Oestrogen-mediated upregulation of the Mas receptor contributes to sex differences in acute lung injury and lung vascular barrier regulation

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In humans, females have a lower incidence of ARDS and female mice are more protected from acute lung injury than their male counterparts. Female protection is partially attributable to endothelial barrier stabilisation *via* the ACE2/Ang(1–7)/Mas axis. https://bit.ly/3fmzgUZ

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ABSTRACT Epidemiological data from the SARS-CoV-2 outbreak suggest sex differences in mortality and vulnerability; however, sex-dependent incidence of acute respiratory distress syndrome (ARDS) remains controversial and the sex-dependent mechanisms of endothelial barrier regulation are unknown. In premenopausal women, increased signalling of angiotensin (Ang)(1–7) *via* the Mas receptor has been linked to lower cardiovascular risk. Since stimulation of the Ang(1–7)/Mas axis protects the endothelial barrier in acute lung injury (ALI), we hypothesised that increased Ang(1–7)/Mas signalling may protect females over males in ALI/ARDS.

Clinical data were collected from Charité inpatients (Berlin) and sex differences in ALI were assessed in wild-type (WT) and Mas-receptor deficient ($Mas^{-/-}$) mice. Endothelial permeability was assessed as weight change in isolated lungs and as transendothelial electrical resistance (TEER) *in vitro*.

In 734 090 Charité inpatients (2005–2016), ARDS had a higher incidence in men as compared to women. In murine ALI, male WT mice had more lung oedema, protein leaks and histological evidence of injury than female WT mice. Lung weight change in response to platelet-activating factor (PAF) was more pronounced in male WT and female $Mas^{-/-}$ mice than in female WT mice, whereas Mas-receptor expression was higher in female WT lungs. Ovariectomy attenuated protection in female WT mice and reduced Mas-receptor expression. Oestrogen increased Mas-receptor expression and attenuated endothelial leakage in response to thrombin *in vitro*. This effect was alleviated by Mas-receptor blockade.

Improved lung endothelial barrier function protects female mice from ALI-induced lung oedema. This effect is partially mediated *via* enhanced Ang(1–7)/Mas signalling as a result of oestrogen-dependent Mas expression.

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Introduction

With an estimated incidence of 86.2 per 100 000 person-years and mortality rates of up to ~43%, acute respiratory distress syndrome (ARDS) presents a major cause of mortality and morbidity in critical care. Defined by bilateral pulmonary infiltrates, lung oedema not fully explained by hydrostatic causes and hypoxaemia [1], ARDS typically develops as a complication of pneumonia or sepsis. Treatment is limited to supportive interventions [2] and prone-positioning in severe ARDS [3], with no effective pharmacological treatment.

Epidemiological studies yield conflicting results for sex differences in ARDS: a couple of studies report lower annual [4] and in-hospital mortality rates [5] in females, a finding that is seemingly supported by emerging data from the recent SARS-CoV-2 outbreak suggesting a higher mortality in men than women [6]. However, other studies in patients at risk detected no significant association of ARDS incidence or mortality with sex [7], or even showed an increased risk in women [8]. Animal models of acute lung injury (ALI) have largely reproduced a relative protection of females over males [9]. In mice, protection was lost in ovariectomised (OVX) females but was restored upon oestrogen replacement, pointing to a mechanism that is at least in part oestrogen-dependent [10]. Protection from ALI was associated with reduced markers of inflammation, suggesting that oestrogen may suppress lung inflammatory responses [10]; however, underlying cellular mechanisms remain unclear. Whether female sex and oestrogen also affect other features of ARDS, such as vascular permeability, has so far not been addressed.

Among the mechanisms proposed to mediate sex-specific differences in cardiovascular disease, signalling *via* the angiotensin-converting enzyme 2 (ACE2)/angiotensin (Ang)(1–7)/Mas axis presents a promising candidate pathway by which female sex and/or oestrogen may protect from incidence and severity of ARDS. In contrast to angiotensin-converting enzyme (ACE), which converts AngI to AngII by removal of a dipeptide, ACE2 removes a single amino acid from AngII to generate the heptapeptide Ang(1–7), which counteracts the vasoconstrictive, proliferative and inflammatory effects of AngII [11], presumably by acting through its G-protein coupled receptor Mas [12]. Our experimental studies and those of others show that pharmacological blockade or genetic deletion of ACE2 aggravates ALI, while intravenous infusion of Ang (1–7) or the Mas agonist AVE0991 attenuates ALI *in vivo* and endothelial leak *in vitro* [13–15]. The latter effects were prevented by the Mas receptor antagonists A779 (Mas-blocker I) or D-Pro^7-Ang(1–7) (Mas-blocker II) [13, 14], substantiating the protective effect of the ACE2 product Ang(1–7) *via* signalling through its receptor Mas. These findings have fuelled translation into a first pilot clinical trial targeting the ACE2/Ang(1–7)/Mas axis for treatment of ARDS [15].

The ACE2/Ang(1–7)/Mas axis has been identified as an important contributor to sex-specific differences in cardiovascular disease with a relative protection of female animals as compared to males or OVX females [16]. With emergent evidence for a beneficial role of the ACE2/Ang(1-7)/Mas axis in both ARDS and the relative protection of premenopausal women from cardiovascular disease, we hypothesised that differences in the ACE2/Ang(1–7)/Mas axis may similarly contribute to a lower incidence and mortality of ARDS in females over males.

