Title: Distinct uterine artery gene expression profiles during early gestation in the stroke-prone spontaneously hypertensive rat

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ABSTRACT

During pregnancy the uterine spiral arteries undergo major vascular remodelling to ensure sufficient uteroplacental perfusion to support the fetus. In pregnancies complicated by hypertensive disorders this remodelling is deficient leading to impaired uteroplacental blood flow and poor maternal and fetal outcomes. The underlying genetic mechanisms for failed vascular remodelling are not fully understood. This study aimed to examine the early-pregnancy associated gene changes in the uterine arteries of spontaneously hypertensive stroke-prone rats (SHRSP) compared to their normotensive counterparts, Wistar-Kyoto rats (WKY). Uterine arteries from gestational day 6.5 WKY and SHRSP were processed for RNA-sequencing, along with virgin, age-matched controls for each strain. Gene expression changes were identified and biological pathways were implicated and interpretated using Ingenuity Pathway Analysis (IPA®). This study found that WKY uterine arteries from early-pregnancy exhibit a gene expression pattern that is suggestive of a pregnancy-dependent reduction in Ca^{2+} handling and RAAS components and an increase in ATP production. In contrast, the expression pattern of pregnant SHRSP uterine arteries was dominated by an elevated immune response and increased production of ROS and downstream effectors of the RAAS. These results suggest that in a rat model, hypertension during pregnancy impacts uterine artery gene expression patterns as early as the first week of pregnancy. The pathway changes involved may underlie or contribute to the adverse vascular remodelling and resultant placental ischaemia and systemic vascular dysfunction observed in SHRSP in late gestation.

KEYWORDS: pregnancy; hypertension; RNA-seq; vascular remodelling; uterine artery.

ABBREVIATIONS

ANGII: Angiotensin 2

AT_1R: Angiotensin Receptor Type 1

ATP: Adenosine Triphosphate

DEG: Differentially Expressed Gene(s)

FPKM: Fragments per Kilobase of Transcript per Million Mapped Reads

GD: Gestational Day

HDP: Hypertensive disorder(s) of pregnancy

IPA: Ingenuity Pathway Analysis

kPa: Active Effective Pressure

lincRNA: Long Non-Coding Intergenic RNA

miRNA: MicroRNA

NP: Non-Pregnant

NOX: NADPH Oxidase

P: Pregnant

PCA: Principal Component Analysis

PKC: Protein Kinase C

RAAS: Renin-Angiotensin-Aldosterone System

RNA: Ribonucleic Acid

RNA-seq: RNA Sequencing

ROS: Reactive Oxygen Species

SHRSP: Stroke Prone Spontaneously Hypertensive Rat

uNK: Uterine Natural Killer Cells

WKY: Wistar Kyoto Rat

INTRODUCTION

Hypertensive complications during pregnancy are abundantly common worldwide, and have been attributed as a leading cause of maternal mortality (1). Hypertensive disorders of pregnancy (HDP) encompass both known hypertension before pregnancy and *de novo* hypertension; diagnosed on or after 20 weeks gestation and includes gestational hypertension, pre-eclampsia, chronic hypertension and superimposed pre-eclampsia (2). The complicated multifactorial nature of these disorders means that there is large variability in clinical presentations and severity (3). Conditions such as pre-eclampsia are major contributors to poor fetal development, resulting in intrauterine growth restriction and premature birth (4). Furthermore, these conditions can impact the health of both the mother and offspring in later life, with the increased risk of development of cardiovascular disease and type 2 diabetes (5-10).

Hypertensive pregnancies share similarities in the vascular dysfunction phenotypes observed in the placental bed and the phenotypes that underpin cardiovascular diseases (11). During normal uncomplicated pregnancies, major uteroplacental vascular remodelling occurs transforming the uteroplacental vessels from narrow, muscular, and vasoactive arteries to more flaccid, open vessels that are less responsive to vasoconstrictive and dilatory agents (12, 13). Maternal blood is then able to freely flow into the intervillous space, thus allowing adequate exchange of nutrients, gases, and waste products between mother and fetus, via the placenta. In HDP, the uteroplacental vasculature fails to remodel sufficiently, thus impairing uteroplacental blood flow. This has the potential to result in fetal growth restriction, placental ischemic-reperfusion damage and increased antiangiogenic and pro-inflammatory factors which are released into the circulation leading to systemic vascular dysfunction (14, 15). Spiral arteries from pre-eclamptic patients have been found to

have shallower remodelling, retain a contractile phenotype and do not show a pregnancy dependent increase in flow mediated vasodilation (16). Furthermore, there is evidence that this remodelling is initiated prior to the invasion of placental-derived trophoblast cells. A study by van der Heijden *et al* in pseudo pregnant mice provides evidence that the initiation of uteroplacental vascular remodelling can occur in the absence of any conceptus material, suggesting that the uterine arteries can be 'primed' by maternal factors in order to respond appropriately to more major placental-dependent remodelling events (17).

Examining the uteroplacental vascular gene expression in these early remodelling stages is key for understanding the vascular responses to HDP. Studies have examined the differential gene expression over gestation in humans and rodents and found pregnancy-dependent changes in the expression of hormone receptors, calcium and potassium channels and growth factors in the uterine artery (13, 18-20). However, studies examining the genetic profile of the uterine arteries response to pathophysiological pregnancy are lacking. The majority of studies investigating gene expression changes associated with abnormal vascular remodelling responses in HDP have focused on term placental or decidual gene expression (21-23). Thus, the mechanisms behind impaired early pregnancy-dependent remodelling are still elusive due to inaccessibility of uterine artery tissue and ethical issues with tissue from early pregnancy time-points. Animal models of HDP can be used to overcome this obstacle.

