Endothelial Cell Vasodilator Dysfunction Mediates Progressive Pregnancy-induced Hypertension in Endothelial Cell Tetrahydrobiopterin Deficient Mice

Running Title: *Chuaiphichai et al*; *Maternal BH4 and Vascular Adaptation to Pregnancy*

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Abstract

Background and purpose: Pregnancy-associated vascular remodeling is essential for both maternal and fetal health. We have previously shown that maternal endothelial cell tetrahydrobiopterin (BH4) deficiency causes poor pregnancy outcomes. Here, we investigated the role and mechanisms of endothelial cell-mediated vasorelaxation function in these outcomes.

Experimental approach: The vascular reactivity of mouse aortas and uterine arteries from non-pregnant and pregnant endothelial cell-specific BH4 deficient mice (*Gch1*$^{fl/fl}$Tie2cre mice) was assessed by wire myography. Systolic blood pressure was assessed by tail cuff plethysmography.

Key results: In late pregnancy, systolic blood pressure was significantly higher (~24 mmHg) in *Gch1*$^{fl/fl}$Tie2cre mice compared with wild-type littermates. This was accompanied by enhanced vasoconstriction and reduced endothelial-dependent vasodilation in both aorta and uterine arteries from pregnant *Gch1*$^{fl/fl}$Tie2cre mice. In uterine arteries loss of eNOS-derived vasodilators was partially compensated by upregulation of intermediate and large-conductance Ca^{2+}-activated K^+ channels. In rescue experiments, oral BH4 supplementation alone did not rescue vascular dysfunction and pregnancy-induced hypertension in *Gch1*$^{fl/fl}$Tie2cre mice. However, combination with the fully reduced folate, 5-methyltetrahydrofolate (5-MTHF), restored endothelial cell vasodilator function and blood pressure.

Conclusions and implications: We identify a critical requirement for maternal endothelial cell *Gch1*/BH4 biosynthesis in endothelial cell vasodilator function in pregnancy. Targeting

vascular *Gch1* and BH4 biosynthesis with reduced folates may provide a novel therapeutic target for the prevention and treatment of pregnancy-related hypertension.

1. Introduction

Hypertensive disorders of pregnancy such as preeclampsia and gestational hypertension are associated with a higher risk of cardiovascular disease for both mother and offspring in later life. Women with these complications during pregnancy have a 4-fold increased risk of developing hypertension [1, 2] and a 2-fold increased risk of developing stroke after pregnancy [1]. Offspring from mothers with hypertensive disorders of pregnancy are also likely to have a higher risk of hypertension and other cardiovascular disease in later in life [3, 4].

The maternal cardiovascular system must undergo significant remodelling to support the growing fetus. By term, the uteroplacental bed receives more than 12% of cardiac output. In order to accommodate this increase in flow, vascular resistance is reduced by outward remodeling and vasodilatation of more proximal uterine arteries, together with trophoblast invasion and remodelling of spiral arteries [5]. Failure of this vascular adaptation is associated with adverse outcomes including pregnancy induced hypertension and pre-eclampsia. Therefore, understanding the mechanisms underlying vascular adaptations and how they impact blood pressure regulations during pregnancy are of great importance.

Nitric oxide (NO), generated by endothelial nitric oxide synthase (eNOS), is a key mediator of vascular adaptation in pregnancy. During pregnancy, enhanced uteroplacental vasodilation is driven by increased NOS-derived NO in humans, mice, and rats [6-9]. Increased uterine artery calibre is associated with both enhanced eNOS expression, activity, and NO bioavailability [10-12]. Loss of eNOS leads to impaired uterine artery remodelling during pregnancy mediated in part by a reduced activation of matrix metalloproteinase in eNOS-knockout mice [13]. NO bioavailability is also central to physiological adaptation in the

more distal uteroplacental circulation with increased expression of eNOS observed in remodelling spiral arteries and in cytotrophoblasts and syncytiotrophoblasts [14]. However, simple strategies attempting to restore or augment NO with NO donors in pre-eclampsia have been disappointing [15]. One possible explanation for these results is the failure to specifically target eNOS uncoupling and consequently altered NO/ROS signalling. To date, the role of eNOS coupling and how uncoupled eNOS impacts on uterine artery remodelling and vascular function during pregnancy is poorly understood.

The generation of NO by eNOS requires the small molecule tetrahydrobiopterin (BH4). Loss of BH4 results in eNOS uncoupling, loss of NO generation and increased production of superoxide and other reactive oxygen species (ROS). Biosynthesis of BH4 is catalyzed by GTPCH (GTP cyclohydrolase 1, encoded by *Gch1*, the rate-limiting enzyme for de novo BH4 biosynthesis). We have previously shown that *Gch1* expression is a key determinant of BH4 bioavailability, eNOS regulation and NO and superoxide generation [16, 17]. Recently, we have demonstrated that maternal endothelial cell BH4 deficiency, due to loss of *Gch1*, leads to progressive hypertension and fetal growth restriction during pregnancy [18]. However, the mechanism mediating these changes and how loss of endothelial cell-specific *Gch1* alters uterine artery function both before and during pregnancy and if these changes are reversible are yet to be answered. To address this question, we assessed vascular function and blood pressure in pregnant and non-pregnant endothelial cell specific *Gch1* knock out mice.

2. Methods

2.1 Generation of Endothelial Cell –Targeted Gch1 Knockout Mice

Endothelial cell–specific BH4 deficient mice and their littermates controls were generated by crossing *Gch1*$^{fl/fl}$ females with *Gch1*$^{fl/fl}$ Tie2cre male mice as described previously [19]. For myeloid cell-specific deletion of *Gch1* mice and their littermates controls, *Gch1*$^{fl/fl}$ females were crossed with *Gch1*$^{fl/fl}$ LysM cre male mice. Mice were housed in ventilated cages (between 4-6 mice per cage of mixed genotypes) in specific pathogen free conditions with a 12-hour light/dark cycle and controlled temperature (20–22°C), and fed standard chow (Teklad global 16% protein diet, Harlan Laboratories) and water ad libitum. Adult female *Gch1*$^{fl/fl}$Tie2cre mice, *Gch1*$^{fl/fl}$ LysM cre and their *Gch1*$^{fl/fl}$ littermates (thereafter referred to as wild-type) on a pure (>10 generations) C57BL6/J background were bred in house and were used for all experiments at 10 to 16 weeks. The generation and phenotyping of the knock-out model were carried out in accordance with the Animal (Scientific Procedures) Act 1986, with procedures reviewed by the clinical medicine animal care and ethical review body (AWERB), and conducted under project licenses PPL 30/3080 and P0C27F69A. Mice were genotyped by polymerase chain reactions using DNA prepared from ear biopsies. For *Gch1*$^{fl/fl}$ genotyping, PCR was performed using the following primers: *Gch1*$^{fl/fl}$-Fw 5'-GTC CTT GGT CTC AGT AAA CTT GCC AGG-3', *Gch1*$^{fl/fl}$-Rv 5'-GCC CAG CCA AGG ATA GAT GCA G-3'. The *Gch1* floxed allele showed a 1030 bp. For F Cre genotyping, PCR was performed using the following primers: Cre Fw 5'-GCA TAA CCA GTG AAA CAG CAT TGC TG-3'. Cre Rv 5'-GGA CAT GTT CAG GGA TCG CCA GGC G-3'. The Tie2cre allele amplified as 280 bp fragment.

2.2 Timed mating

Pregnancy was achieved by mating either virgin female *Gch1*$^{fl/fl}$Tie2cre or *Gch1*$^{fl/fl}$ LysM cre or *Gch1*$^{fl/fl}$ (wild-type) females (aged between 10 to 16 weeks old) with a *Gch1*$^{fl/fl}$ male. Detection of a vaginal plugs indicated successful conception and was taken as 0.5 day of gestation (E0.5). Unless otherwise stated, all tissues were harvested and collected for experiments at either preconception (before timed mating; age-matched controls) or E18.5 day of gestation (late gestation).

2.3 Vasomotor function studies

Vasomotor function in uterine arteries (main branch) and aortas from both non-pregnant (age matched) and pregnant (E18.5) *Gch1*$^{fl/fl}$Tie2cre and wild-type littermates was examined using isometric tension studies in a wire myograph (MultiMyogrph 610M, Danish Myo Technology, Denmark). Briefly, mice were killed by overdose of inhaled isoflurane and vascular rings were isolated from the uterine horns or thoracic aorta. The 2-mm rings were mounted in a wire myograph containing 5 ml of ice-cold Krebs-Henseleit buffer (KHB [in mmol/l]: NaCl 120, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, CaCl2 2.5, NaHCO3 25, glucose 5.5) at 37°C, gassed with 95% O_2/5% CO_2. After allowing vessels to equilibrate for 30 minutes, the optimal resting tension equivalent to 100 mmHg was set. Concentration-response contraction curves were established using cumulative half-log concentrations of U46619 (thromboxane A2 receptor agonist; uterine artery) and phenylephrine (aorta) respectively. Vessels were washed three times with fresh KHB, equilibrated for 20 minutes, and then precontracted to approximately 80-90% of maximal tension with U46619 for uterine arteries or with phenylephrine for aortas. Acetylcholine was used to stimulate endothelium-dependent vasodilatations in increasing cumulative concentrations. Responses were

expressed as a percentage of the pre-contracted tension. Finally, the NO donor sodium nitroprusside (SNP) was used to test endothelium-independent smooth muscle relaxation in the presence of L-NAME. All pharmacological drugs were pre-incubated at least 20 min before the dose-response curves were determined. L-NAME was used at 100 μM, apamin at 50 nM, charybdotoxin at 100 nM, and indomethacin at 10 μM. All drugs used were purchased from Sigma Chemical Company.

2.4 Blood pressure measurement by tail-cuff plethysmography

Systolic blood pressure in conscious wild-type, *Gch1*$^{fl/fl}$ Tie2cre and *Gch1*$^{fl/fl}$ LysM cre mice was determined using the Visitech^{R} computerized tail-cuff plethysmography system (Visitech, USA) following 5 days training and 3 days baseline periods. Experiments were performed between the hours of 8 and 12 am. The animal tails were passed through a cylindrical latex tail-cuff and taped down to reduce movement. Twenty readings were taken per mouse of which the first 5 readings were discarded. The remaining 15 readings were used to calculate the mean systolic blood pressure in each mouse. Systolic blood pressure of plugged *Gch1*$^{fl/fl}$Tie2cre and WT mice were determined throughout gestation (embryonic day E0, E2.5, E5.5, E7.5, E10.5, E12.5, E15.5, E16.5, E17.5, and E18.5).

