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The Importance of Considering Sex Differences in Translational Stroke Research

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Abstract

Stroke is the second leading cause of death worldwide and differences between men and women have been documented in incidence, prevalence and outcome. Here, we reviewed the literature on sex differences in stroke severity, mortality, functional outcome and response to therapies after ischemic stroke. Many of the sex differences in stroke severity and mortality are explained by differences in baseline demographics such as older age in women. However, women account for more stroke deaths, consistently suffer from worse stroke outcomes and are more often institutionalized and permanently disabled than men. These sex differences in functional outcome are equalized after treatment with tissue plasminogen activator (tPA) and women may benefit more from treatment than men. However this may depend on race, as African American women have less of a response to tPA than other groups. Regarding endovascular treatments, the few existing studies that have investigated sex differences in stroke outcome point to equal benefit in both sexes, however, many clinical trials are relatively underpowered to detect sex differences. Further, we considered sex-specific effects in animal models of stroke and present recommendations for the performance of stroke studies in female animals. The male-biased use of research animals is distinguished from the clinical situation where there is a disproportionate and growing female stroke population. Stroke in women is greatly understudied and including both sexes is especially important in both preclinical and clinical studies that evaluate potential stroke therapies.

Keywords

Sex differences; stroke; translational; estrogen; tissue plasminogen activator

Introduction

Stroke affects nearly 17 million men and women every year and accounts for close to 6 million deaths globally [1]. Among stroke survivors a large proportion are permanently disabled [1, 2]. Stroke is a sexually dimorphic disease with well-known sex differences in

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Conflict of interest

The authors declare no conflict of interest.

incidence, prevalence and outcome [2, 3]. Women have worse outcomes after stroke and account for a greater number of stroke deaths compared to men [2, 4], mostly due to longer life-expectancy in women and an older age at the time of stroke. The purpose of this review is to evaluate the importance of considering sex differences in translational stroke research. We present and discuss findings on sex differences in clinical stroke with special emphasis on stroke incidence, severity, mortality, functional outcome and the response to treatments. Considerations were made whether data were adjusted for differences in baseline demographics such as age and risk factors, and social factors. Adjusted analysis is important since the risk factor profile is different in male and female stroke patients with atrial fibrillation and hypertension being more common in women while men more frequently have a history of heart disease, smoking and alcohol use [5-8]. If sex differences remain after demographics and social factors have been controlled this speaks for a true biological difference in stroke outcome between men and women. Lastly, we present some important sex differences in experimental stroke and provide guidance for stroke research in females.

Sex differences in clinical stroke

Stroke incidence

The incidence of stroke is higher in men than in women until an advanced age when the gap starts to narrow and incidence rates in women equal or even surpasses that of men [3, 4, 9-15]. Higher incidence rates have been reported for women at 74-85 years [12] and above 85 years [13, 14]. Interestingly, analysis of data from the Greater Cincinnati/Northern Kentucky study showed higher incidence rates in black and white women under 34 years compared to young black and white men [4, 11]. The higher incidence rates that were observed in younger women in this study may be related to inclusion of subarachnoid hemorrhage patients that are more commonly women [3], and a higher proportion of women of childbearing age that may increase stroke risk due to changes during pregnancy.

Stroke risk in women increases after menopause coinciding with a decline in sex hormones, especially estrogen, pointing to a potentially protective role. This is supported by a study in women that found a significant association between an older age at natural menopause and reduced cumulative stroke incidence [16]. Thus, the more years with estrogen exposure prevents stroke. Attempts to capitalize on the beneficial effects of estrogen for primary or secondary stroke prevention have been largely unsuccessful [17]. In addition, hormonal effects likely cannot fully account for the sex differences in stroke incidence since women are protected until the age of 75-85, well past the menopausal years [11, 12, 14]. Intrinsic biological sex differences and organizational hormonal changes (i.e. permanent effects from previous exposure of sex steroid hormones) are likely major factors in the incidence and response to stroke.

Globally, overall incidence rates have decreased in both men and women during the last two decades [9], although there has been minimal change in elderly patients over 80 [15]. Alarmingly, stroke incidence has increased in younger women aged 30 to 49 years, and a trend was also seen in men [15], which may be due to the increased incidence of obesity and the metabolic syndrome.

Stroke severity

Few studies exist where the primary goal was to investigate whether there are sex differences in initial stroke severity (see Table 1). Two large studies based on the Danish stroke registry of first-ever acute stroke reported that women suffered from more severe strokes than men as assessed by the Scandinavian stroke scale [5, 18]. This sex difference was significant in elderly patients in their early 70s even after age and risk factors were adjusted for [18]. Stroke etiology, marital status and socioeconomic factors were also taken into account [5]. In contrast, Gall et al. did not observe any sex differences in the proportion of severe strokes (National Institutes of Health Stroke Scale, NIHSS >7) after adjusting for confounding factors [19]. Importantly, in this study, pre-stroke function was included in the analysis, and fewer women were living independently prior to their stroke. Higher pre-existing disability in women, especially in elderly women, will influence initial measures of stroke severity. Similar findings were seen in a large Chinese study that included ischemic stroke patients above the age of 75 years [20]. Reid et al. showed a larger proportion of women with severe strokes, that remained when age was adjusted for but not when prestroke handicap and other factors were included in the analysis [21]. Most studies present neurological deficits/stroke severity as part of the unadjusted baseline characteristics. In these unadjusted baseline demographics, no sex differences in stroke severity have been documented in several studies [22-25], although others have reported increased severity in females [26, 27]. One study from Japan reported higher prevalence of severe strokes in women (NIHSS ≥8) [26]. In that study, patients were excluded if they were disabled before the stroke (mRS ≥2), but no age-adjustments were made and women were significantly older. Taken together, it appears that most sex differences in stroke severity are explained by differences in baseline demographics and social factors. Studies show that women are more likely to be disabled, dependent or institutionalized before the stroke [6, 21, 22], even after adjusting for age [28]. Prestroke function is an important predictor of stroke outcome [29] and affects the measurement of stroke severity at admission. Future studies should include not only age and differences in risk factor profiles, but also social factors such as marital and residence status (i.e., living alone) and prestroke function in their analysis.

