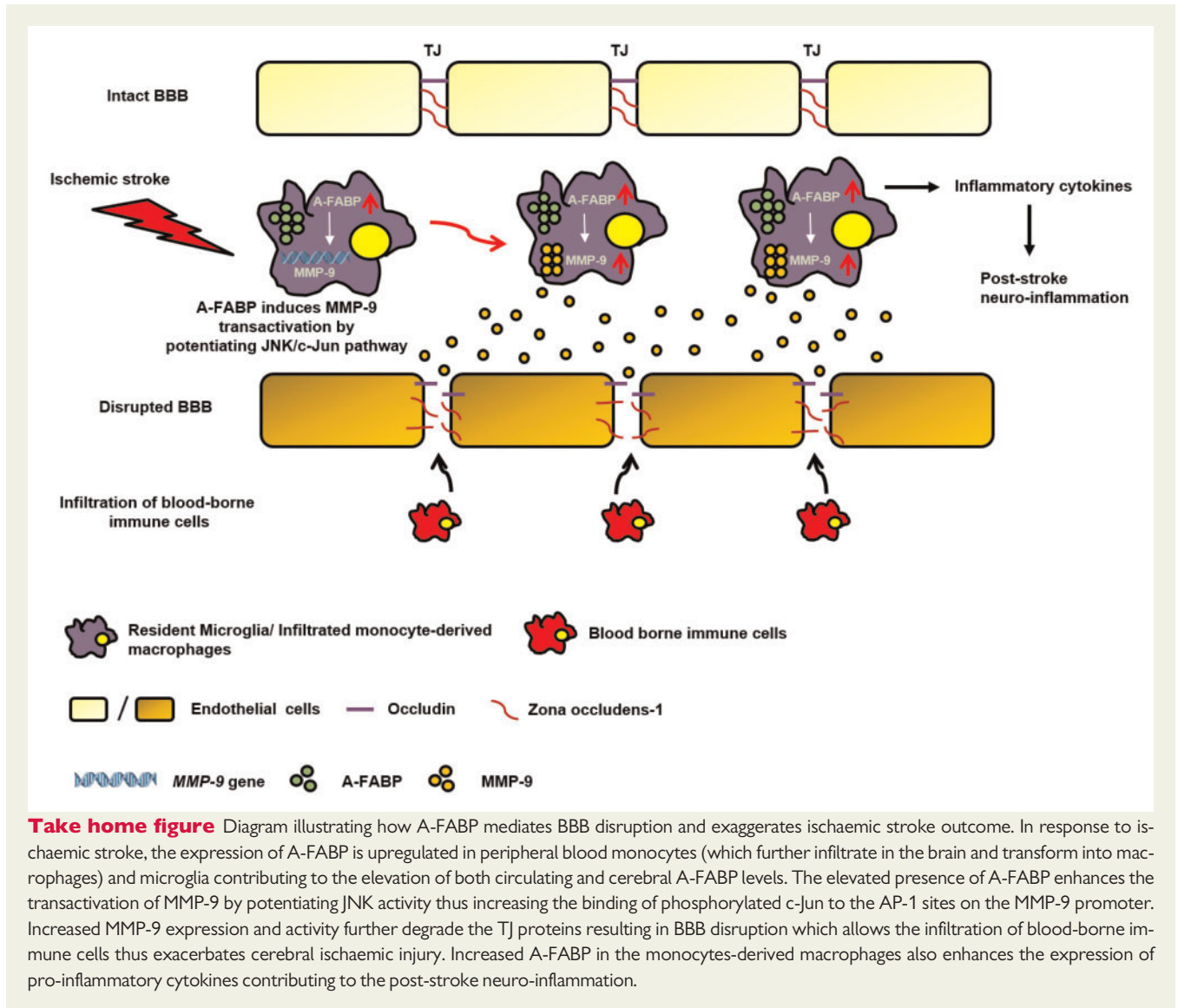
Microglia, the resident macrophages in brain, are activated several minutes after IS and this activation peaks after 2–3 days.[32] Activated microglia exert deleterious effects on the acute phase of IS by releasing cytotoxic factors such as reactive oxygen species (ROS), tumour necrosis factor-alpha (TNF-α), and interleukin 1-beta (IL-1β) leading to BBB disruption and the subsequent recruitment, within 1 day after the stroke, of blood–borne immune cells (including neutrophils, monocytes, and lymphocytes) to the sites of injury; the resulting infiltration is sustained at least for 7 days and underlies the post-stroke inflammation.[33] This evidence supports that the rapidly increased A-FABP expression/presence in microglia contributes to BBB disruption during the acute phase of IS causing cerebral oedema and neuroinflammation. Conversely, it also explains the reduced infiltration of immune cells in the brain of A-FABP KO mice after IS.

