Apolipoprotein-E Controls Adenosine Triphosphate-Binding Cassette Transporters ABCB1 and ABCC1 on Cerebral Microvessels After Methamphetamine Intoxication

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**Background and Purpose**—Methamphetamine is a powerful addictive, which has been associated with ischemic stroke and brain hemorrhage in humans. Whether and how methamphetamine influences the expression of tight junctions and adenosine triphosphate-binding cassette transporters, which have previously been shown to be regulated by apolipoprotein-E (ApoE) under conditions of brain ischemia, was unknown.

**Methods**—C57BL/6J mice received intraperitoneal injections of methamphetamine (3 times 4 mg/kg separated by 3 hours) either alone or in combination with the ApoE receptor-2 inhibitor receptor-associated protein (40 μg/kg) or the inducible nitric oxide synthase inhibitor 1400W (5 mg/kg). Animals were euthanized 3 or 24 hours after methamphetamine exposure. Tissue responses were evaluated with Western blots, immunoprecipitation, and immunohistochemistry using total brain and cerebral microvessel extracts.

**Results**—Methamphetamine induced a transient activation of stress kinases c-Jun N-terminal kinase 1/2 and p38 in the brain parenchyma and increased intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression on cerebral microvessels without inducing loss of tight junction proteins and without inducing IgG extravasation. Methamphetamine transiently increased the expression of the luminal adenosine triphosphate-binding cassette transporter ABCB1 on cerebral microvessels and reduced the expression of the abluminal transporter ABCC1. Elevated expression of ApoE was noted in the brain parenchyma by methamphetamine, activating ApoE receptor-2 on brain capillaries, deactivating c-Jun N-terminal kinase 1/2 and c-Jun, and regulating ABCB1 and ABCC1 expression. Indeed, ApoE receptor-2 and inducible nitric oxide synthase inhibition prevented the ABCB1 and ABCC1 expression changes.

**Conclusions**—Acute exposure to methamphetamine at doses comparable to those consumed in drug addiction does not induce tight junction breakdown but differentially regulates adenosine triphosphate-binding cassette transporters through the ApoE/ApoE receptor-2/c-Jun N-terminal kinase 1/2 pathway. (Stroke. 2012;43:1647-1653.)

**Key Words:** apolipoprotein-E receptor-2 | blood–brain barrier | c-Jun N-terminal kinase 1/2 | drug abuse | endothelium | multidrug resistance | neuropathology | signal transduction | tight junction

**M**ethamphetamine (also called crystal meth or meth) is a psychostimulant and addictive that causes severe health problems mainly due to neurotoxicity, cardiac arrhythmia, and hyperthermia.1,2 In the brain, meth overactivates the dopaminergic system, disrupting synaptic integrity3 and inducing neuronal injury through oxidative stress.4,5 Meth may also induce neuroinflammation6–7 and increase blood–brain barrier (BBB) permeability.8,9 Possibly related to such vascular actions, meth abuse has been shown to be associated with ischemic stroke and intracerebral and subarachnoid hemorrhage in humans.10

The BBB is formed by endothelial cell–cell contacts, the tight junctions,11 which are complemented by transporters on the luminal and abluminal endothelial membranes belonging to the adenosine triphosphate-binding cassette (ABC) transporter and solute carrier families.12,13 Whereas tight junctions are responsible for maintaining paracellular BBB tightness, ABC transporters control brain homeostasis and protect the brain from environmental molecules by eliminating them across the BBB.12,13 In vitro, meth exposure has previously been shown to deregulate tight junction proteins14–16 and impair the expression of a solute carrier, the glucose trans-
porter protein-1. In vivo, the effect of meth on tight junction integrity and ABC transporter expression was unknown. Meth has previously been proposed to act as an ABC transporter substrate.