2.5 Quantitative real-time RT-PCR

Total RNA was extracted using the Ambion Pure Link kit. Reverse transcription was carried out using QuantiTect reverse transcription kit (Qiagen, Hilden, Germany, UK) on 1 μg total cell RNA. Quantitative real-time RT–PCR was performed with an iCycler IQ real-time detection system (BioRad Laboratories, Hercules, USA) using primers and probes from the TaqMan Gene

Expression Assay system (Life Technologies, Loughborough, UK). Gene expression data were normalized to β-actin.

2.6 Determination of Tissue Tetrahydrobiopterin Levels

BH4 and oxidised biopterins (BH2 and biopterin) were determined by high-performance liquid chromatography (HPLC) followed by electrochemical and fluorescence detection, respectively, following an established protocol [20]. Briefly, cell pellets were freeze-thawed in ice-cold resuspension buffer (50 mmol·L^{-1} phosphate-buffered saline, 1 mmol·L^{-1} dithioerythriol, 1 mmol·L^{-1} EDTA, pH 7.4). After centrifugation at 13,200 rpm for 10 min at 4°C, supernatant was removed and ice-cold acid precipitation buffer (1 mol·L^{-1} phosphoric acid, 2 mol·L^{-1} trichloroacetic acid, 1 mmol·L^{-1} dithioerythritol) was added. Samples were vigorously mixed and then centrifuged for 15 min at 13,000 rpm and 4 °C. Samples were injected onto an isocratic HPLC system and quantified using sequential electrochemical (Coulochem III, ESA, Inc.) and fluorescence (Jasco) detection. HPLC separation was performed using a 250-mm ACE C-18 column (Hichrom) and a mobile phase comprised of sodium acetate (50 mM), citric acid (5 mM), EDTA (48 μM), and dithioerythritol (160 μM) (pH 5.2) (all ultrapure electrochemical HPLC grade) at a flow rate of 1.3 ml/min. Background currents of +500 μA and −50 μA were used for the detection of BH4 on electrochemical cells E1 and E2, respectively. 7,8-BH2 and biopterin were measured using a Jasco FP2020 fluorescence detector set at 510 nm excitation and 595 nm emission. Quantification of BH4, BH2, and B was done by comparison with authentic external standards and normalized to sample protein content, determined by the BCA protein assay (Pierce).

2.7 Western blot analysis

Immunoblotting in BMDM were performed to evaluate protein levels of GTPCH (1:10,000 dilution; a gift from S.Gross, Cornell University New York), iNOS (1: 1,100 dilution; Abcam), beta-tubulin (1:20,000; Abcam) followed by appropriate HRP-conjugated secondary antibody (1:10,000-20,000 dilution; Promega). Protein bands were visualised by enhanced chemiluminescence (Super West Pico Chemiluminescence, Thermo Scientific).

2.8 Supplementation of BH4 and BH4 with 5-methyltetrahydrofolate (5MTHF)

WT and *Gch1*$^{fl/fl}$ Tie2cre mice were allocated to normal chow or BH4-supplemented chow (200 mg/kg per day) or BH4 (200 mg/kg per day) +5MTHF (10 mg/kg per day) supplemented chow beginning at 3 days prior timed mating.

2.9 Isolation of murine bone marrow-derived macrophages

Bone marrow was obtained by flushing the femur and tibia of adult mice with PBS as described previously [21]. Briefly, a single cell suspension was prepared by passing the bone marrow through a 70 μm cell strainer. Cells were then cultured in 10 cm nontissue culture treated dishes for 7 days in DMEM:F12 (Invitrogen) supplemented with 100 U/ml penicillin and 100 ng/ml streptomycin (Sigma), 10% (v/v) fetal bovine serum (PAA Laboratories), 5 mM L-glutamine (Sigma), and 10–15% (v/v) L929 conditioned medium at 37 °C and 5% CO_2.

2.10 Stimulation of bone marrow-derived macrophages

Following differentiation cells were harvested and plated into 6-well plates containing serum-free media (Optimem supplemented with 100 U/ml penicillin and 100 ng/ml streptomycin and 0.2% (w/v) low-endotoxin bovine serum albumin (Sigma)). Cells were stimulated with 10 ng/ml IFNγ (Peprotech EC) and 100 ng/ml LPS (Sigma) for 24 h, parallel wells were left unstimulated. After 24 h cell pellets, and cell culture supernatants were collected, or the cells subjected to biochemical analysis.

2.9 Solutions and Drugs

All drugs were obtained from Sigma-Aldrich (Poole, UK) with the exception of BH4 (Schircks Laboratories, Switzerland), 5-MTHF (Merck, Switzeerland). All drugs were dissolved in distilled water, with the exception of indomethacin, which was dissolved in ethanol and U46619, which was dissolved in DMSO and then diluted in physiological buffer for experimentation (pH 7.4 at 37 °C), keeping the final DMSO concentration below 1:1,000 to avoid vehicle-associated artifacts.

2.10 Statistical analysis

All data are reported as mean ± SEM. The experimental unit (n) was defined as a single animal, animals of both genotypes were caged together, and animals of both genotypes were derived from more than one cage in all experiments. Statistical analyses were performed using Graphpad Prism version 9.3.0. (San Diego, USA). Concentration-response curves were fitted to a sigmoidal curve using non-linear regression with the aid of the statistical software GraphPad Prism Version 9.3.0. Data were analyzed for statistical significance using Student's

t-test for unpaired observations. When comparing multiple groups data were analysed by analysis of variance (ANOVA) with Newman–Keuls post-test for parametric data or Kruskal–Wallis test with Dunns post-test for non-parametric data. When more than two independent variables were present a two-way ANOVA with Tukey's multiple comparisons test was used. When within subject repeated measurements were present a repeated measures (RM) ANOVA was used. A value of $P < 0.05$ was considered statistically significant. Data were collected and analyzed with the operator blind of treatment allocation. Randomization was performed by cage.

3. Results

3.1 Loss of endothelial cell Gch1 and BH4 lead to progressive hypertension during pregnancy.

We have previously characterised this mouse model of endothelial cell *Gch1* deficiency [19, 22]. We demonstrated that endothelial cell deletion of *Gch1* resulted in a ~90% reduction in *Gch1* expression and GTPCH protein in endothelial cells leading to a ~90% reduction in BH4 content. A significant reduction in vascular BH4 levels was observed in both conduit and resistance vessels with a ~80% and ~90 reduction respectively [19, 22]. We also previously demonstrated that in both non-pregnant and pregnant mice, BH4 and total biopterin levels in aortas and uterine arteries from *Gch1*$^{fl/fl}$Tie2cre mice were significantly lower compared with that of WT mice [18]. Consistent with our previous report, we observed a small but significant increase in basal systolic blood pressure (~5-7 mmHg) in *Gch1*$^{fl/fl}$Tie2cre mice compared with wild-type littermates (WT: 103 ± 2 mmHg, vs *Gch1*$^{fl/fl}$Tie2cre mice: 110 ± 1 mmHg, $P<0.05$, n=6; Fig. 1A). In wild type mice, systolic blood pressure was unchanged

during pregnancy (baseline WT: 103 ± 2 mmHg, vs late pregnant WT: 103 ± 3 mmHg, n=6; Fig. 1A). In contrast, loss of endothelial cell *Gch1* resulted in a significantly marked increase (~24 mmHg) in systolic blood pressure in later pregnancy (at E18.5 day of gestation) in *Gch1*$^{fl/fl}$Tie2cre mice (Fig. 1A).

As Tie2cre can lead to cre-mediated gene deletion in hematopoietic cells, we next generated a novel mouse of myeloid cell-specific *Gch1* deficiency, the *Gch1*$^{fl/fl}$ LysM cre mouse. In these mice, there is a significant reduction in *Gch1* expression, GTPCH protein and thus BH4 content in myeloid cells (Supplementary Fig. 1). In contrast to the *Gch1*$^{fl/fl}$Tie2cre mouse, no difference in blood pressure was observed between *Gch1*$^{fl/fl}$ LysM cre or *Gch1*$^{fl/fl}$ (i.e. WT) mice either at baseline (WT: 99 ± 2 mmHg, vs *Gch1*$^{fl/fl}$ LysM cre mice: 99 ± 3 mmHg, $P<0.05$, n=7 to 10 animals per group) or at any point during pregnancy (Supplementary Fig. 1). In addition, no difference in either placenta or fetal weight was observed at E18.5 day of gestation between *Gch1*$^{fl/fl}$ LysM cre and wild-type mice.

3.2 Deficiency in endothelial cell Gch1/BH4 during pregnancy leads to impaired vascular function in aortas from pregnant Gch1$^{fl/fl}$*Tie2cre mice.*

First, we determined the physiological requirement for endothelial cell-specific *Gch1*/BH4 biosynthesis in vasomotor function in conduit arteries from both nonpregnant and pregnant *Gch1*$^{fl/fl}$Tie2cre and wild-type mice using wire myography. Isometric tension studies in isolated aortas demonstrated no difference in vasoconstriction responses to phenylephrine prior to pregnancy between the genotypes (Fig. 1B). However, at late stage of pregnancy loss of endothelial cell *Gch1/*BH4 lead to a significant increase in vasoconstriction to

phenylephrine (Fig. 1B). This difference was unlikely due to structural difference between genotypes in pregnant mice as constrictive responses to KCl were similar across all groups (nonpregnant WT: 4.68 ± 0.41mN, vs. nonpregnant *Gch1*$^{fl/fl}$Tie2cre: 5.13 ± 0.32mN, Pregnant WT: 4.35 ± 0.19mN, vs. Pregnant *Gch1*$^{fl/fl}$Tie2cre: 4.74 ± 0.19, $P<0.05$; Fig. 1C). In the presence of L-NAME, vasoconstriction in response to phenylephrine was greatly enhanced in all groups, such that vasoconstrictions were no longer different between wild-type and *Gch1*$^{fl/fl}$Tie2cre mice (Fig. 1D), indicating that the increased vasoconstrictor response in pregnant *Gch1*$^{fl/fl}$Tie2cre aortas is likely mediated by a loss of tonic eNOS-derived vasodilatators.