The stroke-prone spontaneously hypertensive rat (SHRSP) demonstrates an elevated blood pressure throughout gestation with evidence of reduced uteroplacental blood flow and fetal and placental abnormalities conjunctive with common hypertensive complications in human pregnancy (24). Furthermore, SHRSP demonstrate a failure to respond to the cardiovascular demands of pregnancy even in the absence of chronic hypertension. Nifedipine-treated

SHRSP dams did not develop hypertension, yet still demonstrated a failure of uterine artery pregnancy-dependent remodelling and an impaired uteroplacental blood flow compared to the normotensive Wistar-Kyoto rat (WKY) (24), suggesting that the SHRSP impaired remodelling is directed by maternal genetic differences and not pre-existing hypertension. This study aimed to determine the difference in gene expression response to early pregnancy between SHRSP and WKY, in order to identify differential gene expression patterns that may underlie adverse vascular remodelling response to pregnancy.

METHODS

Animals & Mating

All animal procedures were approved by the Home Office according to the *Animals (Scientific Procedures) Act* (1986) (Project Licence 60/9021) and followed *ARRIVE* guidelines. SHRSP and WKY rats (obtained via brother x sister mating in-house at the University of Glasgow) were housed in controlled 12-hour light/dark conditions with a constant temperature (21°C±3°C) with ad libitum access to water and standard diet (rat and mouse No.1 maintenance diet, Special Diet Services). Virgin females of each strain were time mated at 12 weeks (±4 days) of age with stud males of the respective strain, with pregnancy (P) confirmed by the presence of copulation plug (gestational day (GD) 0.5). On GD6.5 dams were euthanized and the uterine artery only isolated and cleaned of connective tissue and adipose tissue for use in either myography experiments or snap-frozen for RNA extraction. Age-matched virgin SHRSP and WKY were used as non-pregnant (NP) controls).

Uterine Artery Myography

Uterine arteries were dissected and prepared for wire myography as previously described (25). Main uterine artery segments only were used for all myography experiments and radial arteries along with any other vessels dissected and discarded. Briefly, after mounting and normalisation to 13.3kPa the arteries contractile and relaxation responses were assessed using dose responses to noradrenaline ($1x10^{-8}$ to 1×10^{-5} mol/L), carbachol and sodium nitroprusside (both 1×10^{-9} to 2×10^{-5} mol/L). A pressure myograph system (Danish Myo Technology) was used to determine external and internal diameters over a range of physiological pressures (10–120 mm Hg). These were then used to calculate the cross-sectional area (μm^2) = $/4\pi$ x ($D_E^2 - D_I^2$), where D_E = external diameter and D_I = internal diameter. Group sizes were between *n*= 5-11.

RNA Sample Preparation and Sequencing

Uterine artery RNA was extracted using the miRNeasy mini kit (Qiagen) according to manufacturer's instructions after homogenisation in Qiazol using TissueLyserII. Total RNA quality was assessed using a eukaryote total RNA picochip on an Agilent Bioanalyzer 2100 (Agilent Technologies, UK). RNA quality was accepted with a RIN >7. RNA was extracted from *n*=3 animals per group.

A minimum of 100ng total RNA was used for RNA-sequencing (RNA-Seq) library preparations. Total RNA libraries were prepared with ribosomal-depleted RNA using the Illumina TruSeq Stranded Total RNA with Ribo-Zero Gold kit (Illumina, USA), following manufacturer's instructions. Sequencing was performed on a NextSeq500® Illumina sequencing system, with paired-end sequencing at a depth of 50 million reads per sample. Adapter and quality trimming of the reads was performed using CutAdapt and Sickle software packages, with FastQC used to ensure suitable sequence quality throughout processing. Reads were aligned using TopHat and the gene annotation build used was the

Ensembl Rnor_6 reference genome, version 81. Differential expression was assessed using DESeq2 software package (Bioconductor 3.6) across four comparisons: WKY NPvP, SHRSP NPvP, WKY NP v SHRSP NP and WKY P v SHRSP P. Principal component analysis (PCA) was performed using Python with sklearn standard scaler. Due to the design of this study, a gene's fold change was expressed relative to non-pregnant expression, thus a negative fold change implies a reduction in expression in the non-pregnant uterine artery compared to pregnant. RNA-seq data was deposited to Annotare 2.0 (Accession No.:E-MTAB-10212).

Pathway Analysis

The differentially expressed protein coding transcripts were investigated using Ingenuity® Pathway Analysis (IPA; Qiagen). This functionally correlated transcript expression profiles from each comparison group to gene expression and highlighted biological relevant pathway changes. The differentially expressed transcripts were filtered with $p_{adj}<0.05$ and to confirm quantifiable expression the fragments per kilobase of transcript per million mapped reads (FPKM) criteria was >1.0. An expression analysis was conducted for each dataset of differentially expressed genes (DEGs) specific to either WKY or SHRSP with common changes removed, to highlight key disease and functional pathway involvement. This was further investigated using a core comparison analysis of the strain-specific datasets to identify differences in canonical pathways and assign activation z-scores for each comparison group. Comparisons were made between non-pregnant (NP) and GD6.5 pregnant (P) for each strain.

Statistical Analysis

All data are presented as mean ± standard error (SEM) unless otherwise stated. No data points were excluded during analysis. Myography data was analysed using area under the

curve compared using one-way ANOVA with Tukey *post-hoc* test. Expression analyses within IPA® were conducted automatically, using the Fishers Exact Test of significance ($p<0.05$) to prevent over-representation.