Stroke mortality

Several studies have assessed sex differences in stroke mortality, either by in-hospital mortality during the acute phase, or more long-term after stroke (see Table 2). Higher in-hospital mortality was found in female stroke patients, but this was not seen after adjusting for confounding variables such as age, race, stroke severity and risk factors [7, 21]. Many studies have found higher acute mortality in women, but most of these did not control for age and stroke severity [24, 27, 30]. The majority of studies examining long-term mortality (month(s) to up to a year after stroke) found no difference between men and women, both in unadjusted analysis [13, 23] or when confounders were controlled for [6, 19, 22, 28]. In addition, sex did not predict stroke death at 100 days or at 1 year follow-up [31, 32]. However, some studies suggest that mortality might be higher in men for stroke patients above 65 [33] or the mid-70s [5, 18, 20]. In contrast, higher case fatality in women at 3 and 12 months was reported in a large nation-wide Swedish study that included 64,746 stroke patients that was independent on activities of daily living (ADL) prior to the stroke; however, no adjustment was made for differences in baseline demographics [8]. Higher case

fatality in women was seen at 6 months in patients from the International Stroke Trial, however, in the logistic regression model when covariates were adjusted for, men were more likely to be dead at 6 months [34]. Thus, it appears that sex differences in stroke mortality are mostly explained by differences in age and baseline demographics rather than biological differences between men and women.

Functional outcome

Functional outcome after stroke is generally measured using the modified Rankins Scale (mRS), the Barthel Index (BI), or is based on the performance ADL such as dressing, toileting and mobility. Whether the patient was discharged home or institutionalized after stroke is commonly quantified indicating the level of disability and the functional outcome in patients. Outcome measures at discharge commonly show worse functional status in women even when confounders have been adjusted for [13, 21, 26, 30] (see Table 3). No significant differences during the acute phase have also been reported [23], but more women than men were discharged to long-term care or nursing homes. Similar findings were noted at long-term follow-ups. Female sex was associated with worse functional recovery at 3, 6 and 12 months and these sex differences were not eliminated when age, baseline or clinical variables were adjusted for [13, 28, 29, 32, 35, 36]. Furthermore, female sex was found as a predictor of disability (BI) and handicap (mRS) [6] and women were more dependent [8, 34]. One study reported that women were 3.5 times more likely to be institutionalized than men after stroke [13]. In contrast, Appelros et al. did not find sex-specific outcomes in mRS; however, the sample size of stroke survivors was small [31]. In a nation-wide study from United States of the “Get With The Guidelines – Stroke” (GWTG-Stroke) population consisting of 383,318 ischemic stroke patients, women were less likely to be discharged home [7]. The women in the GWTG-Stroke study were also less likely to receive care based on seven predefined measures e.g. IV tPA in patients who arrive <2 hours and antithrombotic medication within 48 hours of admission (for the complete list of measures see [7]). Sex differences in quality of care remained after adjustment for potential confounding variables like age, race and risk factors, emphasizing the difficulties in evaluating whether differences are true biological sex differences or dependent on social factors. However, a recent study showed that differences in demographics, prestroke and clinical factors only explained 41% of the sex differences in stroke outcome [29], leaving room for unknown factors to be discovered. In summary, women consistently suffer from worse short and long-term functional outcome than men, and this sex-disparity is not fully explained by the older age in women, prestroke function or comorbidities.

Response to treatments

Biological sex may not only influence stroke severity, functional outcome and mortality, but also impact how patients respond to stroke treatments. Preventive medicines such as aspirin appear to have sex-specific effects. A meta-analysis of randomized controlled trials showed that aspirin reduced the risk of myocardial infarction but not stroke in men, while in women, the scenario was the opposite with reduced risk of stroke and no effect on myocardial infarction [37]. Thrombolysis, predominantly with tissue plasminogen activator (tPA), is one of the treatments that have been analyzed for sex-specific effects. Since the approval of tPA for stroke treatment and documented beneficial effects on functional outcome, it was no

longer justified not to treat eligible patients. Therefore, few randomized placebo-controlled studies on the effect of tPA on stroke outcome exist. One large pooled analysis of randomized trials from the United States, Europe and Australia (n=2,178), found that women benefited more from treatment with tPA than men [38] as treatment nullified the expected gender gap in outcome. In treated women, no sex differences in functional outcome was seen at 3 months, while untreated women had worse outcomes than untreated men, with lower probability of a return to normal or near-to-normal mRS (≤1). Similar findings of no difference in stroke outcome between men and women were reported in a small randomized study with intra-arterial pro-urokinase [39]. Other studies [40-45], as well as a clinical registry study and systematic review [46], have confirmed that men and women treated with tPA had equal functional outcomes at 3 months, suggesting a potentially greater benefit in treated women. In contrast, one study found that men were more likely to have a better functional outcome at 3 months but higher mortality [47]. The study included patients from the “Glycine Antagonist in Neuroprotection” (GAIN) study and patients with early major neurological improvement (<1 h) were excluded which may have impacted the results. In addition, unknown effects from the glycine antagonist cannot be excluded, although no effect on stroke outcome was seen in the trial.