Methods

Full experimental details according to the ARRIVE guidelines, including information on *in vivo*, *ex vivo* and *in vitro* experiments of ALI, and assessment of their endpoints, may be found in the supplementary material. Clinical cases of ARDS were identified by International Classification of Diseases, Tenth Revision (ICD-10) code in the Charité database (years 2005–2016). Research Ethics Board approval EA1/171/15.

Results

Sex difference in ARDS incidence

Of 734 090 inpatients registered at the Charité, Universitätsmedizin (Berlin, Germany) from 2005 to 2016, 2475 were diagnosed with an ICD-10 code of J80 for ARDS, of which 1553 (62.7%) were male *versus* 922 (37.3%) which were female ($p<0.05$). Except for infants (<9 years), females were less common in all age groups, indicating protection from puberty to post-menopausal age (figure 1).

Acute lung injury is attenuated in female mice compared to male mice

We tested for sex-specific differences in the development of ALI with a two-hit model of acid instillation and subsequent overventilation (OV) for 2 h. Compared to controls, male mice developed marked lung oedema as indicated by an increase in wet-to-dry lung weight ratio (figure 2a), vascular leakage evident as increased total protein concentration in bronchoalveolar lavage fluid (BALF) (figure 2b) and extravasation of macromolecular human serum albumin (HSA) (figure 2c). These effects were associated with the accumulation of neutrophils, evident as an increase in myeloperoxidase (MPO) activity (figure 2d) and histological characteristics of lung injury (figures 2e and 2f). In corresponding female mice, all features of

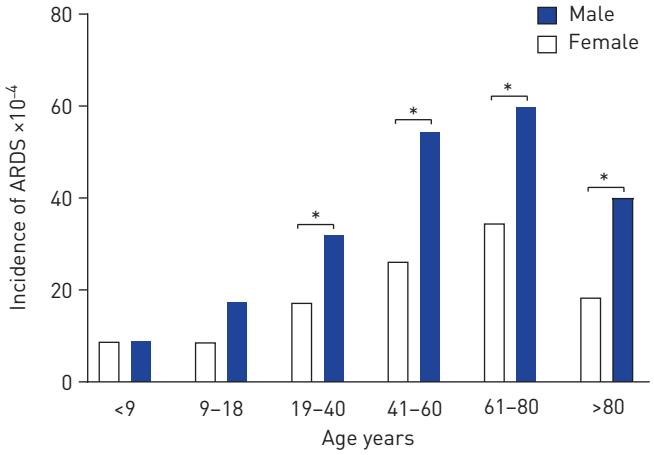

FIGURE 1 Incidence of acute respiratory distress syndrome (ARDS) by age group and sex for inpatients registered at the Charité from 2005 to 2016 (n=734 090). Statistical difference was determined by the Chi-squared test. *: p<0.05.

ALI were normalised (figures 2a–2f), demonstrating a striking protection of female as compared to male mice. Notably, levels for the above parameters did not differ between male and female control mice.

Sex-specific protection from acute lung injury is partially lost in Mas-receptor deficient mice

To probe for a potential contribution of the ACE2/Ang(1–7)/Mas axis in sex-specific protection, we replicated the experiments in Mas-receptor deficient (*Mas*$^{-/-}$) mice. In the control group, *Mas*$^{-/-}$ mice did not differ from their corresponding male and female wild-type (WT) counterparts (figures 2a–2f). Following ALI induction, female *Mas*$^{-/-}$ mice showed less protection compared to *Mas*$^{-/-}$ controls, in that wet-to-dry weight ratio and histological lung injury score increased (figures 2a and 2e) and that BALF protein concentration and histological score exceeded the corresponding values in female WT mice with ALI (figures 2b and 2e). A similar aggravation of ALI was not evident in male *Mas*$^{-/-}$ mice, which rather showed a tendency towards less severe ALI compared to the corresponding WT mice, although this trend only reached significance for parameters of vascular leakage (figures 2b and 2c).

Platelet-activating factor induced vascular permeability is attenuated in isolated lungs of female wild-type mice

As Mas-dependent sex differences mostly related to features of vascular leakage and oedema formation rather than inflammation (MPO activity), we next measured changes in lung weight as a highly sensitive parameter of vascular barrier function following stimulation with platelet-activating factor (PAF), a lipid mediator which causes rapid endothelial barrier failure in the intact lung [17]. Stimulation with PAF significantly increased lung weight gain in isolated lungs of male WT mice, yet not in female WT mice ($p<0.05$). Male *Mas*$^{-/-}$ mice showed less lung weight gain than the male WT controls (figure 3a), while lung weight gain was accelerated in female *Mas*$^{-/-}$ mice (figure 3b), such that male and female knockout lungs no longer differed.

Oestrogen stabilises barrier function in cultured endothelial cells via the Mas receptor

To test for a potential interaction between the female sex hormone oestrogen (E2; 17β-oestradiol) and the Ang(1–7)/Mas axis, which may underlie the partially Mas-dependent barrier protection in female mice, we analysed barrier function in cultured human pulmonary microvascular endothelial cells (HPMECs) *via* transendothelial electrical resistance (TEER) in response to thrombin (figures 4a–4c). In confluent HPMEC monolayers, thrombin stimulation resulted in a rapid reduction in TEER by approximately 15% within 15 min, indicative of inter-endothelial gap formation and endothelial leakage. Pretreatment for 24 h with E2 (1 nmol·L^{-1}) completely prevented the injurious effect of thrombin. Similar protection was achieved when endothelial cells were pretreated with the heptapeptide Ang(1–7) for 30 min prior to thrombin stimulation and combination of both treatments resulted in additive barrier stabilisation (figure 4a).