ABC transporters are actively regulated in the brain under various pathophysiological conditions. In focal cerebral ischemia, our group has previously shown that the transporter ABCB1 (previously: multidrug resistance transporter-1), which is localized on the luminal membrane of brain endothelial cells and extrudes a broad range of substrates—among them also pharmacological drugs—from the vessel into the blood, is upregulated, whereas the transporter ABCC1 (previously: multidrug resistance-associated protein-1), which is expressed on the abluminal endothelial membrane and carries its substrates—among them various cell metabolites—in the opposite direction from the vessel into the brain, is downregulated on cerebral microvessels. ABCB1 and ABCC1 expression were controlled by apolipoprotein-E (ApoE), which deactivates c-Jun N-terminal kinase-1/2 (JNK1/2) and its downstream kinase c-Jun through its receptor ApoER2 (also: low-density lipoprotein receptor-related protein-8), thereby modulating ABC transporter transcription.

Based on these earlier results, we were interested in how an acute meth exposure at doses comparable to those used in drug addiction influences the integrity of tight junctions and ABC transporter expression on brain capillary cells. A deregulation of tight junctions might be relevant with respect to stroke pathogenesis, because it may reveal a mechanism by which meth may provoke cerebral thromboembolism and brain hemorrhage. The regulation of ABC transporters, on the other hand, might unravel a strategy through which the brain protects itself from intoxication.

Materials and Methods

Animal Groups and Methamphetamine Exposure

All animal experiments were done according to the National Institutes of Health guidelines for the care and use of laboratory animals based on guidelines of the local Animal Welfare Committee of the Universidad Complutense de Madrid. Adult male C57BL/6j mice (Harlan, Barcelona, Spain) weighing 25 to 30 g were kept under a homeothermic blanket (Harvard Apparatus). Urethane was chosen as anesthetic because among all known anesthetics urethane has least effects on blood pressure. After systemic intraperitoneal delivery of urethane at doses comparable to those used previously, no changes in blood pressure have been reported. Mean arterial blood pressure was measured using a Laboratory-Trax-4/24T system (World Precision Instruments) through a polyethylene catheter that was implanted into the right femoral artery. One subset of animals was anesthetized 30 minutes before the first (1) vehicle (n=4); or (2) meth (n=5) injection. In these animals blood pressure values were taken at 6-minute intervals before and after each vehicle or meth injection over a duration of 30 minutes, resulting in a total of 5 measurements on each of these animals. These animals were euthanized after the last recording on the same day. To examine longer-lasting effects of meth exposure, an additional subset of animals received 3 consecutive treatments with (1) vehicle (n=4); or (2) meth (n=5). These animals were anesthetized 24 hours after the last injection. During the next 24 minutes, a total of 4 blood pressure measurements were done, of which mean values were recorded.

Microvessel Isolation and Protein Extraction

For total brain homogenates, tissue samples from animals belonging to the same group were pooled (n=4–8; see previously), homogenized, and lysated in 1% NP-40 buffer containing 50 mmol/L Tris-HCl and 150 mmol/L NaCl (pH 7.4) supplemented with 5% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail-2 and sonicated. For microvessel isolation, pooled tissue samples were homogenized in microvessel isolation buffer and supplemented with 5% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail-2. Homogenates were centrifuged at 3200 rpm for 10 minutes at 4°C. The resulting pellets were resuspended in 20% dextran (molecular weight 64 000–76 000; D4751; Sigma) in microvessel isolation buffer. Suspensions were centrifuged at 6500 rpm for 20 minutes at 4°C. The resulting crude microvessel-rich pellets were resuspended in microvessel isolation buffer and filtered through 2 nylon filters of 100-μm and 30-μm mesh size (Millipore, Schwalbach, Germany). Microvessels were stored at −80°C until further use. Isolated microvessels were homogenized in appropriate lysis buffers (see subsequently) supplemented with 5% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail-2. Lysate samples were sonicated over 2 cycles lasting 20 seconds each at 4°C. Protein concentrations were measured using a Bradford assay kit with an iMark microplate reader (Bio-Rad, Hercules, CA). The microvessel fractions produced have previously been shown to exhibit a high degree of purity lacking glial fibrillary acidic protein, which is strongly expressed on astrocytic end feet.