Differences in coagulation and fibrinolysis [48] and a higher prevalence of cardioembolic fibrin-rich occlusions that might be more easily dissolved by tPA [49] have been speculated as contributing factors to the greater benefit seen in women [41]. While higher recanalization rates during the first 72 h were seen in a small cohort of women (n=39) by Savitz et al. [50], a recent study of patients from the CLOTBUST trial did not find sex as a predictor of recanalization in an adjusted analysis [40]. Two other studies with larger sample size (n=205 and n=81) on intra-arterial administration of tPA or urokinase did not find sex differences in recanalization rates [51, 52]. Of note, recanalization might not always lead to reperfusion and studies suggest that reperfusion may be a better predictor of infarct volume and clinical stroke outcome [53, 54]. Sex-specific effects of tPA have also been demonstrated when it is combined with other treatments. Recently, Llull and collaborators published data on female-specific benefit from the combination therapy of alteplase and uric acid [55], one of the first positive neuroprotection trials. This may be due to sex differences in baseline levels of endogenous uric acid, which were lower in women. Women had better functional outcomes after exogenous uric acid administration than men. It has been suggested that women might benefit more from antioxidants such as uric acid, and this merits further investigation in pre-clinical studies. These investigators also found that the combination of alteplase and uric acid reduced the infarct growth (defined by the difference in brain imaging at baseline and 72 hours) compared to alteplase alone, an effect that was only seen in women [55].

The efficacy of tPA may also be influenced by race. Mandava et al. reported that tPA-treated black women had significantly less likelihood of a good outcome (mRS 0-2) compared to white women 3 months following stroke [56]. It was suggested that this differential response to tPA might be due to a higher proportion of blacks presenting with a prothrombotic genotype of plasma inhibitor-1 (PAI-1), an inhibitor of tPA's enzymatic activity [57]. Previous analysis of the NINDS dataset did not show race as a predictor of functional outcome [58] but the study was not powered for subgroup analysis [59]. Therefore, explicit

balancing methods were utilized in the study by Mandava et al. to evaluate the influence on sex and race on tPA outcome independent on differences in baseline characteristics [56]. Taken together, sex differences in response to stroke treatment may be depend on the ethnicity of stroke patients and further studies that carefully consider baseline characteristics are warranted.

Multiple recent trials on endovascular treatments have shown increased rate of functional independence in men and women 90 days after stroke [60-64]. Subgroup analysis by sex performed in two of these studies showed no significant sex differences in the rate of functional independence [64]. A previous study by Lutsep et al. that investigated the effect of sex on early revascularization and outcome at 90 days found similar rates of revascularization in men and women and no differences in functional independence or mortality [65]. In line with those findings, sex was not a predictor of poor neurological outcome (mRS >2) 90 days after endovascular treatment in a small Swedish study [66]. Although studies on sex differences in endovascular treatments are scarce, at this point it does not appear to be a sex-specific response or any reason for treating men and women differently.

Sex differences in experimental stroke

Sex differences are not only a consideration in human stroke, but also are relevant in pre-clinical studies. More than twenty years ago, Hall and authors reported that female gerbils sustained less neuronal damage and better reperfusion cerebral blood flow (CBF) after carotid occlusion compared to males [67]. Similar findings were reported by Alkayed et al. in a study of normotensive and stroke-prone spontaneously hypertensive rats where female animals subjected to transient intraluminal middle cerebral artery occlusion sustained less ischemic brain damage [68]. The sex-dependent effects on infarct volumes were eliminated in ovariectomized (OVX) females indicating a protective effect from female sex hormones. Supporting this hypothesis, OVX female rats treated with estrogen before transient or permanent ischemia restored female protection and led to less ischemic damage [69, 70]. Consistent with a protective role of gonadal steroids, young female mice have smaller infarct than males, while aged reproductively senescent male and female mice have equivalent infarcts after transient focal ischemia [71]. Estrogen's protective effect was also verified in castrated male animals and 16 months old reproductively senescent female rats after transient focal ischemia [72, 73]. However, the beneficial effects of estrogen in stroke seem to be time-sensitive as its neuroprotective and anti-inflammatory effects were lost with delayed estrogen-replacement after ovariectomy [74] or when given to aged animals [75, 76].