Post-ischaemic inflammation plays a key role in the progression of cerebral ischaemia–reperfusion injury.[32] Among the immune cells infiltrating the brain after BBB disruption, monocytes, in particular, differentiate into macrophages and exacerbate inflammation by producing pro-inflammatory cytokines which lead to further brain damage, and result in poor neurological and functional outcome.[34] Monocyte infiltration occurs 4 h after stroke and reaches maximal infiltration within 7 days.[35] In the present murine model of IS, an elevated expression of A-FABP was detected in peripheral blood monocytes. The induction of cerebral A-FABP after IS was mainly attributable to activated resident microglia and infiltrated monocyte-derived macrophages expressing the adipokine. Furthermore, there was no alternation in the expression of A-FABP in peripheral adipose tissues, which are supposed to be the key contributors to circulating A-FABP,[12] after IS. This evidence implied that peripheral monocyte (probably due to the increased influx after BBB disruption) is one of the key cellular origin of cerebral A-FABP after IS. Taken together with the results of BMT experiments and those obtained after A-FABP expression in sorted immune cells from the brain and peripheral blood, it seems reasonable to conclude that microglia and monocyte-derived macrophages expressing A-FABP are the key pathogenic cells exerting local effects contributing to severe stroke outcome.

Cerebral MMP-9 is elevated and dominant in the acute phase after IS and mediates hypoxia-induced cerebral vascular leakage by degrading the TJ proteins occludin and ZO-1.[36] Serum levels of MMP-9 correlate with infarct size, neurological deterioration and mortality.[37,38] MMP-9-deficient mice are protected from both transient and permanent MCAO-induced cerebral ischaemia,[39] as observed in the A-FABP KO mice. The present *in vivo* findings demonstrate that suppression of MMP-9 activity with SB-3CT abolishes the adverse effects of A-FABP overexpression on cerebral ischaemia. They, thus, imply that ischaemia-induced A-FABP is a key regulator which selectively enhances the transactivation of MMP-9 in macrophages (microglia and peripheral monocytes) by potentiating JNK/c-Jun activation resulting in BBB breakdown. Indeed, A-FABP deficiency attenuated the activation of JNK/c-Jun associated with a decreased MMP-9 expression both *in vivo* and *in vitro*. The A-FABP-mediated increase in transcription and expression of MMP-9 were reduced by treatment with the SP600125. These observations are supported by the findings showing that A-FABP is required for the full activation of JNK-signalling in macrophages[25] and that JNK mediates the expression of MMP-9 in murine macrophages.[40] JNK is a shared pathway linking neuronal apoptosis, neuroinflammation, and BBB disruption in IS.[41,42] In the mouse, pharmacological inhibition of JNK activation with SP600125 attenuates ischaemia-induced neuronal apoptosis, neuroinflammation, and BBB leakage.[29] The interaction of A-FABP with the JNK pathway probably is a direct one. However, ischaemia-induced oxidative stress[5] and cytokines such as TNF-α and IL-1β[43] also induce MMP-9 expression after IS. Over-expression of A-FABP, associated with an increased ROS production, induces the expressions of MMP-2 and MMP-9 in arterial smooth muscle cells.[44] In the present experiments, A-FABP induced the expression of inflammatory cytokines in the ischaemic brain. Thus, elevated A-FABP may also exaggerate MMP-9 expression indirectly by enhancing ROS and cytokine production.

In the mouse, treatment with the selective A-FABP inhibitor BMS309403 suppresses LPS-induced activation of JNK/AP-1 and A-FABP expression.[25] Logically, the present *in vivo* findings demonstrate

Take home figure Diagram illustrating how A-FABP mediates BBB disruption and exaggerates ischaemic stroke outcome. In response to ischaemic stroke, the expression of A-FABP is upregulated in peripheral blood monocytes (which further infiltrate in the brain and transform into macrophages) and microglia contributing to the elevation of both circulating and cerebral A-FABP levels. The elevated presence of A-FABP enhances the transactivation of MMP-9 by potentiating JNK activity thus increasing the binding of phosphorylated c-Jun to the AP-1 sites on the MMP-9 promoter. Increased MMP-9 expression and activity further degrade the TJ proteins resulting in BBB disruption which allows the infiltration of blood-borne immune cells thus exacerbates cerebral ischaemic injury. Increased A-FABP in the monocytes-derived macrophages also enhances the expression of pro-inflammatory cytokines contributing to the post-stroke neuro-inflammation.