Western Blotting

Lysates containing 20 μg protein were complemented with 5× sodium dodecyl sulfate loading buffer. Samples were pretreated by heating and processed under reducing conditions except for the ABC transporter blots, for which nonheated samples were loaded under nonreducing conditions to avoid aggregation of these highly glycosylated membrane proteins. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blot analysis using primary antibodies diluted 1:100 for ABCB1 and 1:1000 for all other proteins in 5% skim milk and 0.1 mol/L Tris-buffered saline containing Tween 20%. Blots were digitized, densitometrically analyzed, and corrected for protein.
loading by means of the β-actin or β-tubulin blots. Specifications of the antibodies used are given in the online-only Supplementary Materials and Methods.

Immunoprecipitation Assay
Lysates containing 800 μg protein that had been obtained using a 1% NP-40 lysis buffer containing 150 mmol/L NaCl and 50 mmol/L Tris base (pH 8.0) were supplemented with sodium orthovanadate (final concentration: 1 mmol/L) and complemented with 3 equal volumes of NET buffer (100 mmol/L Tris, 200 mmol/L NaCl, 5 mmol/L EDTA, 5% NP-40, pH 7.4).20 Two micrograms of anti-ApoER2 antibody (Santa Cruz Biotechnology) were added to each sample and incubated overnight at 4°C under slight rotation. The next day 20 μL of protein A/G plus-agarose was added to the samples, which were incubated 1 hour at 4°C. Finally samples were centrifuged for 30 seconds at 15 000 rpm at 4°C. Supernatants were dispersed and pellets washed 3 times in ice cold NET buffer. Twenty microliters of 2× sodium dodecyl sulfate loading buffer was added to each pellet and boiled for 5 minutes followed by a short centrifugation of 2000 rpm at 4°C. Supernatants were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis using sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 10% acrylamide-bis gel followed by Western blot analysis for phosphotyrosine.

Immunohistochemistry for Leukocyte Marker CD45
Twenty-micron cryostat sections obtained from the midstriatum were incubated with 0.3% hydrogen peroxide in 70% methanol in Tris-buffered saline to block endogenous peroxidase. Sections were incubated overnight at 4°C with rat anti-CD45 antibody (1:20; BD Biosciences). The next day, sections were incubated with biotinylated goat anti-rabbit antibody (1:200; Santa Cruz Biotechnology). Immune reactions were revealed with diaminobenzidine tetrahydrochloride (Sigma) using a Vectastain AB kit (Vector Laboratories). Sections were evaluated under a microscope (Axioplan; Zeiss). Twenty-micron cryostat sections obtained from the midstriatum were incubated with 0.3% hydrogen peroxide in 70% methanol in Tris-buffered saline to block endogenous peroxidase. Sections were incubated overnight at 4°C with rat anti-CD45 antibody (1:20; BD Biosciences). The next day, sections were incubated with biotinylated goat anti-rabbit antibody (1:200; Santa Cruz Biotechnology). Immune reactions were revealed with diaminobenzidine tetrahydrochloride (Sigma) using a Vectastain AB kit (Vector Laboratories). Sections were evaluated under a microscope (Axioplan; Zeiss). Immune reactions were revealed with diaminobenzidine tetrahydrochloride (Sigma) using a Vectastain AB kit (Vector Laboratories). Sections were evaluated under a microscope (Axioplan; Zeiss).

Results
Methamphetamine Induces a Parenchymal Stress Response
To evaluate the effect of meth administration on the brain parenchyma, we analyzed the expression and activation of 2 stress activated kinases, JNK1/2 and p38 mitogen-activated protein kinase using antibodies detecting total (nonphosphorylated and phosphorylated) and phosphorylated (ie, activated) JNK1/2 and p38. Interestingly, a robust increase in JNK1/2 (Figure 1A) and p38 (Figure 1B) phosphorylation was detected in total brain lysates at 3 hours after meth administration. This phosphorylation disappeared within 24 hours after meth exposure. In contrast to phosphorylated JNK1/2 and p38, total JNK1/2 and p38 were not influenced by meth (Figure 1A–B). Rectal temperature transiently increased after meth intoxication by approximately 1 to 2.5°C (online-only Supplemental Figure I). Mean arterial blood pressure was not influenced by meth exposure (online-only Supplemental Table I).