There was no difference in endothelium dependent vasodilatation to acetylcholine between the genotypes prior to pregnancy (Fig. 1E and F). However, in late stage of pregnancy, endothelium-dependent vasodilatation to acetylcholine was significantly impaired in pregnant *Gch1*$^{fl/fl}$Tie2cre aortas when compared with aortas from pregnant wild-type mice and aortas from non-pregnant *Gch1*$^{fl/fl}$Tie2cre mice, with a corresponding decrease in maximum relaxation (Emax) to acetylcoline (Fig. 1E and F). In addition, preconstricted tension to phenylephrine for the acetylcholine relaxation curves was not different between groups (Fig. 1G). In the presence of L-NAME, endothelium-dependent vasodilatation to acetylcholine in pregnant and nonpregnant mice of both genotypes was totally abolished (Figure 1H), indicating that eNOS is the major component of ACh-induced vasodilation in the aortas of both pregnant and non-pregnant WT and *Gch1*$^{fl/fl}$Tie2cre mice. Interestingly, we found that pregnancy significantly increased the potency of endothelium-independent vasodilatation to the nitric oxide donor, sodium nitroprusside (SNP) in aortas from both wild-type and *Gch1*$^{fl/fl}$Tie2cre mice when compared to aortas from nonpregnant wild-type mice (Fig. 1I), suggesting that a normal pregnancy is associated with an increased sensitivity to nitric oxide downstream signalling pathway in conduit arteries.

3.3 Loss of endothelial cell Gch1/BH4 during pregnancy caused vascular dysfunction in uterine arteries from pregnant Gch1$^{fl/fl}$Tie2cre mice.

We next determine how loss of endothelial cell *Gch1/*BH4 impacts on pregnancy induced vascular adaptations of the uterine artery. Firstly, the lumen diameter of uterine arteries, as calculated by the length-tension relationship, was significantly increased in pregnancy in both genotypes with no difference between genotypes observed in pregnant or non-pregnant mice (Pregnant WT: 258 ± 12.6 μm, Pregnant *Gch1*$^{fl/fl}$Tie2cre: 276 ± 12.7 μm; Nonpregnant WT 155 ± 6.6 μm, nonpregnant *Gch1*$^{fl/fl}$Tie2cre: 153 ± 3.1*; P<0.05*) (Fig. 2A). This was accompanied by a greater KCl response (~2-fold increased) in uterine arteries from pregnant wild-type and *Gch1*$^{fl/fl}$Tie2cre mice (Pregnant WT: 5.8 ± 0.7 mN, Pregnant *Gch1*$^{fl/fl}$Tie2cre: 5.1 ± 0.4 mN, nonpregnant WT: 2.6 ± 0.3 mN, nonpregnant *Gch1*$^{fl/fl}$Tie2cre: 2.3 ± 0.4; *P<0.05*) (Fig. 2A), indicating that a normal pregnancy is associated with increased lumen size and media thickness of the uterine artery.

In contrast to the aorta, uterine arteries from pregnant wild type mice had a significantly attenuated contractile response to the thromboxane A2 receptor agonist, U46619, (Fig. 2B). Incubation with the eNOS inhibitor L-NAME led to a significant augmentation of the contractile response in uterine arteries from pregnant wild-type mice (Fig. 2C), indicating that the pregnancy induced attenuation was due to increased eNOS-derived NO.

In contrast, uterine arteries from pregnant *Gch1*$^{fl/fl}$Tie2cre mice showed incomplete adaptation to pregnancy with a significantly greater contractile response observed compared with arteries from pregnant wild type mice (Fig. 2B). This difference appeared to be driven in part by a reduced NOS dependent production of vasodilators in uterine arteries from pregnant

Gch1$^{fl/fl}$Tie2cre mice. As in contrast to wild type mice, L-NAME did not alter the vasoconstrictor response in uterine arteries from pregnant *Gch1*$^{fl/fl}$Tie2cre mice (Fig. 2C).

In wild-type uterine arteries, endothelium-dependent vasodilatation to ACh was significantly enhanced in pregnancy compared to nonpregnant wild-type controls (Fig. 2D and E; $P<0.05$). This was accompanied by a significantly enhanced endothelium-independent vasodilatation in response to nitric oxide donor, sodium nitroprusside (SNP), indicating increased vascular smooth muscle sensitivity to downstream NO signalling pathway during pregnancy (Fig. 2G).

In contrast, pregnancy induced enhanced endothelium-dependent vasodilatations was blunted in pregnant *Gch1*$^{fl/fl}$Tie2cre uterine arteries with no difference in endothelial cell mediated vasodilation between pregnancy and non-pregnant uterine arteries from *Gch1*$^{fl/fl}$Tie2cre mice (Fig. 2D and E). In addition, there was no difference in preconstricted tension to U46619 between the groups for acetylcholine relaxation curves (Fig. 2F). This blunted pregnancy induced vascular remodelling was observed despite the presence of pregnancy induced enhanced endothelium-independent vasodilatation in response to nitric oxide donor, SNP (Fig. 2G).

3.4 Contribution of NOS, cyclooxygenase-derived vasodilator, and Endothelium-dependent Hyperpolarization (EDH) in uterine arteries from pregnant mice deficient in endothelial cell BH4.

We next determined the relative contributions of the eNOS-derived vasodilators, cyclooxygenase-derived vasodilator, and EDH in uterine arteries of pregnant mice lacking endothelial cell BH4. Firstly, we found that in the presence of L-NAME, endothelium-dependent vasodilatation was significantly inhibited in uterine arteries from pregnant and

nonpregnant wild-type and nonpregnant *Gch1*$^{fl/fl}$Tie2cre mice, but was unaltered in uterine arteries from pregnant *Gch1*$^{fl/fl}$Tie2cre mice (Fig. 3A and B), adding further evidence to the loss of eNOS-mediated vasodilator function in uterine arteries of pregnant *Gch1*$^{fl/fl}$Tie2cre mice.

In the presence of L-NAME, addition of indomethacin had minimal effect on endothelium-dependent vasodilation in uterine arteries from either pregnant or nonpregnant wild-type and *Gch1*$^{fl/fl}$Tie2cre mice (Fig. 3A and B**)**. In contrast, addition of EDH blockers (combination of apamin and charybdotoxin) totally inhibited the non-eNOS mediated component of endothelium-dependent vasodilation in uterine arteries from both nonpregnant and pregnant mice of both genotypes with a significantly greater augmentation observed in uterine arteries from *Gch1*$^{fl/fl}$Tie2cre mice (Fig. 3A, B, and C**)**. Systematic quantification of the vasodilator responses revealed that in contrast to wild-type mice, uterine arteries from pregnant *Gch1*$^{fl/fl}$Tie2cre mice had a striking loss of eNOS-derived NO and a significant increase in the EDH component (Fig. 3C), suggesting loss of endothelial cell *Gch1*/BH4 leads to a compensatory upregulation of EDH in *Gch1*$^{fl/fl}$Tie2cre uterine arteries. However, this compensatory upregulation was insufficient to fully correct for the loss of eNOS mediated dilators.

To further investigate which specific components of the EDH response is affected in *Gch1*$^{fl/fl}$Tie2cre mice, endothelium-dependent vasodilatation was assessed in the presence of L-NAME and indomethacin with either charybdotoxin or apamin. First, we found that in a normal pregnancy in wild-type mice, intermediate and large Ca^{2+}-activated K^+ channels (charybdotoxin-sensitive component) make up the majority of the EDH component (Fig. 4A and B) with the apamin-sensitive component accountable for approximately a third of the EDH response (Fig. 4A and B). In contrast to wild-type mice, loss of endothelial cell *Gch1*

leads to a significant reduction in the apamin-sensitive component with the charybdotoxin-sensitive component dominating. This effect was seen even when the order of the inhibitors used was changed (Supplementary Fig. 2). Taken together, these observations indicated that loss of endothelial cell BH4 impacts on the ability of endothelial cells to upregulate small Ca^{2+}-activated K^+ channel mediated EDH responses.

3.5 Supplementation with BH4 and 5MTHF, but not BH4 alone, prevents vascular dysfunction and pregnancy-induced hypertension in Gch1$^{fl/fl}$Tie2cre mice.

In our previous study, we showed that treatment of pregnant *Gch1*$^{fl/fl}$Tie2cre mice with BH4 and 5-MTHF was sufficient to restoration BH4 levels and prevented both fetal growth restriction and hypertension in late pregnancy [18]. Given the results of our current study we hypothesized that BH4 and 5-MTHF supplementation is acting to restored blood pressure and fetal growth via normalization of pregnancy induced vascular remodelling in *Gch1*$^{fl/fl}$Tie2cre mice. To address this, we treated *Gch1*$^{fl/fl}$Tie2cre and wild-type mice with either oral BH4 or BH4 with 5-MTHF for 3 days prior to mating, and throughout pregnancy.

We found that oral BH4 supplementation alone was not sufficient to prevent progressive hypertension throughout pregnancy or restore vascular function in uterine arteries from *Gch1*$^{fl/fl}$Tie2cre mice with an enhanced contractile response to U46619 and reduced sensitivity to the endothelium-dependent vasodilatation to acetylcholine still observed in pregnant *Gch1*$^{fl/fl}$ Tie2cre mice compared to their wild type littermates (Fig. 5A, B, C and D).

However, the addition of reduced folate, 5-MTHF to BH4 supplementation resulted in a striking normalization of both the constriction responses to U46619 and relaxation responses to acetylcholine in *Gch1*$^{fl/fl}$ Tie2cre mice, restoring the responses to those observed in wild-type animals (Fig. 5E and F). The combination of 5-MTHF to BH4 oral supplementation just

prior to conception was sufficient to prevent progressive pregnancy induced hypertension in *Gch1*$^{fl/fl}$Tie2cre mice with no difference in blood pressure observed between wild type and *Gch1*$^{fl/fl}$Tie2cre mice from 2.5 days post-conception until the end of the experiment at gestational day 18.5 (Fig. 5C and F).