The potential beneficial effect of estrogen treatment acutely after stroke has also been investigated (see Table 4). Simpkins et al. demonstrated that delivery of estrogen at the beginning of reperfusion, but not after 50 min, significantly reduced the infarct volume [70], while the time window in permanent ischemia was defined to be ≤3 h [77]. It was later demonstrated that male animals benefited from acute postischemic estrogen treatment resulting in improved early reperfusion CBF and attenuated brain damage [78]. Naturally fluctuating estrogen levels in female animals have been linked to the extent of brain damage

[79]. In that study, stroke-prone spontaneously hypertensive rats subjected to ischemic stroke during metestrus had a larger brain damage than rats that were exposed to ischemia and reperfusion during proestrus when estrogen levels are high [79]. Further support for a hormonal origin of sex differences in experimental stroke was elegantly demonstrated by Manwani and colleagues [80]. The four core genotype mice model, in which the testis determining gene *Sry* has been removed from the Y chromosome to an autosome, was utilized to study the contribution of sex chromosomes to stroke outcome [81]. Gonadal males (XXM and XYM) consistently had larger brain infarct volumes than gonadal females (XXF and XYF), and this difference was eliminated when animals were gonadoectomized suggesting hormone-dependency [80]. Some of estrogen's neuroprotective effects have been linked to its anti-inflammatory, anti-apoptotic and vasodilatory properties and are reviewed in detail elsewhere [82, 83]. It should be noted that although the majority of experimental studies favor the beneficial effects of estrogen, there are contrasting studies [84-86], see Table 4 and detailed previous reviews [83, 87]. In summary, young female animals sustain less ischemic damage than young males when subjected to equal types of stroke, an effect that is mainly due to estrogen. Interestingly, middle-aged female mice have been shown to have greater brain damage compared to middle-aged males and young females [71]. Thus, there is a need for studies on sex differences and the contribution of sex hormones to stroke outcome with aging.

Estrogen's beneficial effects have unfortunately not successfully translated to human stroke. Several large randomized placebo-controlled clinical trials of chronic hormone therapy in postmenopausal women have shown either no benefit in preventing heart disease and stroke, or even increased risk [17, 88-91]. The differential effect of estrogen in experimental and clinical studies might originate from the timing of hormone therapy, the age of the women and number of years since menopause [92]. To validate the timing hypothesis of hormone therapy in stroke prevention, Kronos Early Estrogen Prevention Study (KEEPS) trial was initiated where younger women were enrolled and hormone therapy was introduced early after menopause [93]. Although some markers of cardiovascular disease risk such as high- and low-density lipoprotein cholesterol were improved after 48 months of hormone therapy with oral conjugated equine estrogens and progesterone, no differences in atherosclerosis progression as measured by carotid artery intima-media thickness or coronary artery calcium were noted [94]. Previous and current clinical trials have focused on the ability of estrogen to reduce the incidence of stroke, which seems unlikely if estrogen treatment is delayed past the peri-menopausal period. It remains to be investigated whether acute administration of estrogen, as demonstrated in experimental studies, can improve stroke outcome in patients by reducing histological injury or enhancing repair as seen in animal studies [95].

Overall, studies on sex differences in ischemic stroke have mostly been performed in rodents. Studies in higher order species and non-rodent models are scarce. One study in rabbits investigated the effect of endogenous estrogen levels in females and the efficacy of an antiplatelet therapy with aspirin on outcome after embolic stroke [96]. Behavioral outcomes at 24 h were improved in aspirin-treated female rabbits with high endogenous estrogen levels compared to females with low estrogen, suggesting that estrogen improve the efficacy of anti-platelet treatment. Platelet aggregation was compared to previous experiments in males and no sex differences were found. A small study of ischemic stroke in

Rhesus Macaque (n=3 per group) demonstrated no significant difference in the extent of ischemic injury after middle cerebral artery occlusion between males and females, although the variability was larger in females [97]. Studies in higher order species and non-human primates may be of great use in the transition from experimental studies to clinical trials but have the disadvantage of increased research costs and should be carefully designed to answer specific research questions.

Beneficial effects of female sex steroid hormones are not limited to estrogen. Several experimental studies show neuroprotective effects also from progesterone treatment alone or in combination with estrogen in ischemic stroke (see Table 4) and traumatic brain injury, reviewed in detail elsewhere [98, 99]. Acute administration of progesterone reduces ischemic injury and improves neurological function in ovariectomized female rats [100, 101] as well as in young [102-104] and aged rats and mice of both sex [100, 105]. Few existing studies evaluate the chronic effects of progesterone. These either showed no effect [106], increased [107], or reduced [72] stroke injury. In traumatic brain injury, progesterone emerged as a promising candidate for therapy when four phase II clinical trials showed better functional outcomes and decreased mortality [108]. Unfortunately, the recent completion of two large multicenter phase III trials ProTECT III and SyNAPSe showed no effect on mortality and no clinical benefit at 3 and 6 months [109, 110]. However, the potential of progesterone to reduce brain injury is of continued interest and issues with the failed translation of experimental studies into clinical trials has been recently reviewed [108]. The vast majority of preclinical studies demonstrate that sex steroid hormones like estrogen and progesterone can have neuroprotective effects but the experience from clinical trials has been disappointing. Challenges such as dosing, route of administration and time of treatment from stroke onset are likely key factors. Many clinical trials have very wide enrollment windows, as this increases the opportunity to enroll patients; however, the efficacy of any neuroprotective agent is likely to be maximized with early treatment. In addition, changes that occur in the aging brain and vasculature may influence efficacy. Investigators must recognize that stroke is primarily a disease of the elderly, and model the disease appropriately in the laboratory.

