

## What Do Experimental Models Teach Us About Comorbidities in Stroke?

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There is a general consensus that animal models do not perfectly represent human conditions. This is not derived from any specific event, such as an ischemic episode, but rather reflects inherent differences in the physiology, anatomy, and metabolism among different species. Disparities among species also arise from differences in assessing functional recovery in stroke. Humans place a priority in regaining function in speech, cognition, and limb control, but these functions are not always readily translatable in animal models. Moreover, because functional recovery depends on the stroke type and location, induction of an ischemic lesion in a predefined area of an animal model, at best, reflects only a subtype of human stroke. Nevertheless, certain clinical pathophysiology features of stroke, such as acute outcomes and inflammatory/immune responses, are shared in animal models.<sup>1</sup> Combined with complementary knowledge from *in vitro* studies, animal models can provide valuable insight into stroke pathology at a systems level.

Analyses of translational inefficiencies in stroke model systems have led to the identification of underlying issues, which include, but are not limited to, rigor in experimental design, internal and external validity, negative bias, and insufficient statistical power. Together, these inefficiencies have steered collaborative international efforts in preclinical research to overcome these challenges.<sup>2</sup> An additional issue of importance is the insufficient understanding of stroke pathophysiology beyond the acute phase and the primary reliance on infarct volume in reporting acute outcomes. Together with a lack of inclusion of comorbidities in animal models of stroke, the development of clinical protocols based on findings from normal metabolic animals may contribute to translational failures. In light of a recent concerted effort to address the future of translational stroke research,<sup>3</sup> this review focuses on underlying translational issues and discusses insights gained from stroke models inclusive of comorbidities in the preclinical literature.

### Preclinical Animal Models: Limitations

#### Stroke Pathology: A Moving Target With Multiple Components

Despite intense preclinical research efforts, acute neuroprotection-based strategies that are effective in animal models

have shown little or no efficacy in numerous controlled clinical trials (<http://www.strokecenter.org/trials/>).<sup>4</sup> The notion that the inhibition of a single pathway may not be sufficient to overcome multipathogenic events in cerebral ischemia has led to multimodal approaches for stroke therapy.<sup>5</sup> These approaches include transcriptional enhancements that are broadly associated with pro-survival cascades, combined therapy with multiple drugs, or blocking single molecules that are involved in multiple pathologies. There is also a clear indication that multiple pathogenic pathways persist, converge, and change during different stroke stages. For instance, stroke-induced inflammation is followed by inflammation resolution processes,<sup>6</sup> accompanied by altering mononuclear phagocytes from a proinflammatory to reparative phenotype in the brain.<sup>7,8</sup> Given that multiple cellular cascades are activated at a given time and that there is a transition from cellular injury to remodeling at different post-stroke stages, the natural history of stroke pathophysiology during acute, subacute, and chronic recovery stages requires further characterization.

#### Brain Injury Is an Evolving Process

Even though stroke-induced infarction evolves for several days, infarct volume assessment in preclinical studies is routinely performed at a fixed time point, typically 24 to 72 hours poststroke. An additional pathological event that can influence stroke outcome is brain edema.<sup>9</sup> Earlier studies proposed that swelling-corrected infarct volume (indirect infarct volume) represents the extent of brain injury more accurately than swelling-integrated infarct volume (direct infarct volume).<sup>10</sup> This recommendation was based on the premise that the inclusion of swelling overestimates injury size and that brain swelling resolves over time as the infarct matures. However, clinical studies consider stroke-induced brain edema as a life-threatening complication during infarct development.<sup>11</sup> There is a close association between edema and mortality/morbidity in patients with stroke.<sup>12</sup> Although preclinical intervention studies show that injury size covaries with brain swelling,<sup>13,14</sup> Walberer et al argue against interdependence between infarct volume and brain swelling. In their study, an interventional treatment of immunoglobulin reduces infarct without affecting

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brain edema.<sup>15</sup> A direct contributing role of brain edema to infarct development has also been reported. Stroked animals with bilateral craniectomies exhibited much smaller infarct volumes compared with animals with sham craniectomy.<sup>16</sup> Given the fact that edema is a serious complication of ischemic stroke and a prominent event in diabetic and hyperlipidemic stroke,<sup>17–19</sup> reporting brain edema along with infarct size should be considered to more accurately capture acute stroke pathology.