4. Discussion

To the best of our knowledge, this is the first study evaluating the effect of endothelial cell *Gch1* and thus BH4 deficiency and the underlying mechanisms on maternal vascular adaptations in uterine arteries during pregnancy. The key findings are 1) Selective deficiency in maternal endothelial cell *Gch1*/BH4 biosynthesis during pregnancy leads to systemic vascular dysfunction due to loss of eNOS-derived vasodilators in both in aortas and uterine arteries from *Gch1*$^{fl/fl}$Tie2cre mice; 2) In uterine arteries, the vascular impairment is incompletely compensated for by an increase in EDH-mediated vasodilation mediated by an increase in intermediate and large-conductance Ca^{2+}-activated K^+ channels. 3) Oral supplementation of BH4 and 5MTHF, but not BH4 alone, preserves vascular endothelial cell vasodilator function, and thus prevent progressive pregnancy-induced hypertension in mice with endothelial cell *Gch1/BH4* deficiency. Taken together, these findings identify a novel role for endothelial cell *Gch1* and BH4 biosynthesis in vascular adaptations to pregnancy.

Pregnancy is associated with a 10-fold increase in uterine artery blood flow. In order to accommodate this without increases in systemic blood pressure the uterine artery undergoes significant changes with enhanced vasodilation and reduced constriction, leading to reduced vascular resistance and increased blood flow [10, 23]. Nitric oxide is a key mediator of this adaptive response. Plasma NO levels are higher in viable than non-viable pregnancies and

NO is inversely correlated with uterine artery pulsatility index [24]. With loss of eNOS associated with uterine artery dysfunction, reduced placenta nutrient transport and fetal growth restriction [25]. Recently, we have shown that selective deficiency of maternal endothelial cell BH4, by targeted *Gch1* deletion in pregnant mice, is sufficient to cause progressive hypertension during pregnancy and fetal growth restriction [18]. However, the requirement and mechanisms of endothelial cell-mediated vasodilatation function in these outcomes are unknown. In the current study, we have shown for the first time that loss of maternal endothelial specific BH4 is alone sufficient to causes maladaptive uterine artery remodeling with enhanced constrictor and reduced vasodilator response in both aorta and uterine artery. The enhanced constrictor response observed in endothelial cell *Gch1* knockout mice is likely mediated in part by reduced tonic production of eNOS derived vasodilators as the difference between genotypes were abolished in the presence of the NOS inhibitor L-NAME. This is in keeping with clinical studies where acute administration of L-NAME greatly reduced forearm blood flow in pregnant women compared to nonpregnant controls [6, 7]. Hypertension has been shown to be associated with vascular dysfunction, particularly in resistance arteries, the key site for arterial blood pressure regulation. Thus, systemic vascular dysfunction observed in endothelial cell *Gch1* knockout during pregnancy is likely to contribute to increased uteroplacental vascular resistance, progressive pregnancy-induced hypertension and thus fetal growth retardation.

As Tie2cre may lead to cre-mediated gene deletion in hematopoietic cells, we next tested the validity of the pregnancy phenotype being mediated by endothelial cell *Gch1* deletion rather than partial *Gch1* deletion in other cell types. We generated a myeloid cell-specific *Gch1* knockout (*Gch1*$^{fl/fl}$ LysM cre) mouse, in which *Gch1* is specifically deleted in all myeloid cell lineages. We have shown that for the first time that selective deficiency of maternal myeloid

cell *Gch1* and thus BH4, in pregnant mice is not sufficient to cause pregnancy-induced hypertension and fetal growth restriction. These findings indicates that the blood pressure and fetal growth phenotype are driven by loss of *Gch1* in endothelial cells.

Interestingly, prior to pregnancy compensatory changes in eNOS mediated dilation were sufficient to maintain normal vasoconstrictor and dilator responses in endothelia cell *Gch1* knockout mice. We have previously shown in aorta that in the absence of endothelial cell BH4, eNOS become uncoupled and produces H_2O_2 instead of NO, which acts as an endothelium-dependent vasodilator partially compensating for the loss of eNOS derived NO [19, 22]. However, during pregnancy this compensatory mechanism is lost with eNOS mediated vasodilation making minimal contribution to uterine artery vasodilation in endothelial cell *Gch1* knockout mice. Pregnancy is a state of mild oxidative stress [26-28]. Increase expression of antioxidant defenses have previously been observed in pregnancy [29]. Interestingly, catalase levels have been found to be increased in pre-eclampsia as compared to normal pregnancy [30]. It is possible that in this altered redox environment, eNOS derived H_2O_2 is no longer an effective vasodilator in endothelial cell *Gch1* knockout mice.

We show that pregnancy is associated with an increase in EDH mediated vasodilation in uterine arteries. This is consistent with other studies which have shown enhanced EDH mediated dilation in pregnancy [31, 32]. However, EDH upregulation in the absence of endothelial cell BH4, was not sufficient to compensate fully for the loss of eNOS mediated vasodilation in the uterine artery. In uterine arteries from both wild type and *Gch1*$^{fl/fl}$Tie2cre mice, both large and intermediate conductance Ca^{2+}-activated K^+ channels were responsible for the majority of the EDH mediated vasodilation. This is in keeping with previous studies which have shown an increased expression and activity of BK_{Ca} channels [33] and SK_{Ca} channels [32] in uterine arteries of pregnant sheep. Further interrogation of EDH response

showed in pregnant wild type mice, SK_{Ca} channels made up approximately 40% of the EDH response, however, in endothelial cell *Gch1* knockout mice this component was markedly reduced to only 20%. Recent studies have shown metabolic regulation of SK_{Ca} channel in coronary endothelial cells with reduced expression observed in endothelial cells from diabetic arteries [34]. Pregnancy represents a significantly altered metabolic state, further studies interrogating if similar mechanisms are driving reduced SK_{Ca} channel activity in our currently study will be key to address this. These findings reveal that deficiency in endothelial cell BH4 biosynthesis leads to fundamental changes in uterine artery endothelial function and remodeling during pregnancy, with a loss of eNOS-mediated effects that are partially compensated for by an increase in the relative contribution of calcium-activated potassium channels. This observation is in keeping with the prior observation of the important role of BK_{Ca} channels in the uterine artery in pregnancy [35].

Reduced vascular BH4 is a hallmark of multiple cardiovascular conditions [36-39]. Oxidative stress causes oxidation of BH4 to BH2 and B, which are incapable of acting as a cofactor for eNOS leading to uncoupled eNOS. Markers of oxidative stress are present in the placenta and maternal circulation of patients with pre-eclampsia [40] and we have previously shown reduced BH4 in placental extravascular vesicles from women with hypertensive pregnancies [18]. Yet in a landmark clinical trial the antioxidant vitamins C and E failed to prevent the development of pre-eclampsia in high risk pregnancies [41], and simple strategies attempting to restore or augment NO with NO donors in pre-eclampsia have been disappointing [15]. One possible explanation for these results is the failure to specifically target eNOS uncoupling and consequent altered NO/ROS signalling. Thus, augmenting BH4 levels may be a rational therapeutic strategies to treat vascular complication in pregnancy. However, in this study we found that oral BH4 supplementation alone was not sufficient to

prevent both vascular dysfunction and pregnancy-induced hypertension in *Gch1*$^{fl/fl}$Tie2cre mice. We have previously shown that oral supplementation of BH4 is not a consistent approach to increase vascular BH4 levels, either in mice or in patients [18, 42, 43], due to oxidation of BH4 to BH2 and B. Interestingly, vascular supplementation of BH4 can be achieved by combining the BH4 with the 5-methyltetrahydrofoalte. The enzyme dihydrofolate reductase (DHFR) reduces dihydrofolate to the fully reduced folate, tetrahydrofolate, and can also reduce oxidized BH2 to regenerate BH4. We have previously demonstrated that supplementation of BH4 with 5-MTHF, restored BH4 levels in pregnancy [18]. In this study, we show that the combination of BH4 and 5-MTHF is sufficient to prevent vascular dysfunction and pregnancy-induced hypertension in *Gch1*$^{fl/fl}$ Tie2cre mice. 5-MTHF has been shown to be an effective treatment to augment vascular BH4 levels in patients [44], exemplifying the early translational potential of this approach – particularly since folates are already approved for use by pregnant women.

Taken together, this study demonstrated that deficiency in maternal endothelial cell BH4 biosynthesis leads to systemic vascular dysfunction and progressive pregnancy-induced hypertension, which could be reversed by supplementation with BH4 and 5-MTHF. Thus, targeting endothelial cell *Gch1* and BH4 biosynthesis by supplementation with BH4 and 5-MTHF may provide a novel therapeutic target for the prevention and treatment of pregnancy-related hypertension such as pre-eclampsia.