RESULTS

Early Pregnancy did not Alter Uterine Artery Structure or Function

The contractile and relaxation responses of the uterine arteries were not significantly different between strains or pregnancy (Figure 1A-C). There were no significant uterine artery functional changes between NP and GD6.5 in response to increasing luminal pressure (Figure 1D-F). However, there was a strain-dependant response, whereby NP WKY uterine arteries demonstrated a significantly increased external and internal diameter compared to NP SHRSP arteries (* $p<0.05$, ** $p<0.01$ NP WKY vs NP SHRSP; Figure 1D-E). There were no significant differences across pregnancy or strain in cross-sectional area (Figure 1F).

Pregnancy Induces Strain-Specific Changes to Gene Expression as Early as GD6.5

The principal component analysis (PCA) plot (Figure 2A) demonstrates a clear separation between both pregnancy state (PC1: 23.2% variance) and strain (PC2: 16.3% variance). The significant differentially expressed genes (DEGS) were separated by biotype (protein coding, lincRNA, miRNA, small RNA and pseudogenes) and the proportions represented were found to be similar in WKY and SHRSP across pregnancy (Figure 2B). WKY uterine arteries were found to have 552 DEGs, with 173 DEGs upregulated and 379 DEGs downregulated in early pregnancy. SHRSP uterine arteries had 842 pregnancy-specific DEGs, with 180 DEGs upregulated, 662 DEGs downregulated (Table S1). The number of significant DEGs that were novel for each comparison group and those that were common between strains are shown in Figure 2C. The biological relevance of these 188 common DEGs is outlined in Table 1, which details the 15 most significant biological functions predicted by IPA® to be most likely

influenced by pregnancy that are common to both strains achieved via pathway enrichment analysis based on gene expression profiles. All DEGs for both WKY and SHRSP that were altered by pregnancy were visualised by volcano plots, with the most significant genes (-log10p-value ≥ 10) labelled by gene name (Figure 2D).

SHRSP and WKY Dams Show Distinct Differences in Pathway Activation During Early Pregnancy

Ingenuity® Pathway Analysis was used to correlate early pregnancy-dependent changes in gene expression to biologically relevant changes in canonical pathways. Core comparison analysis of pregnancy-dependant DEGs specific to either strain (WKY=364, SHRSP=654, Figure 2C) revealed differences in activation z-score between several predicted canonical pathways and biological functions/diseases (Figure 3). Z-scores, measures of predicted directional activity, were assigned by IPA® based on the pattern of gene expression changes across pregnancy and then ranked using hierarchical clustering. In our data set, an increased z-score implies an increased activation of that pathway in NP uterine arteries compared to GD6.5, thus a pregnancy associated reduction. WKY dams demonstrated a trend towards an increased z-score, thus a reduced activity in pregnant uterine arteries, of several pathways involved in energy production as well as the renin-angiotensin-aldosterone system (RAAS) (Figure 3A). In contrast, the SHRSP group demonstrated negative z-scores and pregnancy associated activation of the aforementioned pathways alongside an increased activation of pathways related to the immune response and cell cycle control (Figure 3A). Whilst WKY and SHRSP shared similarities in activation predictions for apoptosis, morbidity/mortality and organismal death in disease and biological function pathways, although through different mechanisms, SHRSP specific DEGs also influenced the response of several types of immune cells (Figure 3B). Pathways with activation scores that

were different between strains and/or known to affect vessel function were chosen for further analysis.

Early Pregnancy is Associated with Alterations in Inflammatory Response Genes

The increased activation of pathways related to the immune response in SHRSP uterine arteries directed the investigation into gene expression changes in inflammatory response. Both WKY and SHRSP arteries showed differential gene expression that suggests an increased local inflammatory response to pregnancy by GD6.5 (Figure 4). 19 DEGs were common to both strains (Figure 4 & Table S2), however the number of genes influencing pregnancy-associated inflammatory response were greater in SHRSP uterine arteries than WKY (89 vs. 38, respectively) (Figure 4 and Table S3-S4). Taken together, these data suggest hypertensive SHRSP dams experience an elevated immune response to pregnancy compared to WKY.

SHRSP Uterine Arteries have a Pregnancy-Associated Increase in NOX-2 Expression

Pathway analysis also revealed SHRSP uterine arteries are associated with a prediction of increased production of reactive oxygen species (ROS) via NADPH oxidase in early pregnancy (z-score= 1.34 WKY vs. -3.05 SHRSP). The expression of key genes involved in the production of nitric oxide and reactive oxygen species in WKY uterine arteries did not change in response to pregnancy, whilst SHRSP arteries experience a pregnancy-associated increase in expression (Figure 5, Table S5). SHRSP uterine arteries demonstrated an increased expression of NADPH oxidase (NOX) 2 subunits *p22-phox*, *p67-phox*, *gp91* and *p40-phox* in the pregnant SHRSP compared to NP. This suggests an increase in NOX2 expression and therefore ROS production in early pregnancy that does not occur in early WKY pregnancy.

Calcium Signalling Genes were Differentially Altered in WKY and SHRSP Arteries in Response to Pregnancy

IPA revealed gene expression patterns in WKY that suggested a reduction in Ca^{2+} signalling in GD6.5 uterine arteries (Figure 6). There was a significant decrease in expression of *Plcg2*, *Itpr2* and *Myh6* and an increase in *Calm1* expression in pregnant WKY uterine arteries (Table S6). The same expression pattern was not observed in the DEGs from SHRSP arteries (Figure 6B), where there was a decrease in expression of *Plcl1* and *Mylk* and an increase in *Prkcb* expression. This suggests a reduction in Ca^{2+} release and increased sequestering of Ca^{2+} in WKY pregnancy, whereas the DEGs in SHRSP uterine arteries suggest an activation of Ca^{2+} signal transduction in early pregnancy.