### Stroke Pathophysiology in a Comprehensive Context

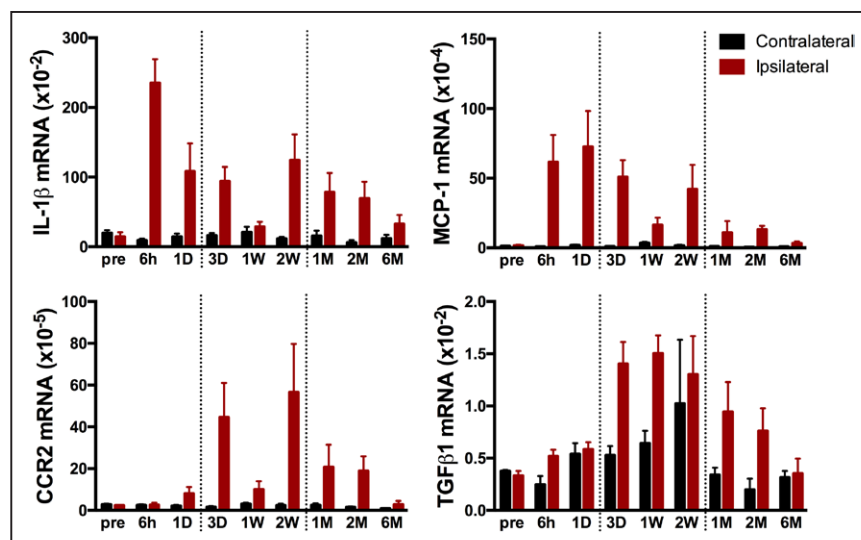
Recurrent translational failures based on neuroprotection strategies may arise from disparities in the outcome analyses between the preclinical and clinical setting. Unlike acute animal studies where the primary outcome is infarct size, injury size is not a final major outcome in patients. In fact, with the exception of those with malignant infarcts, the majority of patients survive and undergo a long road of recovery from functional impairments. Currently there is a limited number of preclinical studies that address chronic recovery. Our unpublished work has revealed the expression of inflammatory markers beyond an acute stage of stroke in mice (Figure). These changes over time suggest a chronic progress of ongoing inflammation and resolution. There is also anatomic distinction of tissue that is involved in acute injury versus recovery. Acute neuroprotection is focused on the injured tissue, whereas recovery largely relies on noninjured tissue in the stroked hemisphere and the contralateral hemisphere.<sup>20</sup> Kim et al<sup>21</sup> reported that poststroke treatment of soy isoflavone in mice did not reduce infarct size and edema, but did improve motor and gait function, suggesting that underlying mechanisms for acute pathology may not overlap with underlying mechanisms for recovery. Thus, the assumption that infarct reduction necessarily leads to functional recovery could be a potential roadblock in translating preclinical outcomes. To facilitate effective translation, comprehensive molecular profiling from acute to recovery stages is required.

### Comorbidity-Modified Stroke Pathology

Comorbidities in humans present as a cluster of risk factors that increase stroke incidence. What is much less appreciated is that comorbidities also alter stroke pathophysiology, lesion development, and recovery in profound ways. Owing to the use of genetic animal models and pharmacological interventions, it is possible to capture certain features of comorbidities. Comprehensive reviews on animal models with comorbidities are available elsewhere.<sup>22,23</sup> We will discuss comorbidity-modified stroke pathology with a special focus on brain swelling, vascular dysfunction, and comorbidity-modified immunity.

### Age and Sex

Aging is a nonmodifiable risk factor for stroke. Reported stroke outcomes in aged animals are equivocal. Compared with young counterparts, aged animals display an early disruption of the blood–brain barrier (BBB), increased brain injury, and delays in functional recovery in focal stroke, as well as more severe lesions in global ischemia.<sup>24–27</sup> In contrast, others have reported that there is an attenuation of infarct volume and edema in aged animals.<sup>28,29</sup> Although the reason for the different outcomes is unclear, blunted AMPK (adenosine monophosphate-activated protein kinase) activation and Na/K/Cl cotransporter expression have been suggested to underlie the reduction of injury size. Sex is another nonmodifiable risk factor that influences stroke outcomes, but the impact of sex is influenced by age.<sup>30</sup> Young females exhibit smaller infarct size, which is attributed to estrogen-induced anti-inflammatory effects.<sup>30–32</sup> The protective effect observed in young females disappears with age, and stroke outcome is worse in aged animals for both males and females.<sup>24,33</sup> Heightened vascular and peripheral inflammation is a common pathophysiological event that underlies sex differences and aging. Because stroke-induced BBB disruption enables infiltration of circulating immune cells into the injured tissue, studies have shown that enhanced cytokine production, oxidative stress, and metabolic burden in astrocytes<sup>34</sup> and increased recruitment of CD8+T cells<sup>35</sup> in aged brain potentiate stroke-induced inflammation. Enhanced peripheral inflammation and