Figure Legends

Figure 1: Blood pressure and vasomotor function in aortas from non-pregnant and pregnant wild-type and *Gch1*$^{fl/fl}$ Tie2cre mice at E18.5 day of gestation.
(**A**) Systolic blood pressure prior to conception and at E18.5 day of gestation, a significant increase in blood pressure was observed in *Gch1*$^{fl/fl}$Tie2cre mice both before conception and at E18.5 compared with their wild type (WT) littermates (*$P<0.05$; n=6 animals per group). (**B**) Vasomotor functions in isolated aortas from non-pregnant (NP) and pregnant (P) mice at E18.5 day of gestation. Vasoconstrictions in response to phenylephrine (PE) was significantly enhanced in aortas from pregnant *Gch1*$^{fl/fl}$Tie2cre mice compared to pregnant wilt-type mice and non-pregnant *Gch1*$^{fl/fl}$Tie2cre mice (*$P<0.05$, WT (P) *vs.* *Gch1*$^{fl/fl}$Tie2cre (P); #$P<0.05$, *Gch1*$^{fl/fl}$Tie2cre (NP) *vs.* *Gch1*$^{fl/fl}$Tie2cre (P); n=6 to 10 animals per group). (**C**) Absolute contraction (mN) in response to KCL in non-pregnant and pregnant mice from both WT and *Gch1*$^{fl/fl}$Tie2cre mice (n=6 to 10 animals per group). (**D**) Vasoconstrictions in response to phenylephrine (PE) in the presence of 100 µM L-NAME in isolated aortas from non-pregnant (NP) and pregnant (P) mice at E18.5 day of gestation of both genotypes. (**E**) Endothelium-dependent vasodilatation in response to acetylcholine (ACh) was markedly impaired in aortas from pregnant *Gch1*$^{fl/fl}$Tie2cre mice compared to pregnant WT and non-pregnant *Gch1*$^{fl/fl}$Tie2cre mice (#$P<0.05$, WT (P) *vs.* *Gch1*$^{fl/fl}$Tie2cre (P); *$P<0.05$, *Gch1*$^{fl/fl}$Tie2cre (NP) *vs.* *Gch1*$^{fl/fl}$Tie2cre (P); n=6 to 10 animals per group). (**F**) EC_{50} and maximum relaxation (%) in response to ACh (*$P<0.05$). (**G**) Preconstricted tension (mN) to PE for each Ach relaxation curve. (**H**) Endothelium-dependent vasodilatations to ACh were totally inhibited in all four groups in the presence of 100 µM L-NAME. (**I**) Endothelium-independent vasodilatations in response to the nitric oxide donor, sodium nitroprusside (SNP) were significantly enhanced in both pregnant wild-type and *Gch1*$^{fl/fl}$Tie2cre mice compared to non-pregnant wild-type and *Gch1*$^{fl/fl}$Tie2cre mice (*$P<0.05$, significant difference between non-pregnant WT and pregnant WT, #$P<0.05$, significant difference between non-pregnant *Gch1*$^{fl/fl}$Tie2cre vs pregnant *Gch1*$^{fl/fl}$Tie2cre mice; n=6 to 10 animals per group).

Figure 2. Effect of endothelial cell *Gch1*/BH4 deficiency on vascular uterine artery function in pregnancy. Vasomotor functions in isolated uterine arteries (UA) from non-pregnant (NP) and pregnant (P) mice at E18.5. (**A**) Uterine artery diameters, as determined by the length-tension relationship at 100 mmHg. A significant increase in diameter was observed with pregnancy in both groups (*$P<0.05$; n=6 animals per group). Vasoconstrictions in response (mN) to KCL response were significantly increased in pregnant UA from both genotypes compared to non-pregnant controls (*$P<0.05$; n=6 animals per group). (**B**) Cumulative dose response curved to the thromboxane A2 mimetic, U46619 in non-pregnant and pregnant arteries from wild-type and *Gch1*$^{fl/fl}$Tie2cre mice (*$P<0.05$, WT (P) *vs.* *Gch1*$^{fl/fl}$Tie2cre (P); WT (NP) *vs.* WT (P); n=6 animals per group). (**C**) Vasoconstriction in response to U46619 in pregnant uterine arteries in the presence or absence of non-selective NOS inhibitor, 100 µM L-NAME (*$P<0.05$, WT (P) control *vs.* WT (P) + L-NAME; #$P<0.05$, WT control (P) *vs.* *Gch1*$^{fl/fl}$Tie2cre control (P); n=6 animals per group). (**D, E and F**) Endothelium-dependent vasodilatation to acetylcholine (Ach) in uterine arteries from non-pregnant and pregnant mice from both genotypes, submaximally constricted with U46619 (*$P<0.05$, WT (NP) *vs.* WT (P); #$P<0.05$, WT (P) *vs.* *Gch1*$^{fl/fl}$Tie2cre (P); n=6 animals per group). (**E**) EC_{50} and maximum vasodilatation in response to ACh (*$P<0.05$; n=6 animals per group). (**G**) Endothelium-independent vasodilatations in response to the nitric oxide donor,

sodium nitroprusside (SNP) were significantly enhanced in both pregnant wild-type and *Gch1*$^{fl/fl}$Tie2cre mice compared to non-pregnant wild-type and *Gch1*$^{fl/fl}$Tie2cre mice (**P<0.05*, significant difference between non-pregnant WT and pregnant WT, *#P<0.05*, significant difference between non-pregnant *Gch1*$^{fl/fl}$Tie2cre vs pregnant *Gch1*$^{fl/fl}$Tie2cre mice; n=6 animals per group).

Figure 3. Contribution of eNOS-derived vasodilators, cyclooxygenase-derived vasodilators, and endothelium-dependent hyperpolarization (EDH) in non-pregnant and pregnant wild-type and *Gch1*$^{fl/fl}$ Tie2cre uterine arteries at E18.5 day of gestation. (**A** and **B**) Endothelium-dependent vasodilatations to acetylcholine (ACh) were determined in the presence of the nitric oxide synthase inhibitor, L-NAME (100 μM) alone, or L-NAME and the cyclooxygenase inhibitor, indomethacin (10 μM), or L-NAME, indomethacin and EDH blockers (apamin and charybdotoxin). (**C**) Percentage contribution of eNOS-derived vasodilators, cyclooxygenase-derived vasodilators and EDH-sensitive components (**P<0.05*; n=6 animals per group).

Figure 4. Contribution of SK_{ca}, IK_{ca} and BK_{ca} channels in pregnant wild-type and *Gch1*$^{fl/fl}$ Tie2cre uterine arteries at E18.5 day of gestation. (**A**) Endothelium-dependent vasodilatations to acetylcholine (ACh) were determined in the presence of the nitric oxide synthase inhibitor, L-NAME (100 μM) and the cyclooxygenase inhibitor, indomethacin (10 μM), or L-NAME, indomethacin and small-conductance Ca^{2+}-activated K^+ channel (SK_{ca}) blocker, apamin (AP; 50 nM), or L-NAME, indomethacin, apamin and non-selectively intermediate and large-conductance Ca^{2+}-activated K^+ channels (IK_{ca} and BK_{ca} , respectively) blocker, charybdotoxin (ChTx; 100 nM). (**B**) Percentage contribution of apamin-sensitive component (SK_{ca}) and charybdotoxin-sensitive component (IK_{ca} and BK_{ca}) in pregnant wild-type and *Gch1*$^{fl/fl}$ Tie2cre uterine arteries at E18.5 day of gestation (**P<0.05*; n=6 animals per group).

Figure 5. Supplementation of BH4 and 5MTHF, but not BH4 alone, restores vascular function and prevents pregnancy-induced hypertension in pregnant mice with endothelial cell *Gch1*/BH4 deficiency. Non-pregnant *Gch1*$^{fl/fl}$Tie2cre and wild-type (WT) littermates were supplemented with BH4 (200 mg/kg/day) alone or BH4 with the fully reduced folate, 5-methyltetrahydrofolate (5-MTHF; 15 mg/kg/day) or control for 3 days before timed-matings, and throughout their subsequent pregnancies. Blood pressure was determined before and throughout pregnancy by non-invasive tail-cuff plethysmography. Vasomotor functions in isolated uterine arteries (UA) from pregnant *Gch1*$^{fl/fl}$Tie2cre and wild-type mice treated with either BH4 alone or BH4+5MTHF or control was assessed by wire myography at 18.5 day of gestation. (**A** and **C**) Oral BH4 supplementation alone was not sufficient to prevent both progressive pregnancy-induced hypertension in *Gch1*$^{fl/fl}$Tie2cre mice (†*P<0.05*, comparing genotype; **P<0.05*, comparing to basal BP in *Gch1*$^{fl/fl}$Tie2cre mice; n=4 to 6 animals per group) and (**B** and **D**) vascular dysfunction in isolated uterine arteries from *Gch1*$^{fl/fl}$Tie2cre mice (**P<0.05*; n=4 to 6 animals per group). (**E** and **F**) The combination of 5-MTHF to BH4 oral supplementation was sufficient to prevent both progressive pregnancy-induced hypertension and vascular dysfunction in UA from pregnant *Gch1*$^{fl/fl}$Tie2cre mice (**P<0.05*; n=4 to 6 animals per group).

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[44] C. Antoniades, C. Shirodaria, N. Warrick, S. Cai, J. de Bono, J. Lee, P. Leeson, S. Neubauer, C. Ratnatunga, R. Pillai, H. Refsum, K.M. Channon, 5-methyltetrahydrofolate rapidly improves endothelial function and decreases superoxide production in human vessels: effects on vascular tetrahydrobiopterin availability and endothelial nitric oxide synthase coupling, Circulation 114(11) (2006) 1193-1201.

6. Acknowledgements

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7. Author contributions

S.C. and K.M.C conceived the study and designed the experiments, with contributions from G.D. Mouse experiments and analyses were done by S.C. with help from G.D., E.M., C.W., and Y.D. Vascular function studies were undertaken by S.C., C.W., and D.A.Y. The manuscript was drafted by S.C., K.M.C., and G.D. All authors discussed the results and had the opportunity to contribute to the manuscript.