Ischemic cell death pathways

Although sex steroid hormones, predominantly estrogen, have been associated with the sex differences in experimental stroke, hormone-independent processes have also been discovered. Activation of different cell death pathways in males and females has received much attention the past years. One of the first studies to demonstrate sexual dichotomy in cell death pathways was performed by Sampei et al. [111]. Neuronal nitric oxide synthase deficient ($nNOS^{-/-}$) male mice had smaller infarcts after permanent middle cerebral artery occlusion compared to wildtype mice, while female mutant mice did not benefit from nNOS deficiency [111]. Subsequent studies have investigated this further and found that males may be more sensitive to oxidative stress followed by an activation of poly (ADP-ribose) polymerase-1 (PARP-1) and translocation of apoptosis-inducing factor (AIF) [reviewed by 112, 113, 114]. On the other hand, cell death in females is initiated by activation of caspases and involves the early release of cytochrome C [reviewed by 112, 113, 114]. The sex-disparities in apoptotic pathways have been found in rat or mouse pups when gonadal

hormone levels are equivalent between the sexes allowing for the study of hormone-independent mechanisms (although hormonal effects cannot be completely excluded) [113, 115], in intact animals [111, 116, 117] and in ovariectomized females with and without estrogen-supplementation [118], suggesting that some of these sex differences are hormone independent. Using genetic knockouts of PARP-1, reduced infarct volume and protection was seen in male pups [115] and adults [116], while no effect or even increased damage was seen in female animals. The potential that sex-specific cell death pathways exist likely impacts the response to treatments and indeed, reduced infarct volume after treatment with a pan-caspase inhibitor was shown in female animals and not in males [116, 118, 119]. The reverse scenario was achieved with a PARP-1 or nNOS inhibitor, where beneficial effects were seen in male mice but female animals had exacerbated stroke damage [117]. The importance of sex-specific effects and studies of sex differences is further emphasized in the case of minocycline, a neuroprotective agent that inhibits PARP-1 signaling [120]. Improved outcomes after minocycline treatment in ischemic stroke patients were reported in one study [121], although no subgroup analysis by sex was performed and there was a low proportion of females (35.1%). In experimental studies, Li et al. found that minocycline was beneficial in male mice with reduced infarct volume and neurological deficits, but not in females [122]. Recently, similar sex-specific effects of minocycline were seen in a clinical study from Iran [123]. Improved clinical outcome as measured by the NIHSS was seen in males at 30, 60 and 90 days after minocycline treatment while no significant effect was seen in females. When male and female data were grouped together, significance was reached at 90 days and the lack of a protective effect in females was not noticed, again highlighting the importance of sex-specific subgroup analysis and designing trials with adequate power to assess sex differences. It should be noted that the sample size in this study was very small (n=53) and NIHSS in the female minocycline group was significantly worse compared to the female control group at admission. Larger studies adjusting for differences in baseline characteristics are warranted to validate these findings.

Sex-specific effects in experimental stroke have been documented for other treatments/targets, e.g. hypothermia [124], erythropoietin [125], G-protein coupled estrogen receptors [126] and the calcium-permeable transient receptor potential M2 (TRPM2) ion channel [127]. In the case of TRPM2 inhibition, smaller infarcts following middle cerebral artery occlusion were only found in male mice and the authors discuss the possibility of PARP-mediated generation of ADPribose that in turn can activate TRPM2, linking TRPM2 signaling to sex specific cell death pathways.

Studying sex differences in stroke

Emerging data on sex differences and the influence of gonadal hormones in experimental stroke make it important to carefully plan and design studies in which female animals are included. A comprehensive guide for research on sex differences has been published by Becker et al. [128]. The authors present strategic guidance and hands-on instructions on how to monitor and characterize the reproductive cycle in female animals. Rodents have a reproductive cycle of 4 to 5 days with rapid changes in female sex hormone levels [129] that can influence studied traits differently depending on the day and time of the day experiments are performed. The use of intact cycling females randomly selected throughout their

reproductive cycle might result in large within-group variations and mask effects if female sex hormones influence the studied traits. There is also a risk of missing sex hormone effects if animals are used for experiments on the same day since female animals tend to cycle together. Since the extent of stroke damage has been linked to hormonal fluctuations during the reproductive cycle [79], depending on the research questions this might impact the results obtained. If the goal is to assess an effect of a potential treatment it is especially important not to have contributing protective effect of estrogen that could skew data if the proportion of females in proestrus (high estradiol levels) is not the same in placebo and treatment groups. One approach is to monitor two to three consecutive reproductive cycles by daily collection of vaginal smears and selecting animals at one or several stages of the reproductive cycle, although this increases the number of animals used. In a study examining a MEK1/2 inhibitor on ischemic stroke outcome, female rats in estrus or diestrus were specifically selected (when estrogen levels are low) and small within-group variations in infarct volume and functional outcome were documented [130]. However, if the intent is to model clinical stroke, the use of reproductively senescent or aged animals is desirable, as stroke outcome is different in young, middle-aged and aged male and female mice and the efficacy of neuroprotective agents differ in the aged brain [71].