To determine whether E2 exerts its barrier-protective effects *via* the Ang(1-7)/Mas signalling pathway, we applied two pharmacologically distinct blockers of the Mas receptor, A779 (Mas-blocker I) and D-Pro^{7}-Ang(1–7) (Mas-blocker II). Both Mas-blockers alone had no effect on the basal TEER response to thrombin (data not shown) but, as expected, blocked the protective effect of Ang(1–7) (figures 4b and 4c). Importantly, both antagonists also attenuated the protective effect of E2 pretreatment, indicating that oestrogen acts at least in part *via* stimulation of the Ang(1–7)/Mas axis.

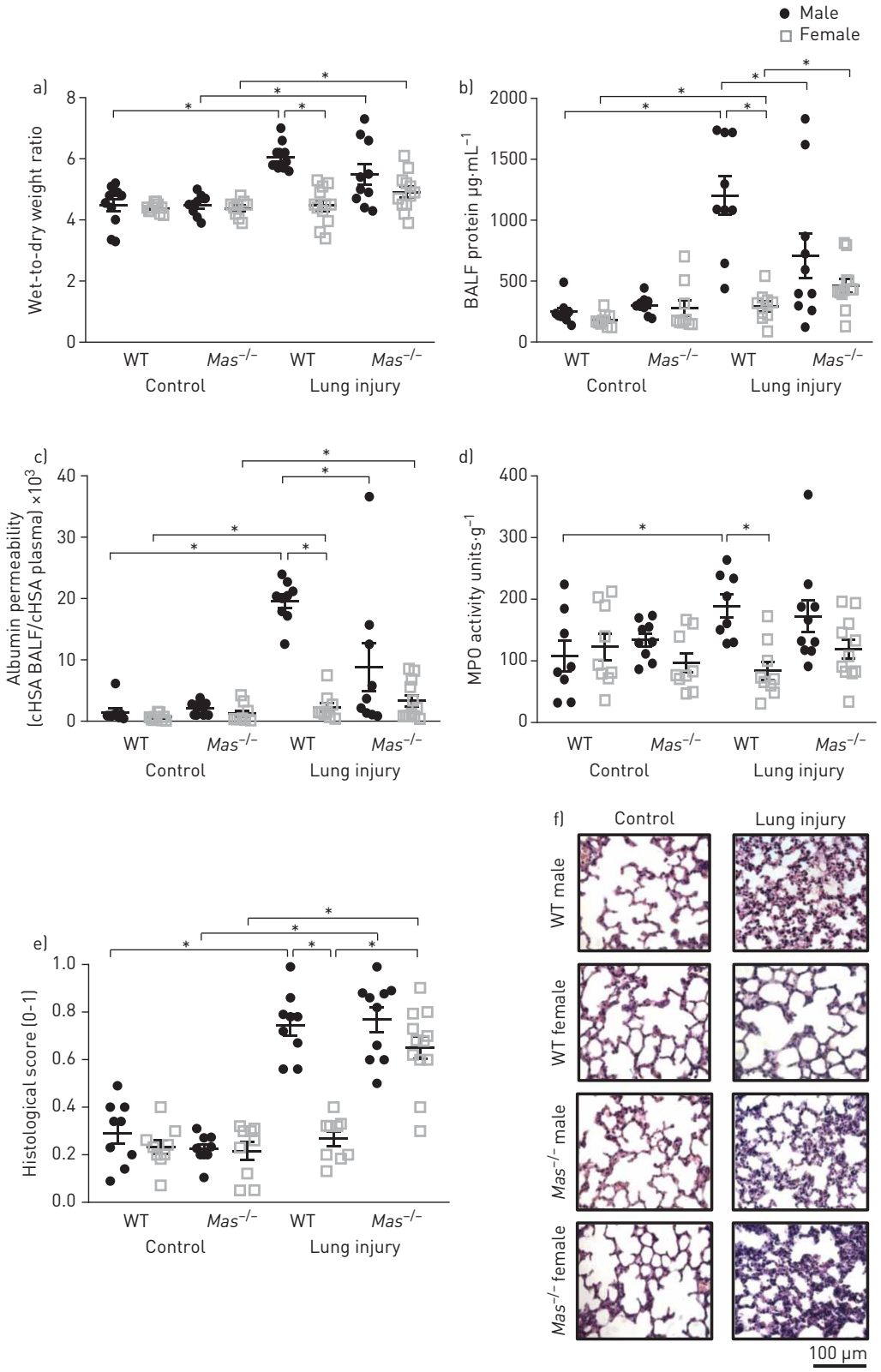

FIGURE 2 Sex-specific differences in severity of acute lung injury (ALI) are partially lost in Mas-receptor deficient (*Mas*$^{-/-}$) mice. Panels are as follows: a) wet-to-dry lung weight ratio; b) protein concentration in bronchoalveolar lavage fluid (BALF); c) human serum albumin (HSA) permeability (determined as the ratio of HSA concentration (cHSA) in BALF over that in plasma); d) lung myeloperoxidase (MPO) activity; and e) histological lung injury score. Representative histological images f) show haematoxylin (H) and eosin (E) stained lung sections for male and female *Mas*$^{-/-}$ mice and corresponding wild-type (WT) mice, which received 2 h of low tidal volume (V_T) ventilation (control mice), as well as male and female *Mas*$^{-/-}$ and corresponding WT mice which received hydrochloric acid instillation and 2 h of overventilation at 20 mL·kg^{-1} V_T (lung injury mice). Data are mean±standard error of the mean (SEM) (from n=8–12 mice each) and statistical difference was determined by Mann–Whitney U-test with Bonferroni correction for multiple comparisons. *: p<0.05.