Figure 1

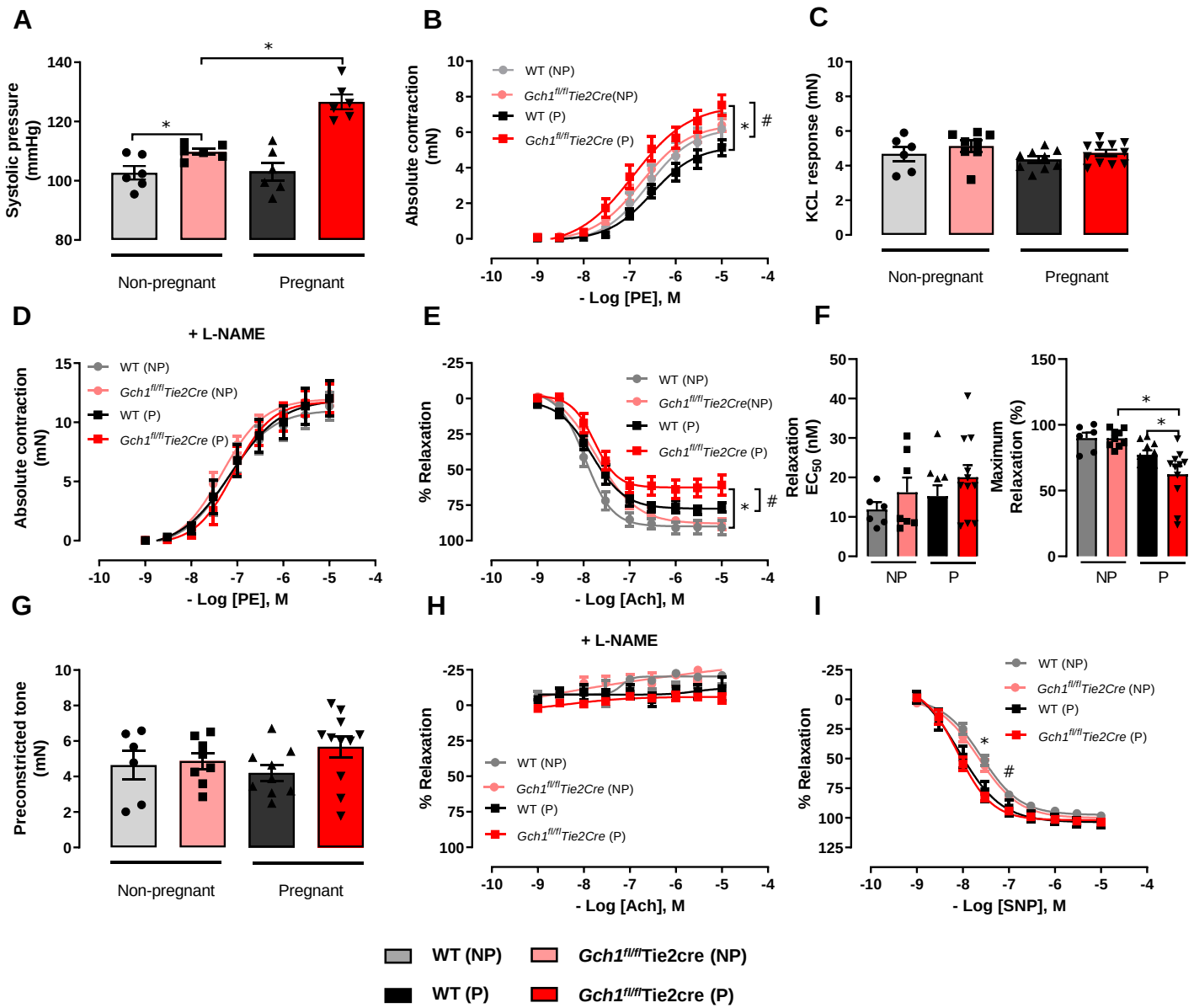

A
Systolic pressure (mmHg)
Non-pregnant
Pregnant
B
Absolute contraction (mN)
WT (NP)
Gch1fl/flTie2Cre(NP)
WT (P)
Gch1fl/flTie2Cre (P)
- Log [PE], M
C
KCL response (mN)
Non-pregnant
Pregnant
D
+ L-NAME
Absolute contraction (mN)
- Log [PE], M
E
% Relaxation
- Log [Ach], M
F
Relaxation EC50 (nM)
Maximum Relaxation (%)
NP
P
G
Preconstricted tone (mN)
Non-pregnant
Pregnant
H
+ L-NAME
% Relaxation
- Log [Ach], M
I
% Relaxation
- Log [SNP], M
WT (NP)
Gch1fl/flTie2cre (NP)
WT (P)
Gch1fl/flTie2cre (P)

Figure 2

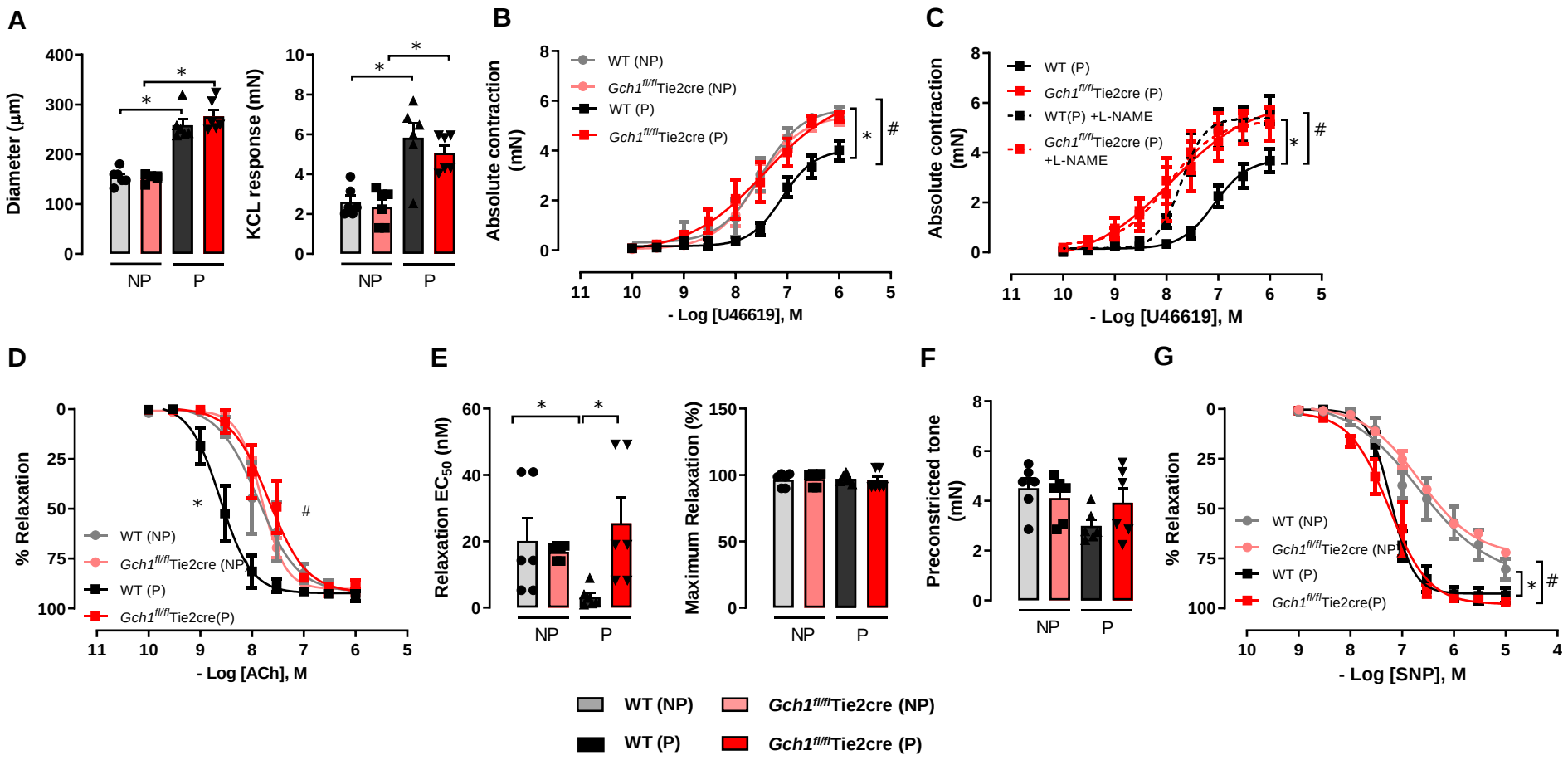

A
Diameter (µm)
KCL response (mN)
NP
P
B
Absolute contraction (mN)
WT (NP)
Gch1fl/flTie2cre (NP)
WT (P)
Gch1fl/flTie2cre (P)
- Log [U46619], M
C
Absolute contraction (mN)
WT (P)
Gch1fl/flTie2cre (P)
WT(P) +L-NAME
Gch1fl/flTie2cre (P) +L-NAME
- Log [U46619], M
D
% Relaxation
WT (NP)
Gch1fl/flTie2cre (NP)
WT (P)
Gch1fl/flTie2cre(P)
- Log [ACh], M
E
Relaxation EC50 (nM)
Maximum Relaxation (%)
NP
P
F
Preconstricted tone (mN)
NP
P
G
% Relaxation
WT (NP)
Gch1fl/flTie2cre (NP)
WT (P)
Gch1fl/flTie2cre(P)
- Log [SNP], M
WT (NP)
Gch1fl/flTie2cre (NP)
WT (P)
Gch1fl/flTie2cre (P)