Methamphetamine Induces Adhesion Molecules in Cerebral Microvessels and Upregulates Tight Junction Proteins Without Provoking IgG Extravasation or Major Leukocyte Infiltration
To investigate how acute meth exposure influences vascular integrity, we studied the expression of adhesion molecules and tight junction proteins in cerebral microvessels and investigated the extravasation of the endogenous tracer IgG in total brain extracts, which upon BBB damage is able to accumulate in the brain parenchyma.21 Meth transiently induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression (Figure 2A–B) and elevated occludin and claudin-5 expression at 3 but not 24 hours in cerebral microvessels (online-only Supplemental Figure IIA–B) but did not influence zona occludens-1 levels (not shown). No IgG extravasation was noted in the brain parenchyma using Western blots (online-only Supplemental Figure IIC). Hematoxylin–eosin stainings revealed that the macroscopical structure of cerebral microvessels remained intact after meth exposure (online-only Supplemental Figure IID). Leukocyte counts in the stria-
tum, as evaluated by CD45 immunohistochemistry, were not significantly increased by meth (online-only Supplemental Figure III).

**Methamphetamine Differentially Regulates ABCB1 and ABCC1 in Cerebral Microvessels**

We next examined how meth influences the expression of the transporters ABCB1 and ABCC1 on cerebral microvessels. Interestingly, ABCB1, which is expressed on the luminal endothelial membrane,\textsuperscript{18} was upregulated (Figure 3A), whereas ABCC1, which is localized on the abluminal endothelial membrane,\textsuperscript{19} was downregulated (Figure 3B) at 3 hours after meth intoxication. ABCB1 and ABCC1 expression returned to baseline levels within 24 hours (Figure 3A–B).

**ApoE Is Upregulated on Meth Exposure, ApoER2 Is Activated, and JNK1/2 and c-Jun Are Deactivated**

Subsequent to brain ischemia, ApoE regulates ABCB1 and ABCC1 expression in an ApoER2/ JNK1/2/c-Jun-dependent way.\textsuperscript{20} To investigate whether ApoE influenced ABC transporters after meth intoxication, we examined ApoE expression in total brain extracts, showing that ApoE was increased at 3 hours after meth exposure (Figure 4A). Subsequent analysis of microvessel extracts revealed that ApoER2 was activated by meth through tyrosine phosphorylation (Figure 4B). Concomitantly, JNK1/2 and c-Jun were dephosphorylated (ie, deactivated; Figure 4C–D).

**Inhibition of ApoER2 and JNK1/2’s Effector iNOS Re-Establish ABC Transporter Expression**

To test whether ApoER2 was indeed responsible for mediating ABC transporter responses to meth exposure, we administered a pharmacological inhibitor of ApoER2, RAP, and an inhibitor of iNOS, 1400W. The iNOS inhibitor was chosen, because iNOS has previously been shown to act as effector of JNK1/2.\textsuperscript{26,27} We found that either RAP or 1400W administration re-established ABCB1 and ABCC1 expression (Figure 5A–B) at the same time restoring JNK1/2 and c-Jun phosphorylation (Figure 5C–D). Rectal temperature was not influenced by RAP and 1400W (online-only Supplemental Figure I). RAP or 1400W alone did not change the expression of ABCB1 or ABCC1 (online-only Supplemental Figure IV).

**Discussion**

Using a mouse model of systemic meth intoxication, we show that acute exposure to meth at doses comparable to those used in drug addiction (doses ingested by consumers are typically in the range of 50–500 mg\textsuperscript{28}) induces a transient parenchymal stress response, reflected by the activation of JNK1/2 and p38 mitogen-activated protein kinase, accompanied by the induction of proinflammatory cell adhesion molecules intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 on cerebral microvessels. Although tight junction proteins occludin, claudin-5, and zonula occludens-1 were expressed at a high level and microvascular permeability for the serum marker IgG was low, a differential regulation of ABC transporters, namely an upregulation of the luminal endothelial transporter ABCB1 and downregulation of the abluminal transporter ABCC1, was observed on cerebral microvessels, which, as we showed, was controlled by ApoE, most probably through its receptor ApoER2 that deactivated the JNK1/2/c-Jun pathway.