**Figure.** Changes of inflammatory marker gene expressions in the poststroke brain. C57BL/6 male mice ( $n=6-8$ ) subjected to 30-minute transient middle cerebral artery occlusion, and gene expressions in the brain were measured by real-time polymerase chain reaction. Dotted lines separate acute (hours to days), subacute (days to weeks), and recovery (weeks to months) phases. CCR2 indicates C-C chemokine receptor type 2; IL-1 $\beta$ , interleukin-1 $\beta$ ; MCP-1, monocyte chemoattractant protein-1; and TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

altered immunity are hallmarks of aging and sex difference, and these features are shared in diabetic, hypertensive, and hyperlipidemic conditions. In this regard, animal models with metabolically compromised comorbidities can capture key pathological features of aging.

### Hypertension

Hypertension is a major risk factor in cardiovascular and cerebrovascular diseases. Widely used animal models are SPSHR (stroke-prone spontaneously hypertensive rats), SHR (spontaneously hypertensive rats) with or without angiotensin, as well as salt diet-induced animals.<sup>22,23</sup> Chronic hypertension impairs autoregulatory responses of cerebral blood flow and causes adaptive structural changes in vasculature.<sup>36</sup> BBB leakage and brain edema have been observed to be associated with chronic hypertension in SPSHR.<sup>37</sup> Hypertensive animals display an increased infarct volume after stroke, but there is no clear evidence that the condition increases stroke-induced brain swelling.<sup>38–40</sup> Whether the edema formed in the hypertensive conditions before stroke blunts stroke-induced brain swelling is unknown. Multiple underlying events associated with exacerbated stroke outcomes in hypertension include generation of 20-hydroxyeicosatetraenoic acid—a cytochrome of P450 metabolites, reactive oxygen species, and oxidative stress—as well as inflammation and disrupted neurovascular units.<sup>40,41</sup> In patients with hypertension, there is an increased number of circulating leukocytes and increased adhesion of leukocytes to endothelial tissue.<sup>42</sup> Consistent with these findings, hypertensive animals also display peripheral inflammation and vascular dysfunction. Animals with hypertension are more susceptible to spontaneous intracerebral hemorrhage and prone to hemorrhagic transformation in response to tPA (tissue-type plasminogen activator) treatment in stroke.<sup>43</sup> Hypertensive animals treated with antihypertensive agents in combination with tPA will address whether this type of intervention can attenuate negative stroke outcomes and hemorrhagic transformation in the hypertensive condition.

### Hyperlipidemia

ApoE KO (apolipoprotein E knock-out) mice receiving a high-fat diet is a model widely used to generate hyperlipidemic condition and has been intensively used for atherosclerosis studies. ElAli et al<sup>44</sup> reported that hyperlipidemic mice have increased BBB permeability and brain edema without increased infarct size. In this study, assessment of injury and edema was not based on volume but area measurements from representative sections, which limits a full estimation of rostrocaudal expansion of brain injury and edema. By altering the fat content in the diet of ApoE KO and normal mice, Kim et al<sup>17</sup> generated different degrees of plasma cholesterol levels and showed that the cholesterol levels are positively linked to the severity of brain injury and swelling. In this study, there was a greater contribution from swelling than infarct volume because brain swelling in these hyperlipidemic mice increased by  $\approx 300\%$ , whereas infarct growth was  $\approx 25\%$  compared with the mice with normal lipid levels. The hyperlipidemia-exacerbated brain swelling was not because of water influx, however, but rather from lipid content. The presence

of numerous lipid-laden foamy macrophages localized in the peri-infarct area accounted for the exacerbated swelling, and blocking lipid uptake through a scavenger receptor CD36 reduced the swelling in these mice. Although the study did not address BBB permeability beyond histological evidence of brain swelling, the presence of lipid-laden macrophages confined in the penumbra suggests that the swelling is partly of a cellular origin.