Figure 3

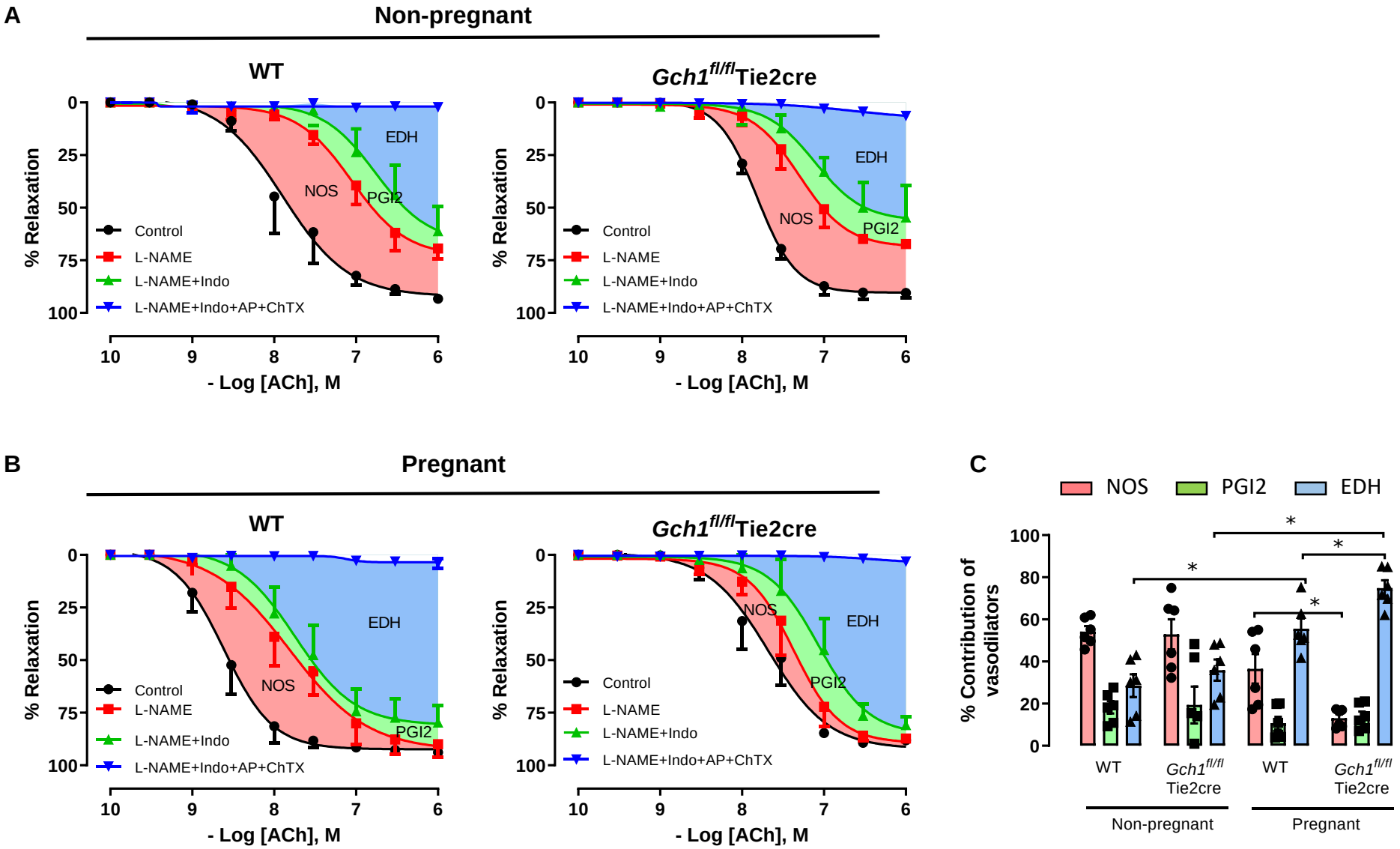

A
Non-pregnant
WT
Gch1fl/flTie2cre
% Relaxation
0
25
50
75
100
10
9
8
7
6
- Log [ACh], M
Control
L-NAME
L-NAME+Indo
L-NAME+Indo+AP+ChTX
NOS
PGI2
EDH
B
Pregnant
C
% Contribution of vasodilators
NOS
PGI2
EDH
WT
Gch1fl/fl Tie2cre
Non-pregnant
Pregnant
*

Figure 4

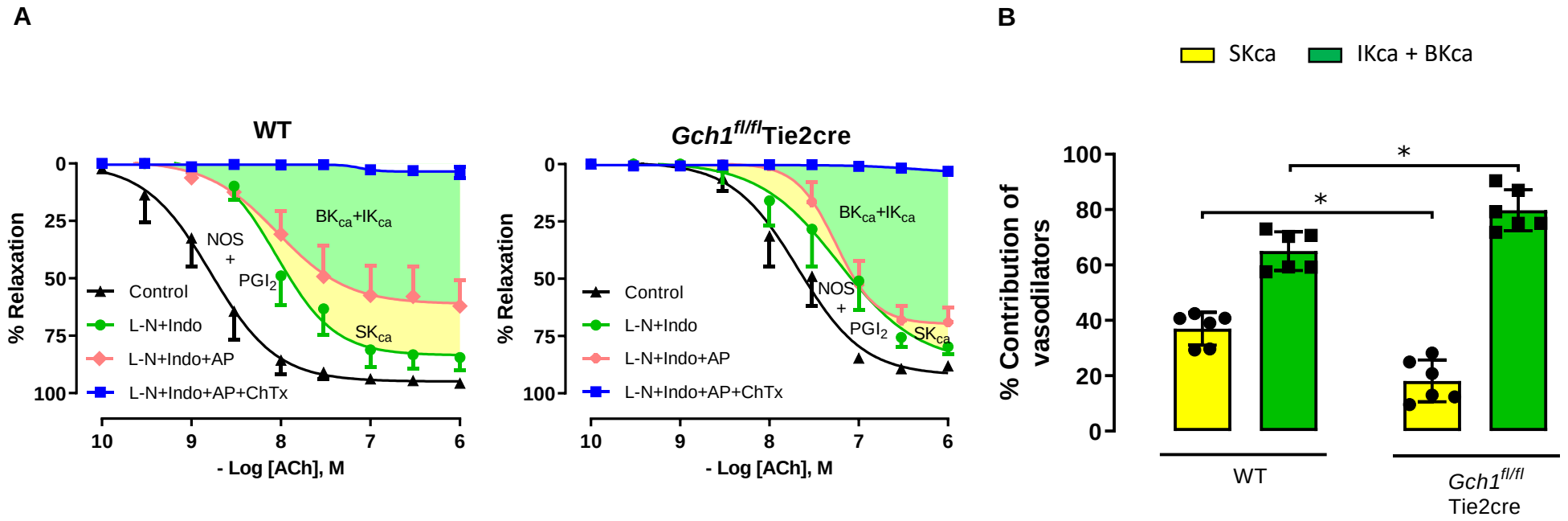
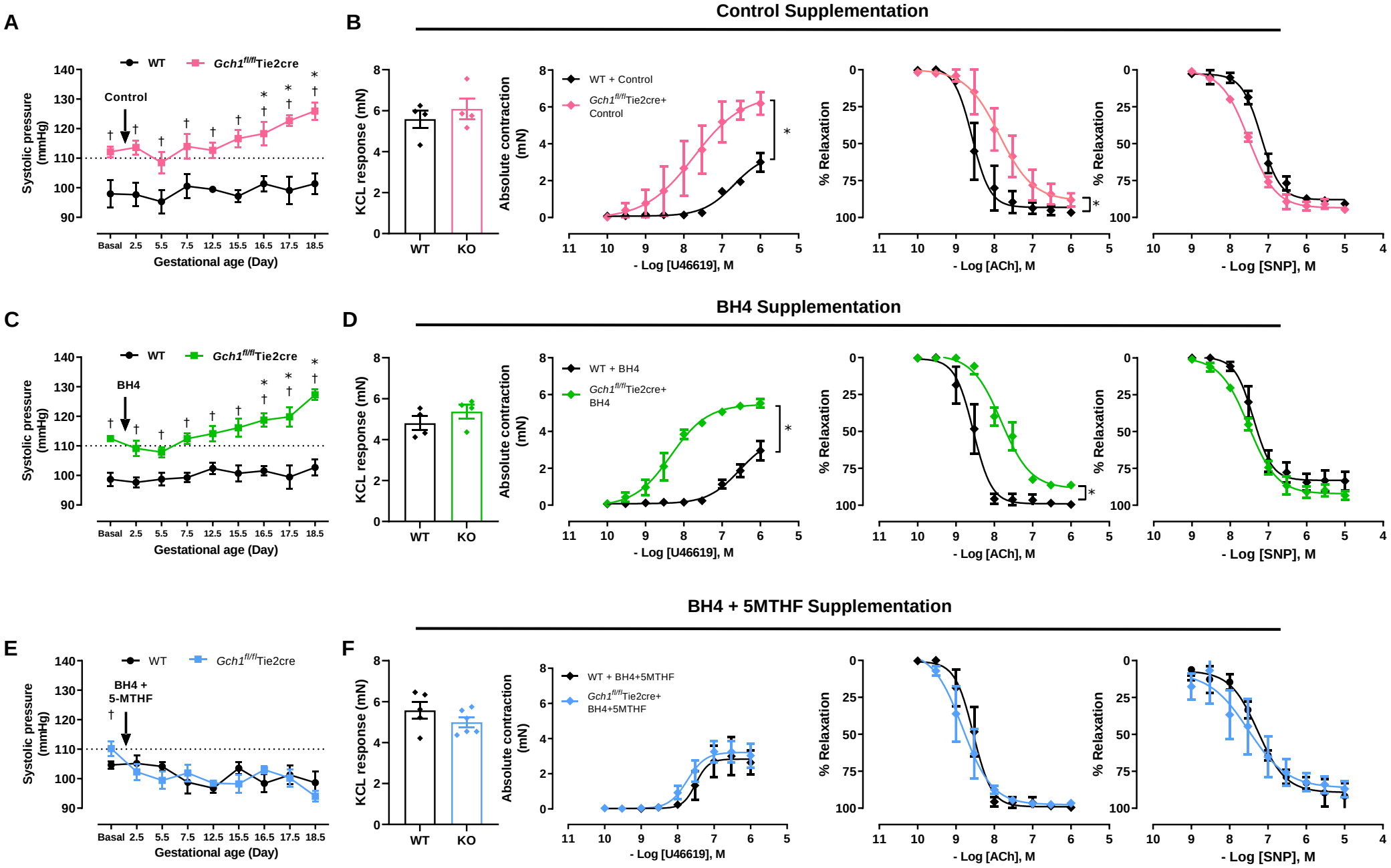
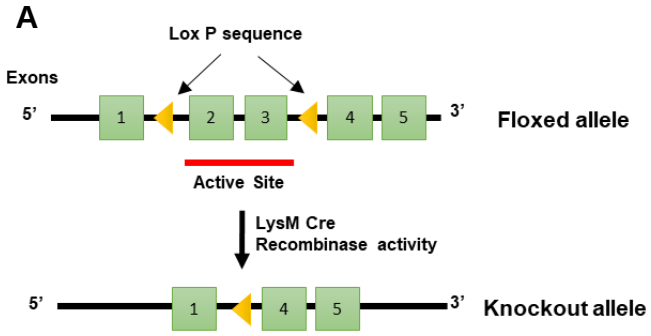
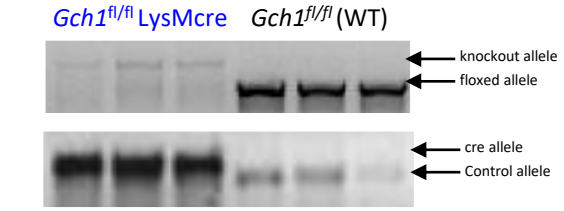

A
WT
Gch1fl/flTie2cre
% Relaxation
0
25
50
75
100
10
9
8
7
6
- Log [ACh], M
Control
L-N+Indo
L-N+Indo+AP
L-N+Indo+AP+ChTx
NOS
+
PGI2
BKca+IKca
SKca
B
SKca
IKca + BKca
% Contribution of vasodilators
0
20
40
60
80
100
*
*
WT
Gch1fl/fl
Tie2cre