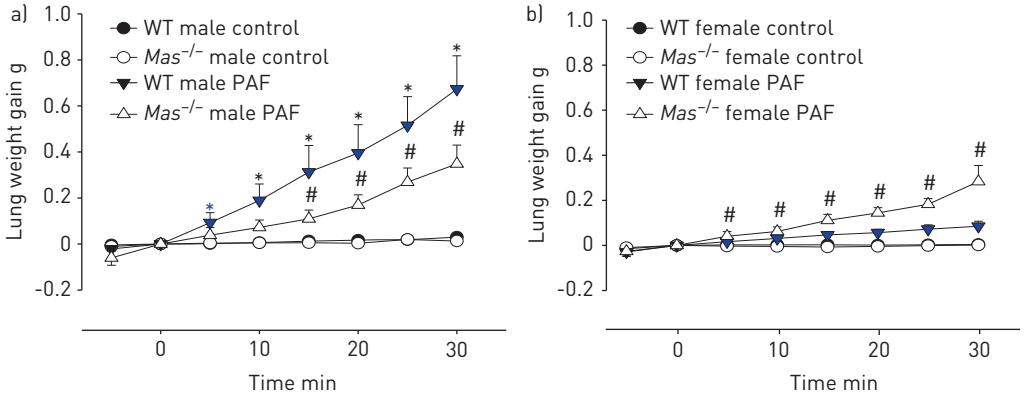

FIGURE 3 Permeability in the lung of female mice is protected from platelet-activating factor (PAF) in a Mas-receptor dependent fashion. Lung weight gain in response to a single 5 nmol bolus of PAF (at time=0 min) was used as a measure of endothelial permeability in isolated perfused lungs. Panels are as follows: a) comparison of control and PAF-stimulated lungs from male wild-type (WT) mice *versus* male Mas-receptor deficient (*Mas*$^{-/-}$) mice; b) comparison of control and PAF-stimulated lungs from female WT mice *versus* female *Mas*$^{-/-}$ mice. Data are mean±standard error of the mean (SEM) (from n=3–6 mice each) and statistical difference was determined by two-way repeated measures ANOVA. *: p<0.05 (comparison of lung weight gain between WT control mice and WT PAF-treated mice or Mas$^{-/-}$ control mice and Mas$^{-/-}$ PAF-treated mice); #: p<0.05 (comparison of lung weight gain between Mas$^{-/-}$ PAF-treated mice and WT PAF-treated mice).

Sex-specific regulation of the ACE2/Ang(1–7)/Mas axis in acute lung injury

To probe for sex-specific differential regulation in the ACE2/Ang(1–7)/Mas axis, we measured the activity of two key enzymes, ACE and ACE2, in the lungs of male and female mice. ACE activity did not differ between male and female WT mice, nor did it differ between control mice and those subjected to ALI (figure 5a). ACE2 activity markedly declined in ALI, yet no sex-specific differences were detectable (figure 5b).

We next tested for gender differences in the expression level of the Mas receptor. In whole lung tissue, expression of the Mas receptor was higher in female mice than in male mice (figure 5c). Specificity of the Mas receptor antibody was confirmed in the lungs of *Mas*$^{-/-}$ mice, which showed only minimal bands (figure 5c). This finding is in line with previous reports [18] and is likely attributable to the sequence