Immunologic mechanisms have been described to account for the negative influence of hyperlipidemia in stroke injury. An early study reported that monocytes from hypercholesterolemic patients have abnormal eicosanoid metabolism and high adhesion ability.<sup>45</sup> In ApoE KO mice, the constituents of the immune system are influenced by comorbid conditions. Hyperlipidemia increases the number of circulating monocytes (monocytosis) that are predominantly of the proinflammatory subset with high LY6C antigen expression.<sup>46</sup> Hyperlipidemic conditions also increase susceptibility to thrombosis and are linked to increased interaction between oxidized lipids and platelets through CD36.<sup>47</sup> In angiotensin-induced hypertensive animals, a loss-of-function study with selective ablation of myelomonocytic cells (reduction of circulating monocytes) showed that proinflammatory monocytes, but not neutrophils, are important in eliciting vascular dysfunction and arterial hypertension.<sup>48</sup> Increased leukocyte and platelet interactions with the cerebrovasculature in ApoE KO mice on a salt diet showed a synergy between hyperlipidemia and hypertension in enhancing inflammatory and prothrombotic properties.<sup>49</sup> Peripheral macrophages in hyperlipidemic mice have elevated expression levels of the inflammatory marker CD36.<sup>17</sup> After stroke, the hyperlipidemic mice also show elevated expression of IL-1 $\beta$  (interleukin-1 $\beta$ ), TNF- $\alpha$  (tumor necrosis factor- $\alpha$ ), MCP-1 (monocyte chemoattractant protein-1), and CCR2 (C-C chemokine receptor type 2) expression in the brain that is CD36 dependent. Using bone marrow transplantation between CD36 wildtype and CD36 KO hyperlipidemic mice, Kim et al<sup>50</sup> showed peripheral (macrophage) CD36 plays a critical role in hyperlipidemia-exacerbated stroke injury and edema. In this study, the effect was abolished in normolipidemic mice, highlighting the difference in stroke pathology between normal and hyperlipidemic conditions.

### Diabetes Mellitus/Hyperglycemia

Experimental diabetic models are typically either genetically modified mice (Zucker rats, *db/db*, or *ob/ob* mice), chemically induced (eg, streptozotocin), or animals fed with high-fat diets.<sup>18,23</sup> As predicted from outcome studies in diabetic patients, diabetic mice showed increased brain swelling and neurological impairment.<sup>18,19,51,52</sup> The enhanced swelling is associated with early BBB disruption and vascular damage,<sup>53,54</sup> endothelial transcytosis, and persistent VEGFR2 (vascular endothelial growth factor receptor 2) expression in the peri-infarct area.<sup>19</sup> Studies also demonstrated increased endothelium–neutrophil interaction and elevated ICAM (intercellular adhesion molecule) expression,<sup>51,55</sup> as well as hemorrhagic transformation with increased MMP (matrix metalloproteinase)-9 activity.<sup>56</sup> Increased vascular dysfunction and BBB permeability are associated with diabetic