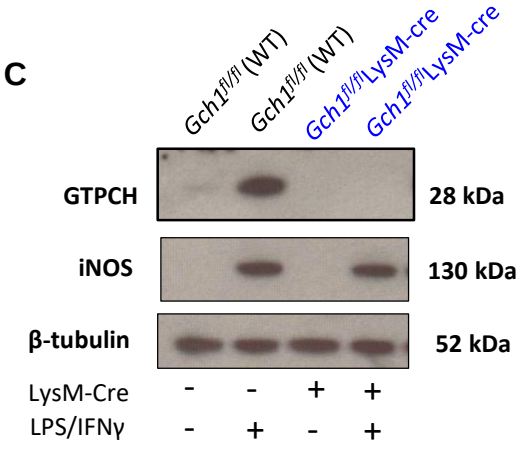
Figure 5

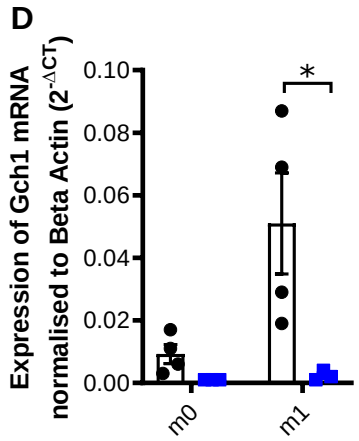

A
B
Control Supplementation
C
D
BH4 Supplementation
E
F
BH4 + 5MTHF Supplementation
WT
Gch1fl/flTie2cre
Control
BH4
BH4 +
5-MTHF
Systolic pressure (mmHg)
Gestational age (Day)
Basal 2.5 5.5 7.5 12.5 15.5 16.5 17.5 18.5
KCL response (mN)
WT
KO
Absolute contraction (mN)
WT + Control
Gch1fl/flTie2cre+ Control
WT + BH4
Gch1fl/flTie2cre+ BH4
WT + BH4+5MTHF
Gch1fl/flTie2cre+ BH4+5MTHF
- Log [U46619], M
% Relaxation
- Log [ACh], M
- Log [SNP], M

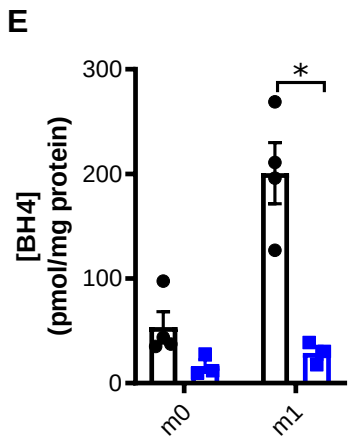
Supplementary Figure 1

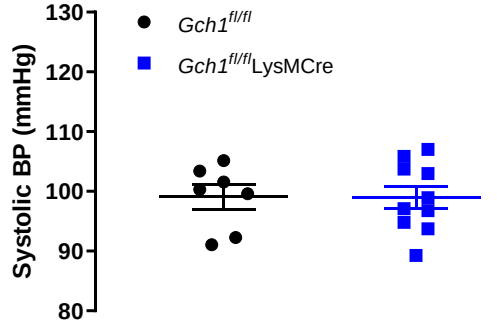


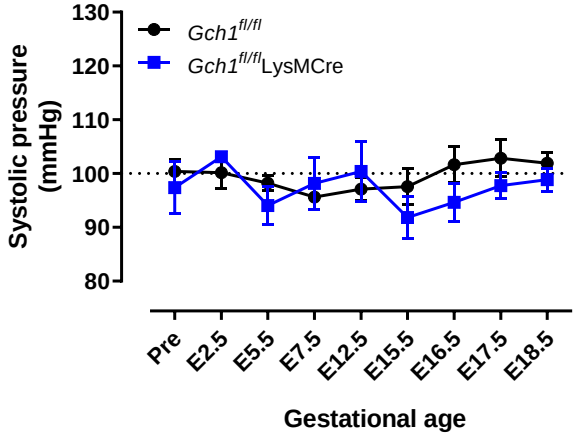


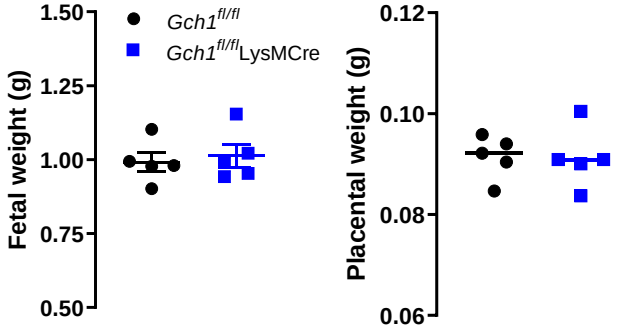






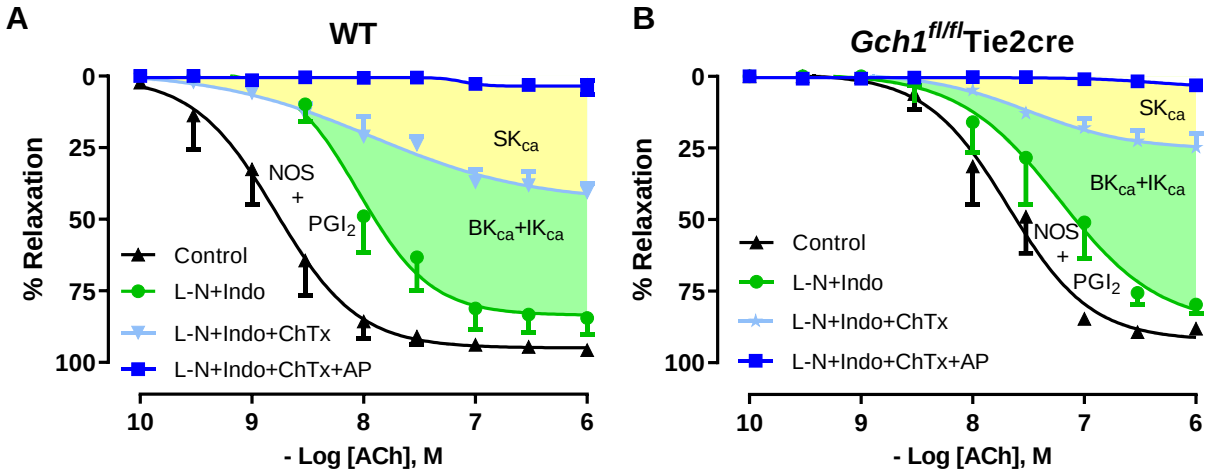






Supplementary Figure 1 Generation and Characterisation of Myeloid Cell-specific *Gch1* Knockout mice. Generation and Characterisation of Myeloid Cell-specific *Gch1* Knockout mice. To generate myeloid cell-specific *Gch1* deletion,*Gch1*$^{fl/fl}$ mice were crossed with LysM cre mice to generate *Gch1*$^{fl/fl}$ LysM cre mice and *Gch1*$^{fl/fl}$ (i.e. WT) littermates as detailed in A) Schematic diagram of LoxP site insertion and the resultant excised allele after crossing with LysM cre line. Bone marrow derived macrophages (BMDM) were cultured from bone marrow. After 7 days the cells were harvested and plated at 1x10^6 cells per well and subject to an overnight stimulation with LPS (100ng/ml) and IFNγ (10ng/ml). After stimulation the cells and untreated control cells were harvested for analysis. B) DNA was extracted from macrophage cell pellets and PCR performed to detect the presence of the floxed and excised knockout allele. *Gch1*$^{fl/fl}$LysM cre BMDM showed efficient excision of the floxed allele, when the cre allele was present. In contrast the knockout allele could not be detected in the absence of cre. C) Western blotting shows a lack of expression of GTPCH protein in *Gch1*$^{fl/fl}$LysM cre macrophages, compared to wild-type (WT) macrophages that show increased expression following activation with LPS and IFNγ for 24 h. Both genotypes of cells showed similar induction of iNOS protein following activation. Equal protein loading was demonstrated by detection of β-tubulin. D) mRNA analysos showed that *Gch1*$^{fl/fl}$LysM cre cells had a significantly reduced expression of *Gch1* mRNA as expected (**P<0.05*; n=3 to 4 per group). E) To determine the efficacy of knockout at a functional level cellular BH4 content was measured by HPLC. *Gch1*$^{fl/fl}$LysMcre BMDM showed a significantly reduced BH4 content (**P<0.05*; n=3 to 4 per group). F) Basal systolic blood pressure, determined by non-invasive tail-cuff plethysmography, was comparable between *Gch1*$^{fl/fl}$LysM cre and wild-type littermate controls (n=7 to 10 animals per group). G) Systolic blood pressure before (prepregnancy) and throughout pregnancy (n=7 to 10 animals per group). (H) Fetal and placental weight at gestation day 18.5 from pregnant *Gch1*$^{fl/fl}$ LysM cre mice and pregnant WT littermate controls (n=5 litters per group).

Supplementary Figure 2



Supplementary Figure 2 Contribution of SK_{ca}, IK_{ca} and BK_{ca} channels in pregnant wild-type and *Gch1*$^{fl/fl}$ Tie2cre uterine arteries at E18.5 day of gestation.
Isometric tension studies of uterine arteries from pregnant WT and *Gch1*$^{fl/fl}$Tie2cre mice at 18.5 days of gestation were examined using a wire myograph. (A) Endothelium-dependent vasodilatations to acetylcholine (ACh) were determined in the presence of the nitric oxide synthase inhibitor, L-NAME (100 μM) and the cyclooxygenase inhibitor, indomethacin (10 μM), or L-NAME, indomethacin and non-selectively intermediate and large-conductance Ca^{2+}-activated K^+ channels (IK_{ca} and BK_{ca} , respectively) blocker, charybdotoxin (ChTx; 100 nM) or L-NAME, indomethacin, chrybdotoxin and small-conductance Ca^{2+}-activated K^+ channel (SK_{ca}) blocker, apamin (AP; 50 nM)(n=6 to 8 animals per group).