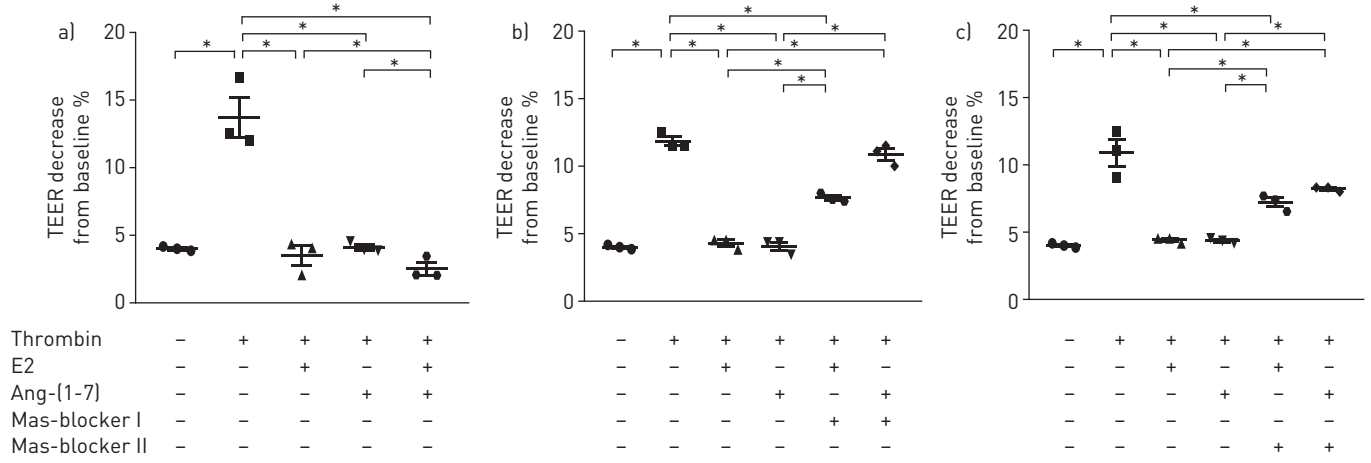

FIGURE 4 Permeability in human pulmonary microvascular endothelial cells (HPMECs) is protected from thrombin-induced barrier leakage in a Mas-receptor dependent fashion, as indicated by changes in transendothelial electrical resistance (TEER) in confluent monolayers of HPMECs. Panels are as follows: a) TEER changes in response to solvent only (control), thrombin (50 µL, 0.5 units·mL^{-1}), thrombin with 24 h pretreatment by 17β-oestradiol (E2) (1 nmol·L^{-1}), thrombin with 30 min pretreatment by angiotensin (Ang)-(1–7) (100 nmol·L^{-1}), or thrombin with both E2 and Ang-(1–7); b) TEER changes in response to solvent only (control), thrombin, thrombin with pretreatment by E2, thrombin with Ang(1–7), or thrombin in the presence of Mas-blocker I (A779) (100 nmol·L^{-1}) with either E2 or Ang(1–7); c) TEER changes in response to solvent only (control), thrombin, thrombin with pretreatment by E2, thrombin with Ang(1–7), or thrombin in the presence of Mas-blocker II (D-Pro^7-Ang(1–7)) (100 nmol·L^{-1}) with either E2 or Ang(1–7). Both receptor blockers were given 15 min before the agonist treatment. Data are mean±standard error of the mean (SEM) (from n=3 each) and statistical difference was determined by one-way ANOVA followed by the Student–Newman–Keuls test. *: p<0.05.