Cerebrovascular effects of meth have already been described previously. In rats and mice, acute meth exposure resulted in an increased permeability to endogenous and...
exogenous BBB tracers. However, such actions were observed at doses far above those consumed in drug addition associated with massive hyperthermia to more than 41.5°C suggesting subsequent lethality, whereas such changes were markedly attenuated under conditions of more subtle hyperthermia. In addition, acute rises in arterial blood pressure lasting over several minutes were found after delivery of higher meth doses. In 1 of those studies delivering meth at doses of 30 mg/kg, a decreased expression of the tight junction proteins occludin, claudin-5 and zonula occludens-1 was noted in the mouse hippocampus 24 hours after exposure. We did not detect arterial blood pressure changes in the current study, and rectal temperature changes were only transient. We observed an elevated expression of occludin and claudin-5 and an unchanged expression of zonula occludens-1. IgG extravasation was low.

Despite absence of overt BBB breakdown, a differential regulation of ABC transporters was noted on cerebral microvessels. The luminal ABCB1, which carries its substrates in direction from the vessel into the blood, was upregulated, occludin and claudin-5 and an unchanged expression of zonula occludens-1. IgG extravasation was low.

Despite absence of overt BBB breakdown, a differential regulation of ABC transporters was noted on cerebral microvessels. The luminal ABCB1, which carries its substrates in direction from the vessel into the blood, was upregulated,
whereas the abluminal ABCC1, which carries its substrates in the opposite direction from the vessel into the brain, was downregulated on brain capillary cells. The expression changes of ABC transporters were controlled by ApoE, which, as we further observed, activated its receptor ApoER2 on cerebral microvessels by tyrosine phosphorylation, thus dephosphorylating and deactivating JNK1/2 and c-Jun. We have previously shown in a model of transient focal cerebral ischemia that ApoE controls ABCB1 and ABCC1 expression on the transcriptional level through the JNK1/2/c-Jun pathway. In that study, we found that either genetic ApoE deletion or pharmacological JNK1/2 blockade prevented the physiological responses of ABC transporters. We now complemented our previous findings with pharmacological ApoER2 and iNOS inhibition data, demonstrating that the ApoE/ApoER2/JNK1/2 pathway is similarly relevant for meth intoxication. Based on our results, ApoE that is released from the brain parenchyma might act as a sensor for brain injury in a variety of pathophysiological states, transmitting stress signals to the endothelial cells through ApoER2, which as we previously reported is constitutively expressed on the abluminal endothelial membrane.

Through this ApoER2 signal, ABC transporters are regulated in a way that facilitates the removal of toxins from the brain tissue. We did not perform biodistribution experiments in the present study, yet we have previously shown in focal cerebral ischemia that alterations in ABCB1 and ABCC1 expression are accompanied by profound changes in the brain-to-blood distribution of ABC transporter substrates. ABCC1 is a broad-spectrum transporter, which binds a large variety of environmental and also pharmacological compounds. ABCB1 has more restricted substrate-binding properties. ABCC1 was shown to have strong affinity to amphipathic molecules and brain metabolites among those Phase II degradation products generated as a consequence of oxidation processes. At the murine BBB, such metabolites require detoxification and removal across the BBB. By upregulating the luminal ABCB1 and downregulating the abluminal ABCC1 transporter, the brain possesses an efficient detoxification mechanism. Strengthening this ABC transporter response might represent a promising strategy that might allow the prevention of meth-induced brain injury.

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Disclosures
None.