**Table. Comorbidity-Modified Stroke Pathology and Outcome in Experimental Animal Models of Stroke**

	Comorbidity	Type of Stroke	Comorbidity-Modified Pathology	Stroke Outcome	Reference
BBB disruption	Aged rats	tMCAO	Early disruption, BBB permeability↑	Delayed functional recovery, neuronal damage↑	24
	Young female rats	tMCAO	Preserving blood flow effect	Injury↓	32
	Young and aged, male and female mice	tMCAO	BBB permeability ↓(female) or ↓(male)	Injury ↓(male), injury ↑(female), edema↓	65
	SHR/salt diet	Permanent MCAO	No change in BBB disruption, cerebral blood flow reduction↑	Injury↑	38
	SHR and SPSHR	tMCAO	Regional cerebral blood flow reduction↑	Injury↑	41
	T2D rats (diabetic diet+Stz)	tMCAO	BBB disruption↑	No change in injury	53
	T1D rats (Stz)	tMCAO	BBB permeability↑ (hippocampus)	Cognition↑, neuro- and oligo-dendrogenesis↑	66
	Acute hyperglycemic rats	tMCAO	Early BBB disruption	Injury↑, edema↑, rescued by HMGB1 antagonist	54
	ApoE KO mice, HFD	tMCAO	BBB permeability↑	No change in injury, edema↑	44
Vascular dysfunction	SHR	Photothrombosis	ICAM-1, VCAM-1, no change in P-selectin expression	Injury↑	39
	T2D mice (db/db)	tMCAO	ICAM-1↑	Injury↑, edema↑, mortality↑	51
	Zucker diabetic fatty rats	tMCAO	Soluble ICAM↑, neutrophil–endothelial interaction↑	Injury↑, edema↑	55
	T1D rats (Stz)	tMCAO	Vascular thrombosis	Brain repair (neuro- and oligo-dendrogenesis)↑	66
	T1D mice (Stz)	Photothrombosis	Endothelial transcytosis↑, no change in tight junction, VEGFR2↑, synaptic structure↓(penumbra)	No change in injury, VEGFR2 inhibition rescued BBB permeability and function	19
	T1D mice (Stz)	tMCAO	sEH↑ (cerebral vessels)	Injury↑, rescued by sEH antagonist	57
Immune/inflammation	Aged mice	tMCAO	Brain CD8 T cells↑, anti-inflammatory microglia↑	NA	35
	Aged rats	Photothrombosis	Astroglia response↓	Injury↑, motor deficit↑	26
	Multiparous (vs virgin) female mice	tMCAO	Immunosuppressive microglia phenotype before stroke	Injury↓, behavior↑	67
	SHR	Photothrombosis	Leukocyte infiltration↑	Injury↑	39
	Hypertensive mice (angiotensin)	Spontaneous intracerebral hemorrhage	Oxidative stress↑, MMP-9 activity↑	Stroke incidence↑	68
	T2D mice (db/db)	tMCAO	Proinflammatory genes↑, macrophage/neutrophil extravasation↑	Injury↑, edema↑, mortality↑	51
	Zucker diabetic fatty rats	tMCAO	Neutrophil↑	Injury↑, edema↑	55
	T1D mice (Stz)	tMCAO	MMP-9 activity↑	No change in injury, hemorrhagic transformation↑	56
	Acute hyperglycemic rats	tMCAO	HMGB1↑(cerebrospinal fluid)	Injury↑, edema↑, rescued by HMGB1 antagonist	54
	T2D mice (db/db)	Hypoxic ischemia	Delayed expression of proinflammatory cytokine	Wound healing↓	61
	Goto-Kakizaki rats	tMCAO	MMP-2 activity↑	Injury↓, hematoma formation↑	58
	T2D mice (diabetic diet+Stz)	tMCAO	Blunted acute inflammatory response	Injury↑, edema↑	18
	ApoE KO mice, HFD	tMCAO	Peripheral granulocytes↑	Injury↑	69
	ApoE KO mice, HFD	tMCAO	Neutrophil infiltration↑	Injury↑, behavior↑	70
ApoE KO mice, HFD	tMCAO	CD36 on monocytes/macrophages↑	Injury↑, edema↑	17	

(Continued)

Table. Continued

	Comorbidity	Type of Stroke	Comorbidity-Modified Pathology	Stroke Outcome	Reference
Pathway/ molecules	Aged rats	Global ischemia	NA	Injury↑, ischemic incidence↑	27
	Aged mice	tMCAO	AMPK activation↑ at baseline and blunted in response to stroke	Injury↓	29
	Aged mice	tMCAO	Na/K/Cl cotransporter expression↓	Edema↓	28
	Young male and female mice	tMCAO	Estrogen-mediated inducible nitric oxide synthase expression↓	Injury↓	71
	SHR	Thromboembolic stroke	NA	Hemorrhagic transformation↑	43
	T1D mice (Stz)	tMCAO	Mitochondrial dysfunction	No change in injury, hemorrhagic transformation↑	56
	T1D mice (Stz)	tMCAO	No functional reorganization in secondary region for sensorimotor restoration	No change in injury, behavior↓	60

ApoE KO indicates apolipoprotein E knock-out; BBB, blood–brain barrier; HFD, high-fat diet; HMGB, high mobility group box protein; ICAM, intercellular adhesion molecule; NA, not available; sEH, soluble epoxide hydrolase; SHR, spontaneously hypertensive rat; SPSHR, stroke-prone spontaneously hypertensive rat; Stz, streptozotocin; T1D, type 1 diabetes mellitus; T2D, type 2 diabetes mellitus; tMCAO, transient middle cerebral artery occlusion; VCAM, vascular cell adhesion molecule; and VEGFR2, vascular endothelial growth factor receptor 2.

conditions. Diabetes mellitus-enhanced brain swelling is derived from enhanced VEGF (vascular endothelial growth factor) signaling, and an anti-VEGF strategy is effective in attenuating brain swelling.<sup>19</sup> Despite the prominent effect on brain swelling, whether diabetes mellitus can change infarct volume is not clear because several studies have reported either increase,<sup>18,51,55,57</sup> decrease,<sup>58</sup> or no change<sup>19,53,56,59</sup> in infarct volume. Because infarct volume is exacerbated by brain edema in large territorial stroke,<sup>16</sup> the injury severity may explain the difference such that diabetes mellitus-exacerbated swelling in a confined space drives infarct growth in large territory infarcts but not in smaller strokes.