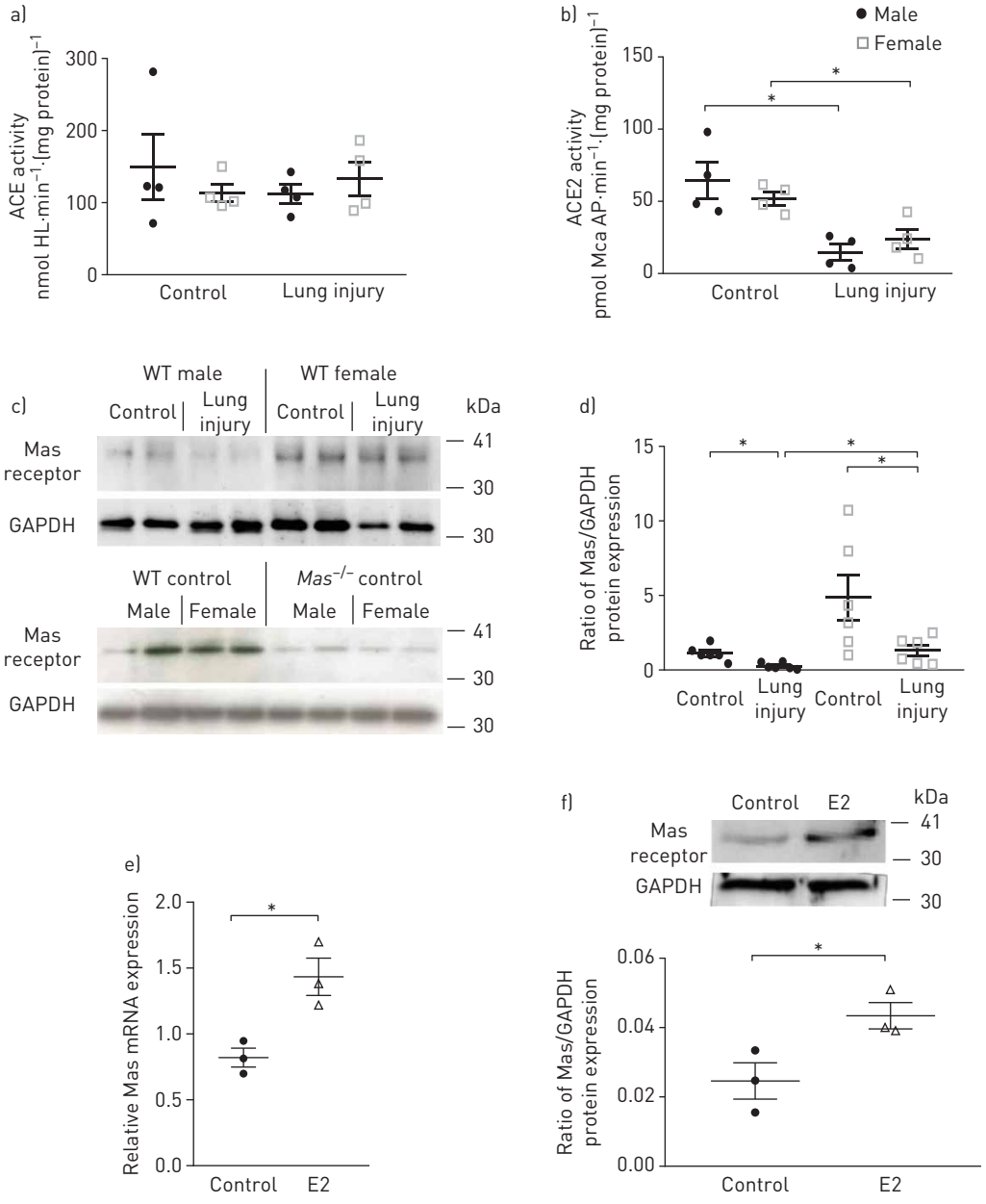

FIGURE 5 Sex-specific regulation of the angiotensin-converting enzyme 2 (ACE2)/angiotensin (Ang)-(1–7)/Mas axis in acute lung injury (ALI). Panels are as follows: a) angiotensin-converting enzyme (ACE) activity for wild-type (WT) mice; b) ACE2 activity from male and female WT mice under control conditions or with ALI induced by hydrochloric acid instillation and overventilation (OV); c) representative Western blots and d) Mas-receptor expression in the lungs of male and female WT mice under control and ALI (hydrochloric acid instillation and OV) conditions. Western blots from male and female WT and Mas-receptor deficient (*Mas*$^{-/-}$) mice (under control conditions) are included to demonstrate antibody specificity. Data are mean±standard error of the mean (SEM) (from n=4–6 mice each) and statistical difference was determined by Mann–Whitney U-test and Bonferroni correction for multiple comparisons. e) Quantitative PCR and f) Western blot analysis show increased Mas-receptor expression in human pulmonary microvascular endothelial cells (HPMECs) following stimulation by 17β-oestradiol (E2) (1 nmol·L^{-1} for 24 h) or solvent (control) (different lanes in f) are from independent cultures). Data are mean±SEM (from n=3 each) and statistical difference was determined by Mann–Whitney U-test and Bonferroni correction for multiple comparisons. HL: L-histidyl-L-leucine; Mca AP: Mca-Ala-Pro-Lys(Dnp)-OH; GAPDH: glyceraldehyde 3-phosphate dehydrogenase. *: p<0.05.

similarity between Mas and many other receptors of the Mas-like family, generating background traces due to minor cross reactivity. ALI reduced the expression of Mas in both males and females, yet Mas expression remained approximately five-fold higher in females than in males (figures 5c and 5d). These findings suggest that higher Mas expression may contribute to improved barrier function in females and

that loss of Mas may play an important role in lung vascular leakage independent of sex. Mas receptor expression was consistently increased by E2 in cultured HPMECs at the mRNA (figure 5e) and protein (figure 5f) level, suggesting that E2 stimulates signalling *via* the ACE2/Ang(1–7)/Mas axis by increasing expression of the Mas receptor rather than increased formation of its ligand.

Protection from acute lung injury is lost in ovariectomised mice

To verify the contribution of E2 in female endothelial barrier protection, we next investigated the effect of ALI in OVX mice. Compared to sham-operated female mice, OVX mice developed more pronounced lung injury in the two-hit ALI model, as indicated by an increased wet-to-dry lung weight ratio (figure 6a), BALF protein concentration (figure 6b) and histological lung injury score (figure 6e). No significant differences in HSA ratio or MPO activity were detected between OVX mice and sham mice under control and ALI conditions (figures 6c and 6d). Importantly, Mas receptor expression was more profoundly reduced by ALI in OVX mice compared to sham-operated mice (figure 6f), consolidating the notion that female sex and oestrogen confer endothelial barrier protection at least in part *via* an upregulation of the Mas receptor.

Discussion

Here, we confirm a higher prevalence of ARDS in men *versus* women in a large patient database. These epidemiological data were reproduced in a preclinical model which showed a striking protection of the lung endothelial barrier in female as compared to male mice. This protection was in part attributable to increased signalling *via* the ACE2/Ang(1–7)/Mas axis, as protection was partially lost in 1) $Mas^{-/-}$ female mice *in vivo*; 2) isolated lungs of $Mas^{-/-}$ female mice *ex vivo*; and 3) E2 pretreated HPMECs in the presence of the Mas receptor antagonists A779 (Mas-blocker I) or D-Pro^{7}-Ang(1–7) (Mas-blocker II). The protective role of female sex on lung endothelial barrier function was in part mediated *via* the female sex hormone oestrogen, as protection was lost in OVX female mice. Meanwhile, pretreatment with E2 (the predominant oestrogen in terms of serum levels and oestrogenic activity), replicated barrier protection in a Mas-receptor dependent manner *in vitro*. Increased signalling *via* the ACE2/Ang(1–7)/Mas axis in female mice, as opposed to male mice, was attributable to increased pulmonary expression of the Mas receptor rather than to increased ACE2 activity. Upregulation of the Mas receptor was replicated in E2 pretreated HPMECs, establishing a direct link between female sex and the ACE2/Ang(1–7)/Mas axis. Taken together, these findings suggest that female sex confers endothelial barrier protection from ALI at least in part *via* an oestrogen-dependent upregulation of the Mas receptor.

A previous meta-analysis of critical care patients with ARDS identified marked differences in sex-specific prevalence, with 61% men *versus* 39% women [19]. In addition, a higher risk of ARDS development for critically ill males, as compared to females, was subsequently replicated in a sepsis cohort [20] and also seems to apply to the recent SARS-CoV-2 outbreak [6]. Other studies have also reported a higher risk for women to develop ARDS following critical traumatic injury [8] and a higher mortality in female ARDS patients in a tertiary care hospital in India [21]. The results from our clinical data analyses, with a male to female distribution of 62.7% *versus* 37.3%, closely match and thus confirm the previous meta-analysis and the experience from the present COVID-19 pandemic, consolidating a marked sex difference in the prevalence of all-cause ARDS.

This effect was replicated in a murine ALI model with female mice showing protection for all hallmarks of the disease (*i.e.* barrier failure, inflammation and tissue injury) [22]. This finding is in line with a prior animal study demonstrating sex-specific protection from ALI [10]. In the past, this effect was attributed primarily to the immunomodulatory effects of oestrogen [10], or to improved alveolar fluid clearance secondary to an E2-mediated upregulation of the epithelial sodium channel (ENaC) [23].