WKY vessels also demonstrated a pregnancy-dependant reduction in the expression of RAAS genes leading to a predicted decrease in Ca^{2+} release downstream of the AT_1R (Figure 7A). In contrast, SHRSP pregnant vessels demonstrated an increase in expression of genes involved in RAAS signalling in the uterine artery (Figure 7B), thus a predicted activation of pathways downstream of AT_1R that results in the production of ROS and vasoconstriction (Table S7).

SHRSP and WKY Dams Show Distinct Differences in Genes Related to Energy Production During Early Pregnancy

WKY uterine arteries demonstrated an overall increase in the expression of genes related to oxidative phosphorylation in pregnant relative to non-pregnant arteries, with a z-score of -2.53. The z-score for SHRSP was 0, indicating that this pathway is not involved in the early adaptation to pregnancy in this strain. IPA® revealed the DEGs were related to increased expression of components of complexes I, III, IV and V in WKY dams across the first 6 days of gestation (Table S8; Figure 8A). This change in expression was not observed in SHRSP uterine arteries.

DISCUSSION

This study focussed on specific changes in the gene expression profile of uterine arteries in WKY and SHRSP rats from non-pregnant to GD6.5. It complements many other studies that have examined differential gene expression across pregnancy in both humans and rodents (10, 26-28). However, to our knowledge, it is the first to examine expression changes in early pregnancy, prior to functional and structural changes in the uterine arteries in a model of chronic hypertension. We identified differential expression of 364 pregnancy-specific genes in WKY and 654 in SHRSP. These specific DEGs in WKY were predicted to be involved in pathways related to increased energy production and reduced calcium signal transduction, whilst SHRSP-specific changes were predicted to influence the immune response and activate pathways leading to vasoconstriction. Despite similarities in pregnancy-associated gene expression in both strains, our data suggests that uterine arteries in SHRSP dams demonstrate an altered genetic response in early pregnancy that may lead to maladaptive changes and failed priming of the arteries and thus, deficient remodelling.

We have previously shown that the SHRSP exhibits deficient uterine artery remodelling, reflected by altered vascular structure and function and uteroplacental blood flow at GD18.5 (24). This was not the case at the early pregnancy (GD6.5) time point examined in the current study, with uterine arteries responding to vasoactive substances and having similar structure to WKY. From the prediction analysis performed using IPA® we found both SHRSP and WKY dams shared gene expression changes indicating a necessary vascular response to pregnancy is conserved between strains. The biological functions associated with the 188 pregnancy-specific DEGs in common between WKY and SHRSP were associated

with processes involved in vascular remodelling. However, out-with this set of shared genes in common, the pregnancy-dependant specific gene expression changes were observed to differ greatly between WKY and SHRSP suggesting an additional stress response present in SHRSP only. This may be in part due to pre-existing hypertension in this strain. However, it is worth noting that in previous studies amelioration of the SHRSP dam's pre-existing hypertension only partially resolves their observed abnormal uterine artery remodelling (24). We have previously shown that treatment with nifedipine prior to and throughout pregnancy in the SHRSP whilst controlling their blood pressure - and as a result limiting the known direct effects of hypertension on oxidative stress and inflammatory cell activation (30, 32, 33) - did not improve measures of uterine artery function including assessment via myography and Doppler ultrasound *in vivo* at GD18.5 (24).

We chose to further investigate gene expression changes related to the inflammatory response pathway, given the known importance of uterine-specific natural killer (uNK) cells, decidual macrophages and other maternal immune cells in coordinating appropriate trophoblast invasion into the spiral arteries (29), and the association between an exaggerated inflammatory response in the placental bed of hypertensive pregnancies (30). Whilst both strains demonstrated an increased local inflammatory response to pregnancy, this was much greater in the SHRSP suggesting hypertensive SHRSP dams experience an abnormally elevated immune response in response to pregnancy, as early as GD6.5. Indeed, an abnormal immune response has been implicated in early gestation as an initiating factor that may interfere with crucial interactions between uterine natural killer cells and trophoblasts, resulting in systemic inflammation, impaired placentation and dysregulated vascular remodelling (31). An array of factors can contribute to impairment of the immune response, one of which is reactive oxygen species (ROS) produced by NADPH oxidase (NOX).

348 In macrophages, ROS released to the extracellular space by membrane-bound NOX in
349 chronic inflammatory conditions has been shown to impair the function of T-cells and NK
350 cells (32). Oxidative stress, or ROS accumulation, produced by NOXs is also known to play a
351 role in the development and maintenance of hypertension (33). IPA® predicted an increase
352 in ROS production via increased expression of NOX2 in SHRSP but not WKY uterine arteries
353 in early pregnancy. This suggests that hypertensive SHRSP dams experience an increase in
354 ROS production in early pregnancy which may contribute to the elevated immune response
355 as well as abnormal uterine artery function observed in later pregnancy.
356 Mitochondrial function is crucial in early pregnancy as in the first trimester there is a heavy
357 reliance on glucose utilisation for the increased production of ATP to meet the increased
358 maternal and fetal energy requirements (34). Analysis of the proteome in placental tissue
359 from normotensive and pre-eclamptic pregnancies demonstrates an association between
360 pre-eclampsia, inflammation, and mitochondrial dysfunction (35). In our data there was an
361 overall increase in the expression of genes involved in oxidative phosphorylation in pregnant
362 WKY uterine arteries. However, SHRSP uterine arteries did not demonstrate any gene
363 expression changes in the pathway across pregnancy. This implies that in early hypertensive
364 pregnancy either energy production is already at its peak preventing further increases or it
365 is unable to respond to the demands of pregnancy. This inability to adapt to changing
366 energy requirements may contribute to a dysregulated immune response, impaired later
367 vascular remodelling or predicted changes in calcium signalling and transduction.
368 Given the importance of Ca^{2+}-signalling in a myriad of functions required to complete a
369 healthy pregnancy, together with studies demonstrating a decrease in serum Ca^{2+} in
370 hypertensive pregnancies (36), we chose to investigate expression changes in the α-
371 adrenergic signalling pathway in this study. The DEGs in WKY uterine arteries indicate a