References
21. ElAli A, Hermann DM. Liver X receptor activation enhances blood–brain barrier integrity in the ischemic brain and increases the abundance of


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Supplementary Materials and Methods

Antibodies

The following antibodies were used for the Western blotting and immunoprecipitation studies: Antibodies against ApoE (sc-6385), ABCB1 (sc-8313), claudin-5 (sc-28670), VCAM-1 (sc-8304), β-tubulin (sc-9104) and phospho-tyrosine (PY20; sc-508) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Antibodies against total JNK1/2 (9252), phosphorylated JNK1/2 Thr183/Tyr185 (9255), total c-Jun (9165), phosphorylated c-Jun Ser63 (9261), total p38 mitogen-activated protein kinase (MAPK) (9212), phospho-p38 MAPK Thr180/Tyr182 (9211) and β-actin (4967) were obtained from Cell Signaling (Danvers, MA, U.S.A.). The antibody against occludin was from Invitrogen (71-1500), the antibody against ApoER2 (ab52905) from Abcam (Cambridge, UK), the antibody against ICAM-1 (BAF796) from R&D systems (Wiesbaden-Nordenstadt, Germany), and the antibody against ABCC1 (ALX-801-007-c250) from Alexis Biochemicals (San Diego, CA, U.S.A.).
Legends to Supplementary Figures

Suppl. Fig. 1. Methamphetamine increases rectal temperature that is not affected by the ApoER2 inhibitor RAP and the inducible NO synthase (iNOS) inhibitor 1400W. Rectal temperature was continuously monitored during the experiments. Data are means±SD (n=5-10 animals/group). **p<0.01 for Meth compared with vehicle; ††p<0.01 for Meth/ RAP compared with vehicle; ‡‡p<0.01 for Meth/ 1400W compared with vehicle. Note the absence of differences in rectal temperature between the Meth, Meth/ RAP and Meth/ 1400W groups.

Suppl. Fig. 2. Methamphetamine increases occludin and claudin-5 expression in cerebral microvessels, and does not provoke IgG extravasation. (A, B) Western blot analysis using brain capillary extracts demonstrating that occludin and claudin-5 are increased at 3 hours after Meth exposure. Note that occludin and claudin-5 levels returned to baseline values after 24 hours. (C) Western blot analysis using total brain extracts showing absence of extravasated IgG in the brain parenchyma after Meth exposure. Ischemic tissue from mice submitted to 30 min middle cerebral artery occlusion followed by 24 hours reperfusion was used as positive control. (D) Hematoxylin-eosin staining showing the absence of any changing in the macroscopical structure of cerebral microvessels. Data are means±SD (n=4 Western blots). *p<0.05/ **p<0.01 compared with vehicle.

Suppl. Fig. 3. Leukocyte infiltration is not increased upon Meth exposure. Immunohistochemistry for leukocyte marker CD45 showing negligible changes of brain leukocyte infiltration after Meth exposure. A representative microphotograph is also shown. Data are means±SD (n=5 animals/group). No significant differences between groups were found.
Suppl. Fig. 4. The ApoER2 inhibitor RAP and inducible NO synthase (iNOS) inhibitor 1400W alone do not affect ABB1 and ABCC1 expression. (A, B) Western blot analysis using brain capillary extracts demonstrating that (A) ABCB1, and (B) ABCC1 expression do not differ among otherwise healthy mice that received either vehicle, RAP or 1400W 3 hours before. Data are means±SD (n=4 animals/group). No significant differences between groups were found.
Supplementary Figure 2

(A) Western blot analysis of β-tubulin and Occludin expression in cells treated with vehicle, meth, 3h, and 24h. Significance is indicated by an asterisk. The bars represent percent of control.

(B) Western blot analysis of β-tubulin and Claudin-5 expression in cells treated with vehicle, meth, 3h, and 24h. Significance is indicated by an asterisk. The bars represent percent of control.

(C) Western blot analysis of IgG heavy chain in cells treated with vehicle, meth, 3h, and 24h.

(D) Immunohistochemical staining for IgG heavy chain in cells treated with vehicle, meth, 3h, and 24h.
**Supplementary Table.** Mean arterial blood pressure (in mmHg) in mice receiving methamphetamine (Meth) injections

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<th>12 - 18 min</th>
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<td></td>
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<td><strong>Third injection</strong></td>
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No significant differences between groups. Data are means±SD (n=4-5 mice/group).

NA, not applicable.