In contrast, sex-specific differences in lung endothelial permeability have so far not been addressed. Here, we identified a marked stabilisation of the endothelial barrier in the lungs of female mice compared to male mice, evident as reduced wet-to-dry weight ratio and extravasation of protein and HSA into the BALF *in vivo*, and as reduced weight gain in response to PAF *in situ*. The latter finding highlights that vascular barrier stability is an inherent feature of the female lung and, specifically, the endothelial cell layer, and is not secondary to sex-specific activation of immune cells (which are absent in the isolated lung). Endothelial barrier protection in female mice was predominantly attributable to the female sex hormone oestrogen, as protection was largely lost in OVX mice *in vivo*, while pretreatment with E2 conferred protection from endothelial barrier failure *in vitro*. Taken together, our preclinical data demonstrate a stabilising effect of female sex on the lung endothelial barrier that is partly mediated by oestrogen. However, these findings are not entirely congruent with our clinical data, which show that the protective effect of female sex extends beyond menopause. Hence, sex differences in ARDS are likely multifactorial and may involve oestrogen-dependent and oestrogen-independent effects. Consistent with this notion we previously showed that, in the absence of E2, endothelial cells from female donors still

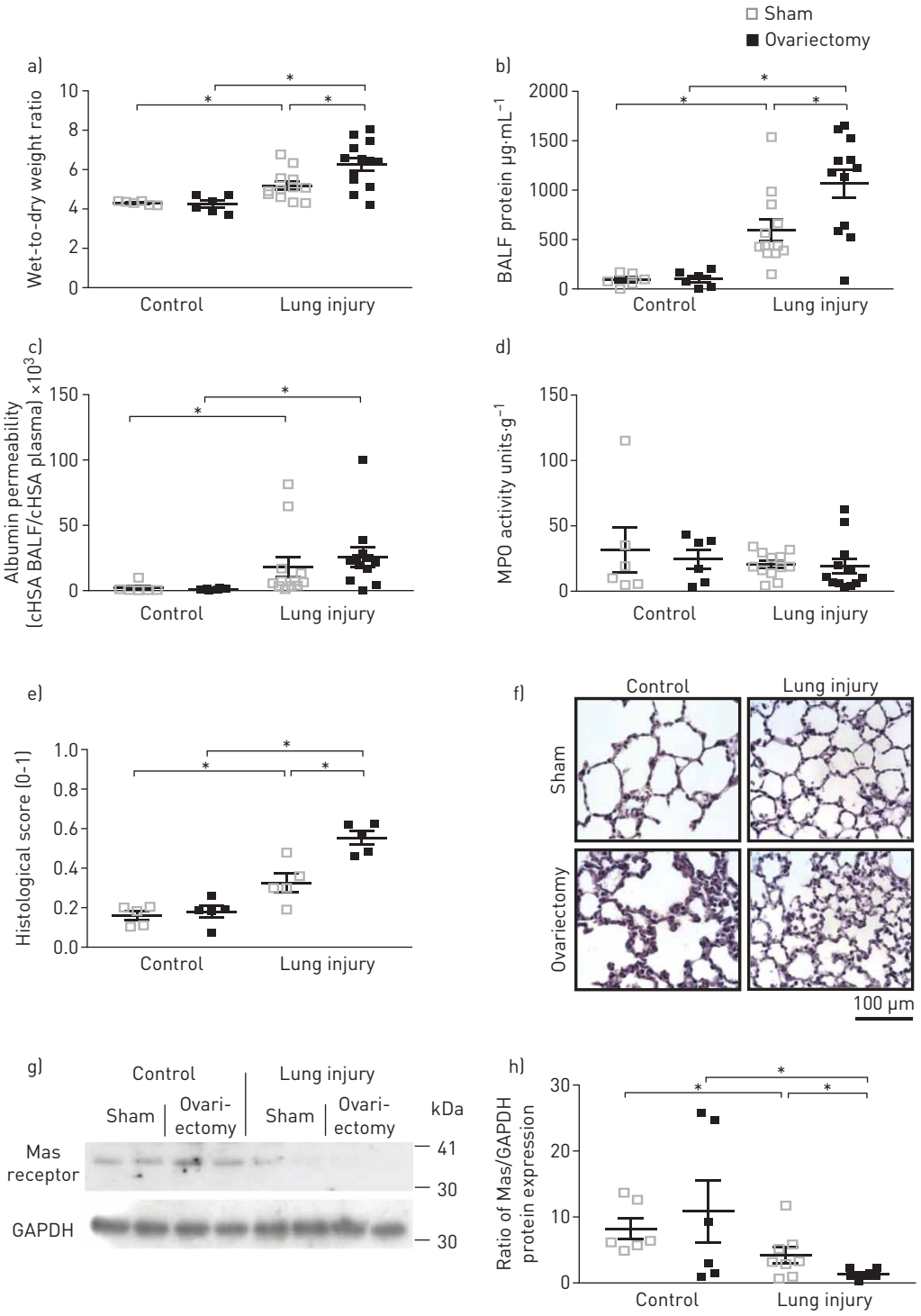

FIGURE 6 The protective effect of female sex in acute lung injury (ALI) is lost in ovariectomised (OVX) mice. Panels are as follows: a) wet-to-dry lung weight ratio; b) protein concentration in bronchoalveolar lavage fluid (BALF); c) human serum albumin (HSA) permeability (determined as the ratio of HSA concentration (cHSA) in BALF over that in plasma); d) lung myeloperoxidase (MPO) activity as a measure of neutrophil invasion; e) histological lung injury score; f) representative histological images showing haematoxylin (H) and eosin (E) stained lung sections for OVX and sham-operated female wild-type (WT) mice under control or ALI (induced by hydrochloric acid instillation and overventilation (OV)) conditions; g) representative Western blot and h) Mas-receptor expression in the lungs of OVX and sham-operated female WT mice under control and ALI (hydrochloric acid instillation and OV) conditions. Data are mean±standard error of the mean (SEM) (from n=5–12 mice each) and statistical difference was determined by Mann–Whitney U-test with Bonferroni correction for multiple comparisons. GAPDH: glyceraldehyde 3-phosphate dehydrogenase. *: p<0.05.

show a higher cell viability and express higher levels of genes indicative for immune responsiveness compared to male donors [24].