pregnancy-associated reduction in Ca^{2+} release and sequestering. This may contribute to the decrease in peripheral vascular resistance and blood pressure normally experienced during pregnancy. Gopalakrishnan *et al* highlighted that the typical transcriptome response to pregnancy, in the rat uterine artery, involves the downregulation of calcium signalling and vascular smooth muscle contraction pathways (37). In contrast, SHRSP uterine arteries demonstrated an increased expression of *Prkcb*, a member of the PKC family which is known to mediate vascular contraction independently of intracellular Ca^{2+} (38, 39) along with an expression pattern suggesting an increased overall activation of the Ca^{2+} signalling pathway. This suggests a potential mechanism for the increased contractile response observed in later gestation in SHRSP uterine arteries (24).

Normal pregnancy is associated with an up-regulation of RAAS and concurrent increased resistance to angiotensin II (ANGII), whilst HDPs (particularly pre-eclampsia) are associated with an increased sensitivity to ANGII and suppression of the RAAS (40, 41). IPA® analysis of the RAAS pathway revealed a pregnancy-dependant decrease in expression of RAAS genes in WKY uterine arteries and an increased expression of genes downstream of angiotensin receptor 1 (AT_1R) in SHRSP uterine arteries. These data suggest that whilst there is evidence of a decrease in Ca^{2+} release in WKY uterine arteries, in SHRSP arteries the pathways downstream of AT_1R have an increased activation, leading to increased ROS production and vasoconstriction. However, animal models with excessive RAAS activation also result in pre-eclampsia like symptoms and there is evidence that demonstrates a role for autoantibody activation of AT_1R and its downstream effectors leading to vasoconstriction (40). Interestingly, these downstream effectors include NOX and other genes that were also implicated in the increased production of ROS and immune response demonstrated in SHRSP uterine arteries in early pregnancy.

This study sought to examine the specific differences in gene expression in response to early pregnancy in the normotensive WKY and hypertensive SHRSP uterine artery. Taken together, our data provides evidence that hypertension during pregnancy results in distinct gene expression changes in pathways influencing uterine artery vascular function that are not seen in normotensive pregnancy. These pathway changes may underlie or contribute to the adverse vascular remodelling and resultant placental ischaemia and systemic vascular dysfunction in later stages of gestation seen in hypertensive disorders of pregnancy in rodents. In the absence of human uterine artery samples from an early gestational time point, the results here may lend some insight into maladaptive responses in human hypertensive pregnancies.

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REFERENCES

1. Say L, Chou D, Gemmill A, Tunçalp Ö, Moller AB, Daniels J, et al. Global causes of maternal death: a WHO systematic analysis. Lancet Glob Health. 2014;2(6):e323-33.
2. Brown MA, Magee LA, Kenny LC, Karumanchi SA, McCarthy FP, Saito S, et al. The hypertensive disorders of pregnancy: ISSHP classification, diagnosis & management recommendations for international practice. Pregnancy Hypertens. 2018;13:291-310.
3. Cunningham MW, LaMarca B. Risk of cardiovascular disease, end-stage renal disease, and stroke in postpartum women and their fetuses after a hypertensive pregnancy. Am J Physiol Regul Integr Comp Physiol. 2018;315(3):R521-R8.
4. Stojanovska V, Scherjon SA, Plösch T. Preeclampsia As Modulator of Offspring Health. Biol Reprod. 2016;94(3):53.
5. Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet. 1986;1(8489):1077-81.
6. Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. Lancet. 1989;2(8663):577-80.
7. Barker DJ, Gluckman PD, Godfrey KM, Harding JE, Owens JA, Robinson JS. Fetal nutrition and cardiovascular disease in adult life. Lancet. 1993;341(8850):938-41.
8. Wilson BJ, Watson MS, Prescott GJ, Sunderland S, Campbell DM, Hannaford P, et al. Hypertensive diseases of pregnancy and risk of hypertension and stroke in later life: results from cohort study. BMJ. 2003;326(7394):845.
9. Sattar N, Ramsay J, Crawford L, Cheyne H, Greer IA. Classic and novel risk factor parameters in women with a history of preeclampsia. Hypertension. 2003;42(1):39-42.
10. Hubel CA, Snaedal S, Ness RB, Weissfeld LA, Geirsson RT, Roberts JM, et al. Dyslipoproteinaemia in postmenopausal women with a history of eclampsia. BJOG. 2000;107(6):776-84.
11. Sattar N, Greer IA. Pregnancy complications and maternal cardiovascular risk: opportunities for intervention and screening? BMJ. 2002;325(7356):157-60.
12. Pijnenborg R, Vercruysse L, Hanssens M. The uterine spiral arteries in human pregnancy: facts and controversies. Placenta. 2006;27(9-10):939-58.
13. Osol G, Mandala M. Maternal uterine vascular remodeling during pregnancy. Physiology (Bethesda). 2009;24:58-71.
14. Burton GJ, Woods AW, Jauniaux E, Kingdom JC. Rheological and physiological consequences of conversion of the maternal spiral arteries for uteroplacental blood flow during human pregnancy. Placenta. 2009;30(6):473-82.
15. Robertson WB, Brosens I, Dixon HG. The pathological response of the vessels of the placental bed to hypertensive pregnancy. J Pathol Bacteriol. 1967;93(2):581-92.
16. Lyall F, Robson SC, Bulmer JN. Spiral artery remodeling and trophoblast invasion in preeclampsia and fetal growth restriction: relationship to clinical outcome. Hypertension. 2013;62(6):1046-54.
17. van der Heijden OW, Essers YP, Spaanderman ME, De Mey JG, van Eys GJ, Peeters LL. Uterine artery remodeling in pseudopregnancy is comparable to that in early pregnancy. Biol Reprod. 2005;73(6):1289-93.
18. Pastore MB, Jobe SO, Ramadoss J, Magness RR. Estrogen receptor-α and estrogen receptor-β in the uterine vascular endothelium during pregnancy: functional implications for regulating uterine blood flow. Semin Reprod Med. 2012;30(1):46-61.
19. Mishra JS, Gopalakrishnan K, Kumar S. Pregnancy upregulates angiotensin type 2 receptor expression and increases blood flow in uterine arteries of rats. Biol Reprod. 2018;99(5):1091-9.
20. Hu XQ, Song R, Romero M, Dasgupta C, Huang X, Holguin MA, et al. Pregnancy Increases Ca^{2+} Sparks/Spontaneous Transient Outward Currents and Reduces Uterine Arterial Myogenic Tone. Hypertension. 2019;73(3):691-702.
21. Lian IA, Toft JH, Olsen GD, Langaas M, Bjorge L, Eide IP, et al. Matrix metalloproteinase 1 in pre-eclampsia and fetal growth restriction: reduced gene expression in decidual tissue and protein expression in extravillous trophoblasts. Placenta. 31. England2010. p. 615-20.