Much attention has been given to the effect estrogen in ischemic stroke, but androgens in males also affect stroke incidence and outcome. In men, levels of testosterone decline with aging which has been associated with increased risk of stroke and low levels may worsen stroke outcome [131]. In preclinical studies androgens have a detrimental effect after stroke in young male mice but are beneficial if given to aged animals that have an age-related decline in endogenous sex steroid [131]. Gonadectomy provides a tool for studying the effect of sex steroid hormones in stroke but do not recapitulate the overall aging phenotype as the loss of gonadal hormones is abrupt and complete, and the animals are still young, with young vasculature and brain. Chemical induction of menopause in female animals by 4-vinylcyclohexene diepoxide (VCD) better mimics the natural decline in female hormones and can be useful [132], but the use of aged animals is likely to be the most translationally relevant, despite their high cost. One should also be aware of the possibility of difference in body size between males and females, and between ovariectomized females treated with placebo versus estrogen, that can affect behavioral analysis and all tests should be performed in sham male and female animals and in experimental animals before initiating experiments if possible.

Similar to experimental studies of stroke, it is important to perform subgroup analysis by sex in clinical studies and take into account whether women included in the study are pre- or postmenopausal or are on hormone therapy, which is far less common now compared to a decade ago, due to the findings of the WHI. Most female stroke patients are over 70 years of age making this less relevant, but with the increase in stroke incidence in younger women, or during the peri-partum period, hormonal factors need to be considered. In addition, epidemiological studies of sex differences in stroke outcome should take into account not only differences in basic clinical variables and risk factors, but also pre-stroke function and living situation. This is especially important as social isolation has negative effects on stroke mortality and morbidity [133]. Factors such as depression are also understudied and seem to have a differentially negative impact on women. When the number of variables included in

multivariate analysis increases, larger numbers of patients are needed, and unfortunately many studies are underpowered. However epidemiological studies help us define factors that may be important to stroke outcome, which then can be further evaluated in experimental studies where age and risk factors can be controlled and targets can be directly manipulated.

Concluding remarks

Sex differences in stroke have received more attention over the past decade but are still largely understudied. Although existing literature on sex differences in stroke severity and mortality are somewhat contradictory, women consistently suffer from worse functional outcomes and have high levels of long-term disability. Out of the number of fatal strokes in the United States during 2010, 67% were in women, a number that is expected to increase even further in the coming years [4]. Furthermore, sex differences in stroke outcome are not fully explained by differences in baseline demographics, including age. Other contributing factors such as epigenetics, immune responsiveness, inflammation and chromosomal contributions to ischemic sensitivity have yet to be investigated in detail. Studying sex-specific effects is especially important in preclinical and clinical studies of potential stroke therapies. If treatments that only have been evaluated in one sex in experimental studies are to be tested clinically, there is a risk of adverse effects in one sex cancelling out beneficial effects in the other sex. This further highlights the importance of subgroup analysis by sex in clinical stroke studies. Lastly, there is a disconnect between a male-bias in research animals used for experimental studies and the disproportionate large population of women among stroke patients [134]. Not only is the sex of research animals important, but sex matters when studying cell lines *in vitro* as demonstrated by differential susceptibility of cultured XY and XX neuronal cells to cytotoxic insults [134, 135]. Stroke in females is understudied and given the growing stroke burden in women this is the direction where more research is needed. The importance of studying comorbidities in stroke has already been underscored and it is imperative that this applies to both sexes.

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Table 1

Sex differences in stroke severity

Patients	Inclusion	Location	Period	Sex difference in severity	Variables controlled for	Reference
79,617	IS, ICH	Multicenter, Denmark	2003-2012	♀> ♂, aged over 70	Multivariate (socioeconomic position, marital status)	[5]
26,818	IS	Multicenter, Denmark	2000-2007	♀> ♂, aged over 75	Multivariate	[18]
1,316	IS, ICH, SAH, UNDETM	Melbourne, Australia	1996-1997, 1997-1999	No	Multivariate (occupation, prestroke living situation)	[19]
810	IS, ≥75 yrs	Tianjin, China	2009-2011	No	Multivariate	[20]
2,725	IS, ICH	Halifax, Canada	1996-2006	No	Multivariate (prestroke handicap)	[21]
4,046	IS, ICH, SAH, TIA	Ontario, Canada	2004-2005	No	Unadjusted	[22]
3,323	IS, ICH, SAH, UNDETM	Multicenter, Canada	2001-2002	No	Unadjusted	[23]
537	IS, ICH	Providence, USA	2010-2012	No	Unadjusted	[24]
505[a]	IS	Multicenter, USA	NA	No	Unadjusted	[25]
6,236	IS (independent prestroke)	Multicenter, Japan	1999-2013	♀> ♂	Unadjusted	[26]
1,581	IS, ICH, SAH UNDETM	Barcelona, Spain	1995-2002	♀> ♂	Unadjusted	[27]

Multivariate analysis commonly include age, stroke severity, stroke subtype and risk factors, other variables of importance that were adjusted for are given in brackets. IS = Ischemic stroke, ICH=Intracerebral hemorrhage, SAH=Subarachnoid hemorrhage, UNDETM=Undetermined