Hyperglycemia and insulin resistance are hallmarks of diabetes mellitus. In addition, the diabetic condition is linked to endothelial dysfunction and chronic low-grade systemic inflammation.<sup>60</sup> The exacerbation of stroke outcome in *db/db* mice is associated with increased extravasation of macrophages/neutrophils and exacerbated proinflammatory gene expression.<sup>51</sup> Zucker diabetic fatty rats also have larger infarct volumes and edema and worse neurological function after ischemia–reperfusion.<sup>55</sup> Comorbidity-modified immune mechanisms also include deregulated immune responses in diabetic stroke. Poststroke inflammatory responses and scar formation are delayed in diabetic *db/db* mice.<sup>61</sup> Peritoneal macrophages from diabetic mice show a blunted inflammatory response on lipopolysaccharide stimulation and have reduced MCP-1 expression in early poststroke brain.<sup>18</sup> These studies support a view that these comorbidities may be associated with blunted or delayed inflammation during the acute phase, which in turn triggers prolonged inflammatory responses in the recovery phase in the poststroke brain and exacerbates stroke outcomes. Despite the fact that inflammation causes cellular demise, rapid and orchestrated inflammatory responses are critical for subsequent tissue repair and remodeling.<sup>62</sup> Thus, the impact of diabetes mellitus (and other comorbid conditions) on the dichotomous role of inflammation during acute and recovery stages should be investigated.

### Obesity in Stroke Recovery

Obesity precipitates the development of metabolic disorders, including dyslipidemia, insulin resistance, diabetes mellitus, and metabolic syndrome. These conditions are often accompanied by low-grade systemic inflammation and increased circulating cytokines, and they adversely affect stroke incidence and acute outcomes.<sup>60</sup> An overwhelming number of clinical studies suggest, however, that obese patients experience reduced mortality and better recovery when exposed to the same disease conditions—a phenomenon referred as the obesity paradox.<sup>63</sup> Factors that reduce mortality are of particular importance in patient care. Although understanding and defining the mechanisms underlying this paradox in stroke is clinically relevant, the mechanisms by which obesity can benefit poststroke survival are not clear at present. As shown in the Figure, persistent expression of pro- and anti-inflammatory mediators months after stroke indicates that ongoing inflammation and resolution processes continue throughout different poststroke stages. Furthermore, evidence suggests a beneficial role for proinflammatory immune cells on chronic recovery.<sup>64</sup> Thus, with the recognition of a dichotomy of immune cells participating in acute inflammation and chronic remodeling processes, chronic outcome studies in obese animals that take other comorbid conditions into account should provide insight into the underlying mechanisms of the obesity paradox in stroke.

### Perspective

Stroke induces multiple death and survival signaling over time. The temporal changes are marked by different molecular expression profiles at different poststroke stages and elicit specific biological/pathological responses at the cellular and molecular levels. Clinical and animal studies show that comorbid conditions affect peripheral inflammatory, vascular, and immune responses, as well as increase the incidence of stroke and influence stroke outcome. The identification and understanding of anatomic/structural differences in humans

and animals will allow for the selection of proper preclinical comorbid models to reflect human conditions. Moving forward, recognizing the presence of peripheral adaptations associated with metabolically dysfunctional states will be an important consideration in evaluating stroke-induced brain injury, edema, and functional recovery. Given the recent preclinical studies showing disparities in stroke pathology among normal and comorbid conditions as summarized in the Table, future studies should aim to address the natural history of stroke pathophysiology in a comprehensive context of stroke stages in normal and metabolically compromised conditions. Together, this will provide a platform to define potential therapeutic windows for either blocking or promoting targets of interest to reduce acute impairment and enhance repair/recovery processes. These concerted efforts will enable us to move toward the development of clinically effective treatment strategies in stroke.<sup>65-71</sup>

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### Disclosures

None.

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## What Do Experimental Models Teach Us About Comorbidities in Stroke? Sunghee Cho and Jiwon Yang

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