22. Herse F, Lamarca B, Hubel CA, Kaartokallio T, Lokki AI, Ekholm E, et al. Cytochrome P450 subfamily 2J polypeptide 2 expression and circulating epoxyeicosatrienoic metabolites in preeclampsia. Circulation. 2012;126(25):2990-9. doi: 10.1161/CIRCULATIONAHA.112.127340. Epub 2012 Nov 15.
23. Wang Y, Fan H, Zhao G, Liu D, Du L, Wang Z, et al. miR-16 inhibits the proliferation and angiogenesis-regulating potential of mesenchymal stem cells in severe pre-eclampsia. FEBS J. 2012;279(24):4510-24. doi: 10.1111/febs.12037. Epub 2012 Nov 22.
24. Small HY, Morgan H, Beattie E, Griffin S, Indahl M, Delles C, et al. Abnormal uterine artery remodelling in the stroke prone spontaneously hypertensive rat. Placenta. 2016;37:34-44.
25. Morgan HL, Butler E, Ritchie S, Herse F, Dechend R, Beattie E, et al. Modeling Superimposed Preeclampsia Using Ang II (Angiotensin II) Infusion in Pregnant Stroke-Prone Spontaneously Hypertensive Rats. Hypertension. 2018;72(1):208-18.
26. Lash GE, Schiessl B, Kirkley M, Innes BA, Cooper A, Searle RF, et al. Expression of angiogenic growth factors by uterine natural killer cells during early pregnancy. J Leukoc Biol. 2006;80(3):572-80.
27. Lash GE, Pitman H, Morgan HL, Innes BA, Agwu CN, Bulmer JN. Decidual macrophages: key regulators of vascular remodeling in human pregnancy. J Leukoc Biol. 2016;100(2):315-25.
28. Robson A, Lash GE, Innes BA, Zhang JY, Robson SC, Bulmer JN. Uterine spiral artery muscle dedifferentiation. Hum Reprod. 2019;34(8):1428-38.
29. Mor G, Cardenas I, Abrahams V, Guller S. Inflammation and pregnancy: the role of the immune system at the implantation site. Ann N Y Acad Sci. 2011;1221:80-7.
30. Barakonyi A, Miko E, Szereday L, Polgar PD, Nemeth T, Szekeres-Bartho J, et al. Cell death mechanisms and potentially cytotoxic natural immune cells in human pregnancies complicated by preeclampsia. Reprod Sci. 2014;21(2):155-66.
31. Taylor EB, Sasser JM. Natural killer cells and T lymphocytes in pregnancy and pre-eclampsia. Clin Sci (Lond). 2017;131(24):2911-7.
32. Yang Y, Bazhin AV, Werner J, Karakhanova S. Reactive oxygen species in the immune system. Int Rev Immunol. 2013;32(3):249-70.
33. Paravicini TM, Touyz RM. NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities. Diabetes Care. 2008;31 Suppl 2:S170-80.
34. Thornburg KL, Jacobson S-L, Giraud GD, Morton MJ. Hemodynamic changes in pregnancy. Seminars in Perinatology. 2000;24(1):11-4.
35. Xu Z, Jin X, Cai W, Zhou M, Shao P, Yang Z, et al. Proteomics Analysis Reveals Abnormal Electron Transport and Excessive Oxidative Stress Cause Mitochondrial Dysfunction in Placental Tissues of Early-Onset Preeclampsia. Proteomics Clin Appl. 2018;12(5):e1700165.
36. Adamova Z, Ozkan S, Khalil RA. Vascular and cellular calcium in normal and hypertensive pregnancy. Curr Clin Pharmacol. 2009;4(3):172-90.
37. Gopalakrishnan K, Kumar S. Whole-Genome Uterine Artery Transcriptome Profiling and Alternative Splicing Analysis in Rat Pregnancy. Int J Mol Sci. 2020;21(6).
38. Gutiérrez A, Contreras C, Sánchez A, Prieto D. Role of Phosphatidylinositol 3-Kinase (PI3K), Mitogen-Activated Protein Kinase (MAPK), and Protein Kinase C (PKC) in Calcium Signaling Pathways Linked to the α(1)-Adrenoceptor in Resistance Arteries. Front Physiol. 2019;10:55.
39. Ringvold HC, Khalil RA. Protein Kinase C as Regulator of Vascular Smooth Muscle Function and Potential Target in Vascular Disorders. Adv Pharmacol. 2017;78:203-301.
40. Verdonk K, Visser W, Van Den Meiracker AH, Danser AH. The renin-angiotensin-aldosterone system in pre-eclampsia: the delicate balance between good and bad. Clin Sci (Lond). 2014;126(8):537-44.
41. Hussein W, Lafayette RA. Renal function in normal and disordered pregnancy. Curr Opin Nephrol Hypertens. 2014;23(1):46-53.