NA=Not available

[a]prospective

Table 2

Sex differences in stroke mortality

Patients	Subtypes	Location	Period	Sex difference in mortality	Variables controlled for	Reference
79,617	IS, ICH	Multicenter, Denmark	2003-2012	♀< ♂ at1 wk and 1 mo (> 70 yrs)	Multivariate (socioeconomic position, marital status)	[5]
4,499[a]	IS, ICH, SAH	Multicenter, Europe	1993-1994	No, 3 mo	Multivariate (living situation, prestroke handicap)	[6]
383,318	IS	Multicenter, USA	2003-2008	No, at discharge	Multivariate	[7]
64,746	IS, ICH, UNDETM (independent prestroke)	Multicenter, Sweden	2008-2010	♀> ♂, 3 and 12 mo	Unadjusted	[8]
1,136[b]	IS, ICH, SAH	Framingham, USA	1948-2005	No, 30-, 90-180d	Unadjusted	[13]
26,818	IS	Multicenter, Denmark	2000-2007	♀< ♂ (> 78 yrs)	Multivariate	[18]
1,316	IS, ICH, SAH	Melbourne, Australia	1996-1997, 1997-1999	No, 28 d	Multivariate (occupation, prestroke living situation)	[19]
810	IS ≥75 yrs	Tianjin, China	2009-2011	♀< ♂ at 12 mo, no sex diff. at 36 mo	Multivariate	[20]
2,725	IS, ICH	Halifax, Canada	1996-2006	No, at discharge	Multivariate (prestroke handicap)	[21]
4,046	IS, ICH, SAH, TIA	Ontario, Canada	2004-2005	No, 30 d	Age, subtype, comorbidity index	[22]
3,323	IS, ICH, SAH, UNDETM	Multicenter, Canada	2001-2002	No, 6 mo	Unadjusted	[23]
537	IS, ICH	Providence, USA	2010-2012	No, at discharge	Unadjusted	[24]
1,581	IS, ICH, SAH UNDETM	Barcelona, Spain	1995-2002	No, at discharge	Unadjusted	[27]
19,547	IS, ICH	Multicenter, Sweden	2001	No, 7-,28- or 90d	Age	[28]
2,566	IS, ICH, SAH, TIA	Michigan, USA	2002	No, at discharge	Multivariate (prestroke ambulatory status)	[30]
377	IS, ICH	Orebro, Sweden	1999-2000	No, 1 y	Multivariate	[31]
1754[a]	IS[c]	Multicenter, Germany	1998-2000	No, 100 d	Multivariate	[32]
44,832	IS, ICH, UNDETM	Ontario, Canada	1993-1995	♀> ♂, 30 d and 1 y (above 65 yrs)	Multivariate	[33]
17,370	IS	Int. Stroke Trial, Multicenter, worldwide	1992-1996	♀< ♂, 6mo	Multivariate (level of consciousness)	[34]

Multivariate analysis commonly include age, stroke severity, stroke subtype and risk factors, other variables of importance that were adjusted for are given in brackets. IS = Ischemic stroke, ICH=Intracerebral hemorrhage, SAH=Subarachnoid hemorrhage, UNDETM=Undetermined

[a] prospective

[b] Number of incident strokes in the Framingham original and offspring cohorts

[c] prestroke mRS<4 and non-intubated

Table 3

Sex differences in functional outcome

Patients	Subtypes	Location	Period	Sex differences in functional outcome	Variables controlled for	Reference
4,499[a]	IS, ICH, SAH	Multicenter, Europe	1993-1994	♀> ♂ disability (BI<15) and handicap (mRS>1) at 3 mo	Multivariate (prestroke handicap, level of consciousness)	[6]
383,318	IS	Multicenter, USA	2003-2008	♀< ♂ discharged to home	Multivariate	[7]
64,746	IS, ICH, UNDETM (independent prestroke)	Multicenter, Sweden	2008-2010	♀ predictor of dependency at1 y	Multivariate	[8]
1,136[b]	IS, ICH, SAH	Framingham, USA	1948-2005	♀> ♂ likely to be institutionalized at 3-6 mo	Multivariate (prestroke handicap)	[13]
2,725	IS, ICH	Halifax, Canada	1996-2006	♀< ♂ excellent outcome at discharge (BI≥95)	Multivariate (prestroke handicap)	[21]
3,323	IS, ICH, SAH, UNDETM	Multicenter, Canada	2001-2002	♀> ♂ discharged to long-term care	Multivariate (marital status, prestroke living situation, consciousness, comorbidity index)	[23]
537	IS, ICH	Providence, USA	2010-2012	♀< ♂ discharged to home	Unadjusted	[24]
6,236	IS (independent prestroke mRS 0-1)	Multicenter, Japan	1999-2013	♀> ♂ poor functional outcome at discharge	Multivariate (poststroke treatments)	[26]
19,547	IS, ICH	Multicenter, Sweden	2001	♀> ♂ institutional living at 3 mo	Multivariate (level of consciousness)	[28]
2,566	IS, ICH, SAH, TIA	Michigan, USA	2002	♀> ♂ poor function at discharge (mRS)	Age, prestroke ambulatory status	[30]
377	IS, ICH	Örebro, Sweden	1999-2000	No sex diff in risk of dependency at 1 yr	Lack of effect in univariate	[31]
1754[a]	IS[c]	Multicenter, Germany	1998-2000	♀> ♂ incomplete functional recovery at 100d	Multivariate	[32]
17,370	IS	Multicenter, worldwide	1992-1996	♀>♂, poor outcome at 6 mo	Multivariate (level of consciousness)	[34]
373[a]	IS, ICH,SAH, TIA[d]	MI, USA		♀>♂ dependent at 3 mo	Multivariate (prestroke ambulatory status)	[35]

Multivariate analysis commonly include age, stroke severity, stroke subtype and risk factors, other variables of importance that were adjusted for are given in brackets. IS = Ischemic stroke, ICH=Intracerebral hemorrhage, SAH=Subarachnoid hemorrhage, mRS=modified Rankin Scale, UNDETM=Undetermined

[a] prospective

[b] Number of incident strokes in the Framingham original and offspring cohorts

[c] prestroke mRS<4 and non-intubated

[d] life expectancy >6 mo and not discharged to hospice.