that post-administration, after MCAO-surgery, of the BMS309403 improves the outcome of IS by attenuating MMP-9 expression and curtailing BBB disruption. Therefore, in the brain, microglia and monocytes-derived macrophages expressing A-FABP, on the one hand, increase MMP-9 transactivation to exaggerate BBB disruption while, on the other hand, the adipokine is a key mediator of the inflammatory response in macrophages by forming a positive feedback loop with JNK/AP-1 signalling[25] and modulates cytokine production[45,46] contributing to post-stroke neuroinflammation.

In line with the animal findings, the clinical findings demonstrate in patients that circulating active MMP-9 levels correlate with those of circulating A-FABP, and that within 24 h of stroke onset a positive correlation is obvious between circulating levels of A-FABP and infarct volume, which further supports the pathological role of A-FABP and its underlying mechanism in mediating BBB disruption and exaggerating cerebral ischaemic injury. Combined with the observations that the circulating levels of A-FABP are elevated in obese subjects and type 2 diabetic patients[12] and that the expression of A-FABP in

microglia contributes to obesity-induced neuro-inflammation,[47] the present findings may provide a plausible explanation for why obesity and type 2 diabetes are major risk factors of IS. In support of this interpretation, a positive correlation also exists between circulating A-FABP levels and arterial stiffness, a clinical hallmark of atherosclerosis, in patients with hypertension, type 2 diabetes and in geriatric adults.[48,49] Increased arterial stiffness is associated with early stroke onset, stroke incidence and is an independent predictor of post-stroke outcome including poor collateral circulation associated with increased infarct size[17] and increased risk of haemorrhagic transformation in acute IS.[16,18] Treatment with the angiotensin II type 1 receptor blocker olmesartan, reduces arterial stiffness in hypertensive patients and decreases the circulating levels of A-FABP.[50] Likewise, positive associations exist between circulating MMP-9 and arterial stiffness.[51] Genetic variations in MMP-9 may be involved in the development of arterial stiffness.[52] High circulating MMP-9 levels in the acute phase of cerebral infarction are an independent predictor of haemorrhagic transformation in all stroke subtypes.[53] Together with

the present findings showing genetic ablation and pharmacological inhibition of A-FABP attenuates intracerebral haemorrhages in mice after MCAO, this evidence strongly supports the pathogenic role of A-FABP-MMP-9 axis in arterial stiffness which may also contribute to the pathogenesis of IS and its outcome. Atherosclerosis is a key risk factor of IS.[13] Atherosclerotic plaque rupture causes the formation of blood clots which may impede blood flow or, after the rupture, may form emboli travelling to the brain or other parts of the body.[54] A-FABP regulates the inflammatory response and cholesterol accumulation in macrophages contributing to foam cell formation; atherosclerotic lesions express high levels of A-FABP.[55] Increased expression of active MMP-9 in macrophages favour rupture of atherosclerotic plaques.[56] Thus, the evidence that A-FABP enhances the expression of MMP-9 (and its activity) in macrophages suggests that the effect of elevated levels of the latter on plaque rupture is attributable to the increased presence of the former. In addition to alleviating IS outcome, treatment with BMS309403 reduces formation of atherosclerotic lesions in ApoE KO mice[55] and in the pig.[40] Taken in conjunction with the clinical data showing a positive correlation between circulating A-FABP and active MMP-9, the present findings support the notion that inhibition of A-FABP, by reducing MMP-9 expression, may be a potential therapeutic strategy not only for the acute treatment of patients with IS but also for the prevention of recurrent stroke in patients with atherosclerosis.

In summary, the present study uncovered that, in mice, A-FABP is a key mediator of IS because it exaggerates MMP-9 mediated disruption of the BBB (*Take home figure*). Genetic ablation or pharmacological inhibition of A-FABP alleviates the consequences of IS injury. The clinical findings also support a regulatory role of A-FABP on MMP-9 expression and its detrimental effect on IS outcome. Collectively, the current data define A-FABP, beyond its established role as biomarker, as a novel target for the treatment of IS.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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