TABLE 1

Table 1: The top 15 biological functions and diseases associated with the 188 DEGs associated with pregnancy in both SHRSP and WKY.

Biological Diseases & Functions	-log(*p*-value)
Cell Cycle	13.119
Cellular Assembly & Organisation	12.827
DNA Replication, Recombination & Repair	12.827
Cancer	11.570
Organismal Injury & Abnormalities	11.570
Reproductive System Disease	11.570
Cell Death & Survival	10.535
Protein Synthesis	8.391
Neurological Disease	8.156
Cellular Movement	8.099
Cardiovascular System Development & Function	7.533
Cellular Development	7.255
Cellular Growth & Proliferation	7.255
Gastrointestinal Disease	7.253
Hepatic System Disease	7.223

Biological Diseases & Functions were determined using Ingenuity Pathway Analysis (IPA®) pathway enrichment analysis based on gene expression. The diseases & functions that the 188 in-common DEGs are most likely to influence are listed at the top, with a greater -$\log_{10}$(*p*-value) suggesting increased likelihood of involvement in the uterine arteries adaptation to pregnancy.

FIGURE LEGENDS

Figure 1 Isolated uterine artery function and structure was analysed ex vivo by wire and pressure myography in response to varying concentrations ($_{1x}10^{-9}$ – $_{2x}10^{-5}$M) of vasoactive stimuli and varying pressures along a gradient of 10-110mmHg. There was no significant difference in the pregnancy-dependent or strain responses to noradrenaline (A), carbcachol (B) or sodium nitroprusside (SNP) (C). External (D) and internal (E) diameters were significantly increased in NP WKY uterine arteries compared to NP SHRSP arteries (*p<0.05, **p<0.01). There were no significant differences in uterine artery cross-sectional area (F). Data presented as mean ± SEM and analysed using one-way ANOVA with Tukey's *post hoc* test; n=5-11. NP= non-pregnant.

Figure 2 (A) The principal component analysis of non-pregnant (NP) and GD6.5 pregnant (P) uterine artery gene expression from WKY and SHRSP rats, showing the ordination of all samples and the top two principle components, PC1 (Pregnancy variance = 23.2%) and PC2 (Strain variance = 16.3%). (B) The biotype profiles demonstrate the proportions of the different biotypes represented by the significantly differentially expressed genes. lincRNA = long non-coding RNA, miRNA = microRNA. (C) Venn diagram showing the number of DEGs specific to SHRSP (654) or WKY (364), or in common between strains (188), across pregnancy determined by IPA®. (D) The DEGs altered by pregnancy in WKY and SHRSP represented by volcano plots. p-values were adjusted for multiple comparisons and transformed by $-\log_{10}$. DEGs with a $-\log_{10}$(p-value) ≥10 are labelled by gene name; n=3/group.

Figure 3 The core comparison pathway analysis of canonical pathways (A) and those related to biological functions/diseases (B) of non-pregnant (NP) and GD6.5 (P) uterine artery gene expression from WKY and SHRSP rats. Pathways were ranked by hierarchical clustering and assigned z-scores as area measures of the predicted direction of activity such that a positive score was associated with increased pathway activation in NP compared to GD6.5 uterine arteries, thus a pregnancy associated reduction; n=3/group.

Figure 4 Predicted interaction network of the inflammatory response in non-pregnant (NP) or GD6.5 (P) uterine arteries in SHRSP and WKY dams. The genes in common (same direction, similar fold-change) are shown in the lower semi-circle. There were more genes involved in this response in SHRSP uterine arteries than WKY. Here, green represents an increased expression and red a

583 decreased expression in pregnant (GD6.5) vs non-pregnant uterine arteries;
584 *n*=3/group.
585
586 **Figure 5** Predicted expression network for the production of nitric oxide and
587 reactive oxygen species (ROS) in non-pregnant (NP) or GD6.5 (P) uterine
588 arteries in WKY (A) and SHRSP (B) dams. No changes were detected in WKY
589 uterine arteries. SHRSP arteries showed an increased expression of four NOX2
590 subunits, PKC=protein kinase C, PP1/PP2a=protein phosphatase complex 1/2a
591 and PI3K= phosphatidylinositol kinase complex 3. NOX2= NADPH oxidase 2.
592 Here, green represents an increased expression, red a decreased expression,
593 grey no change and a green/red representing conflicting expression in
594 pregnant (GD6.5) vs non-pregnant uterine arteries; *n*=3/group.
595
596 **Figure 6** Predicted expression network for adrenergic calcium signalling in non-
597 pregnant (NP) or GD6.5 (P) uterine arteries in WKY (A) and SHRSP (B) dams.
598 WKY demonstrated a predicted pregnancy-dependant reduction in expression
599 of PLC= phospholipase C, ITPR2= Inositol 1,4,5-triphosphate receptor type 2
600 and myosin= Myosin heavy chain 6, alongside an increased expression of
601 Calm1= calmodulin 1. SHRSP showed a pregnancy-dependant increase in the
602 expression of Prkcb= protein kinase C beta and a reduced expression of Mylk=
603 myosin light chain kinase and PLC= phospholipase C. Here, green represents an
604 increased expression, red a decreased expression, grey no change and a
605 green/red representing conflicting expression in pregnant (GD6.5) vs non-
606 pregnant uterine arteries; *n*=3/group.
607
608 **Figure 7** Predicted expression network for renin-angiotensin-aldosterone
609 system (RAAS) signalling in non-pregnant (NP) or GD6.5 (P) uterine arteries in
610 WKY (A) and SHRSP (B) dams. WKY demonstrated a predicted pregnancy-
611 dependant reduction in expression of PLCγ = phospholipase C gamma, ITPR2=
612 Inositol 1,4,5-Trisphosphate receptor type 2, MEKK1= Mitogen-activated
613 protein kinase kinase kinase 1. SHRSP showed a pregnancy-dependant increase
614 in the expression of SHP-1= Protein tyrosine phosphatase non-receptor type 6,
615 NOX = NADPH oxidase, PKC= protein kinase C, PAK= p-21 activated kinase 1,
616 PI3K= Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma.
617 Here, green represents an increased expression, red a decreased expression,
618 grey no change and a green/red representing conflicting expression in
619 pregnant (GD6.5) vs non-pregnant uterine arteries; *n*=3/group.
620
621 **Figure 8** Predicted expression network for oxidative phosphorylation signalling
622 in non-pregnant (NP) or GD6.5 (P) uterine arteries in WKY (A) and SHRSP (B)