Table 4

Effects of estrogen and progesterone in experimental stroke

Species/model	Sex/age	Manipulation	Effect on outcome	Reference
Rat/pMCAO	Female/adult	OVX+/−E, replacement at OVX or at stroke onset, OVX 1 wk pre-MCAO	↓Infarct with E replacement during OVX	[69]
Rat/MCAO	Female/adult	OVX+/−E, replacement 24 h pre- or 40-90 min post-MCAO, OVX 1 wk pre-MCAO	↓Infarct with E replacement 24 h pre- or 40 min post-stroke	[70]
Rat/MCAO	Male, female/16 months	Male, female+E or P, replacement7 days pre-MCAO	↓Infarct in E and P treated females. No difference in infarct between males and females.	[72]
Rat/MCAO	Male/adult	Intact;Intact+chronic E (7-10 d pre-MCAO), Intact+acute premarin (30 min prestroke);CAST;CAST+E,	↓Infarct in all E treated animals; CAST no effect on infarct	[73]
Mouse/MCAO	Female/adult	OVX+/−E, immediate or 10 wks delayed replacement	↓Infarct and pro-inflammatory cytokines by immediate E	[74]
Mouse/MCAO	Female/adult, aged (22 mo)	Adult OVX+/−E, Aged+/−E	↓Infarct by E in adult, no change in aged. Larger infarct in aged than in adult.	[75]
Mouse/MCAO	Male,female/aged 20 mo	Intact+chronic E (17-20 mo of age), Intact +acute E at 20 months. MCAO was performed at 20.5 mo	↓Infarct by chronic E, no effect by acute E in females. Benefit in males by chronic and acute E.	[76]
Rat/MCAO	Female/adult	Intact;OVX+/−E, replacement post-MCAO	↓Infarct when E was given ≤3h post-stroke	[77]
Rat/MCAO	Male/adult	Intact+premarin at reperfusion	↓Infarct by premarin.	[78]
Rat WKY, SHRSP/pMCAO	Female/adult	Animals were in metestrus or proestrus at time of MCAO	↓Infarct in SHRSP in proestrus compared to estrus	[79]
Rat WKY, SHRSP/pMCAO	Female/adult	OVX+/− E, MCAO 2 wks later	↑Infarct in WKY by E, no effect in SHRSP	[84]
Rat/pMCAO	Female/adult	OVX+/− E, MCAO 2 wks later	↑Infarct by E, no effect neurological function	[85]
Rat/MCAO (ET-1)	Female/adult, reproductively senescent (9-11 mo)	Intact, OVX+/−E	↑Infarct by E in reproductively senescent, ↓ in adult. Larger infarcts in reproductively senescent than in adult	[86]
Mouse/MCAO	Male,female/Adult (3 mo)	Intact female,OVX+/−E, Male+/−E	↑Neurogenesis and functional outcome by E	[95]
Mouse/MCAO	Female/adult, aged (12 mo)	Adult OVX +/− P, Aged +/− P	↓Infarct by P in aged, no effect in OVX. P improved motor function in	[100]

Species/model	Sex/age	Manipulation	Effect on outcome	Reference
			OVX, no effect in aged.	
Rat/MCAO	Female/adult	OVX+/−P, pre-MCAO or pre- and post-MCAO	↓Infarct by P treatment pre- and post-MCAO	[101]
Mouse/MCAO	Male/adult	Intact +/−P post-MCAO	↓Infarct by P and improved motor function	[102]
Rat/MCAO	Male/adult	Intact +/− P, pre- or post-MCAO treatment	↓Infarct by P and improved motor function when given pre- or post-MCAO	[103]
Rat/pMCAO	Male/adult	Intact+/−P post-MCAO	↓Infarct by P and improved motor function	[104]
Rat/pMCAO	Male/aged (24 mo)	Intact+/−P post-MCAO	↓Infarct by P and improved motor function	[105]
Mouse/MCAO	Female/adult	OVX+/−P pre-MCAO	No effect on infarct by P	[106]
Rat/MCAO	Female/adult	OVX+/chronic P (7-10 d pre-MCAO) or acute P (30 min pre-MCAO)	↑Infarct with chronic P	[107]
Rat/MCAO	Female/aged (14-18 mo)	OVX+/−E, P or E+P; replacement 2 mo pre-MCAO	↓Infarct by E and E +P, not by P alone	[136]
Rat/MCAO	Male, female/adult	OVX+/−E, P or E+P post-MCAO; OVX 2-3 wks pre-MCAO	↓Infarct and improved functional outcome in all hormone-treated groups	[137]

MCAO=Middle cerebral artery occlusion, OVX=Ovariectomy, E=17β-estradiol, pMCAO=Permanent middle cerebral artery occlusion, P=Progesterone, WKY=Wistar Kyoto, SHRSP=Spontaneously hypertensive stroke prone, ET-1=Endothelin-1, CEE=Conjugated equine estrogens, MPA=Medroxyprogesterone, CEP=Conjugated equine estrogens and medroxyprogesterone