Part of the E2-dependent barrier protection in our preclinical model was mediated *via* increased signalling through the ACE2/Ang(1–7)/Mas axis, as ALI signs were aggravated in female mice deficient for the Mas receptor. These effects related primarily to features of vascular permeability, while MPO activity as a measure of neutrophil infiltration was unchanged. Although these findings point to a role for the Mas receptor in endothelial barrier regulation, the *in vivo* phenotype of the global Mas knockout mouse cannot be attributed to a specific cell type. The role of the Mas receptor in sex-specific endothelial barrier protection was therefore further consolidated in isolated mouse lung from female $Mas^{-/-}$ mice, which showed increased lung weight gain in response to PAF as compared to the corresponding WT mice. Similarly, barrier protection by E2 pretreatment in thrombin-treated HPMECs was attenuated by Mas receptor antagonists. Increased signalling *via* the ACE2/Ang(1–7)/Mas axis has been shown by us and others to have barrier protective effects in ALI [13, 14, 25], presumably *via* a set of signalling pathways that include, amongst others, PI3K/AKT signalling [26] and endothelial nitric oxide formation [13]. A critical role for the ACE2/Ang(1–7)/Mas axis in sex-specific differences has previously been documented in cardiovascular diseases of the systemic circulation (though not the pulmonary circulation). Induction of systemic hypertension by AngII is attenuated in intact female rats as compared to males or OVX females, yet this protection is abrogated by the Mas receptor antagonist A779 (Mas-blocker I) [27]. In hypertensive rats, renal Ang(1–7) levels are lower in males as compared to females [28]. Conversely, female mice on a high-fat diet have higher plasma levels of Ang(1–7), yet this effect is lost after they are OVX [29].

Lack of ACE2/Ang(1–7)/Mas signalling in $Mas^{-/-}$ mice reduced the sex-specific differences in wet-to-dry weight ratio and BALF protein concentration in ALI by ∼70%. This effect was only partially attributable to aggravated barrier failure in female $Mas^{-/-}$ mice, but was also due to a tendency for barrier stabilisation in male $Mas^{-/-}$ mice relative to their WT equivalents. This barrier stabilisation was consistently evident, both in isolated lungs as reduced weight gain in response to PAF and *in vivo* as reduced BALF protein and HSA extravasation. The potential mechanisms by which Mas signalling may aggravate ALI in male mice remain to be elucidated. Notably, in addition to its established protective effects, Mas signalling has also been implicated in several potentially injurious pathways, such as endothelial nitric oxide synthase (eNOS) uncoupling [30], or disease processes such as renal fibrosis [31]. What determines whether Mas-dependent signalling triggers protective or injurious effects remains unclear so far, but may potentially involve Mas ligands other than Ang(1–7) and/or biased agonism *via* the Mas receptor, as well as modulation of Mas-receptor signalling by posttranslational modifications or by interaction with other receptors or downstream pathways. In line with this notion, the findings of our present study suggest that female *versus* male sex, or sex hormones, regulate cellular responses not only *via* Mas receptor expression but presumably also *via* modulation of signalling pathways downstream of the Mas receptor, or *via* parallel signalling pathways that interact with Mas-receptor signalling.

The detrimental effects of Mas-receptor deficiency in female ALI were particularly prominent in histological assessment by a composite lung injury score [22], potentially because of an amplification effect due to the cumulative nature of the score. However, sex-specific differences in albumin extravasation were affected by Mas-receptor deficiency to little or no degree. This seeming discrepancy may be explained by the fact that albumin leakage across the stimulated lung microvascular endothelium is largely the result of active transcytosis rather than paracellular gap formation, which may not be regulated by Mas-receptor signalling [32]. Previous studies have located the ACE2 gene on the X chromosome [33], suggesting that ACE2 expression and hence Ang(1–7) formation may be genetically increased in females as compared to males. No difference in ACE2 activity was detected between male and female lung tissue in the present study. Instead, the protective effect of female sex was largely abrogated in OVX mice, suggesting that protection was conferred by a hormonal rather than genetic mechanism. We found the Mas receptor to be approximately five-fold upregulated in both healthy and injured lungs of female mice compared to their respective male counterparts, an effect that was likely mediated by oestrogen as Mas-receptor expression was more drastically reduced *in vivo* by ALI in OVX mice compared to sham-operated female mice and upregulated *in vitro* by exogenous E2 treatment in HPMECs. Similar higher expression of the Mas receptor in females compared to males has previously been demonstrated in various models of systemic cardiovascular disease associated with a relative protection of the female sex [28, 34]. This suggests that increased Mas expression suffices to confer protection *via* increased ACE2/Ang(1–7)/Mas signalling, just as deficiency of Mas aggravated ALI in the present study. Importantly, sex-specific differences in Mas expression seem to be equally evident in humans, in that Mas mRNA abundance is higher in decidual explants collected from women carrying a female as compared to a male fetus [35]. Higher expression levels of the Mas receptor in females compared to males may imply that female ARDS patients could potentially profit more from ACE2/Ang(1–7) treatment than males, in personalised therapy, a concept that

presently escapes experimental testing in preclinical murine models given the modest degree of injury in untreated females.

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