623 dams. WKY demonstrated a predicted pregnancy-dependant increase in
624 expression of components of complexes III, IV and V. WKY also demonstrated a
625 conflicting expression in complex I. No changes were detected in SHRSP
626 uterine arteries. Here, green represents an increased expression, red a
627 decreased expression, white no change and a green/red representing
628 conflicting expression in pregnant (GD6.5) vs non-pregnant uterine arteries;
629 *n*=3/group.

FIGURE 1

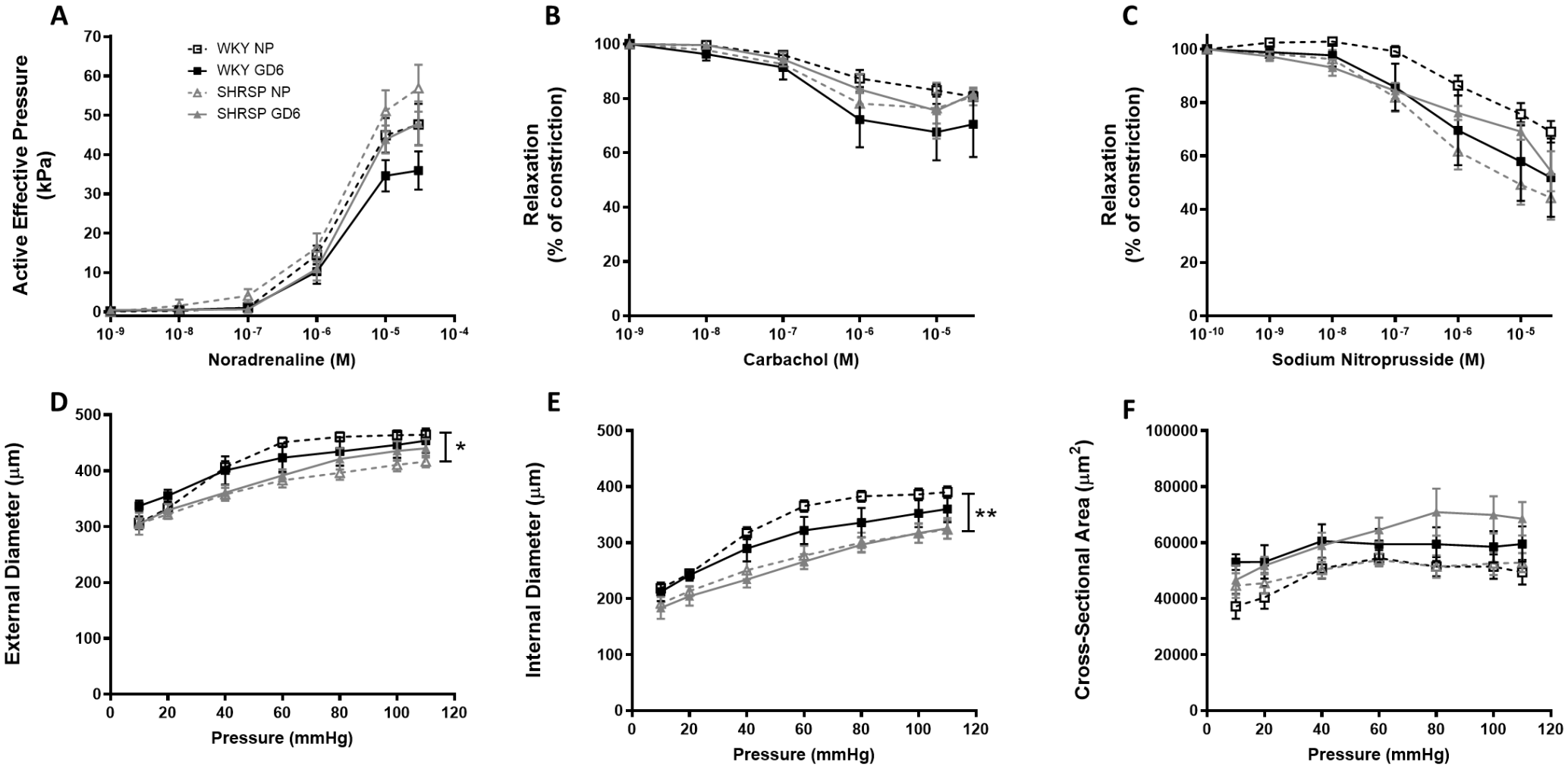


FIGURE 2

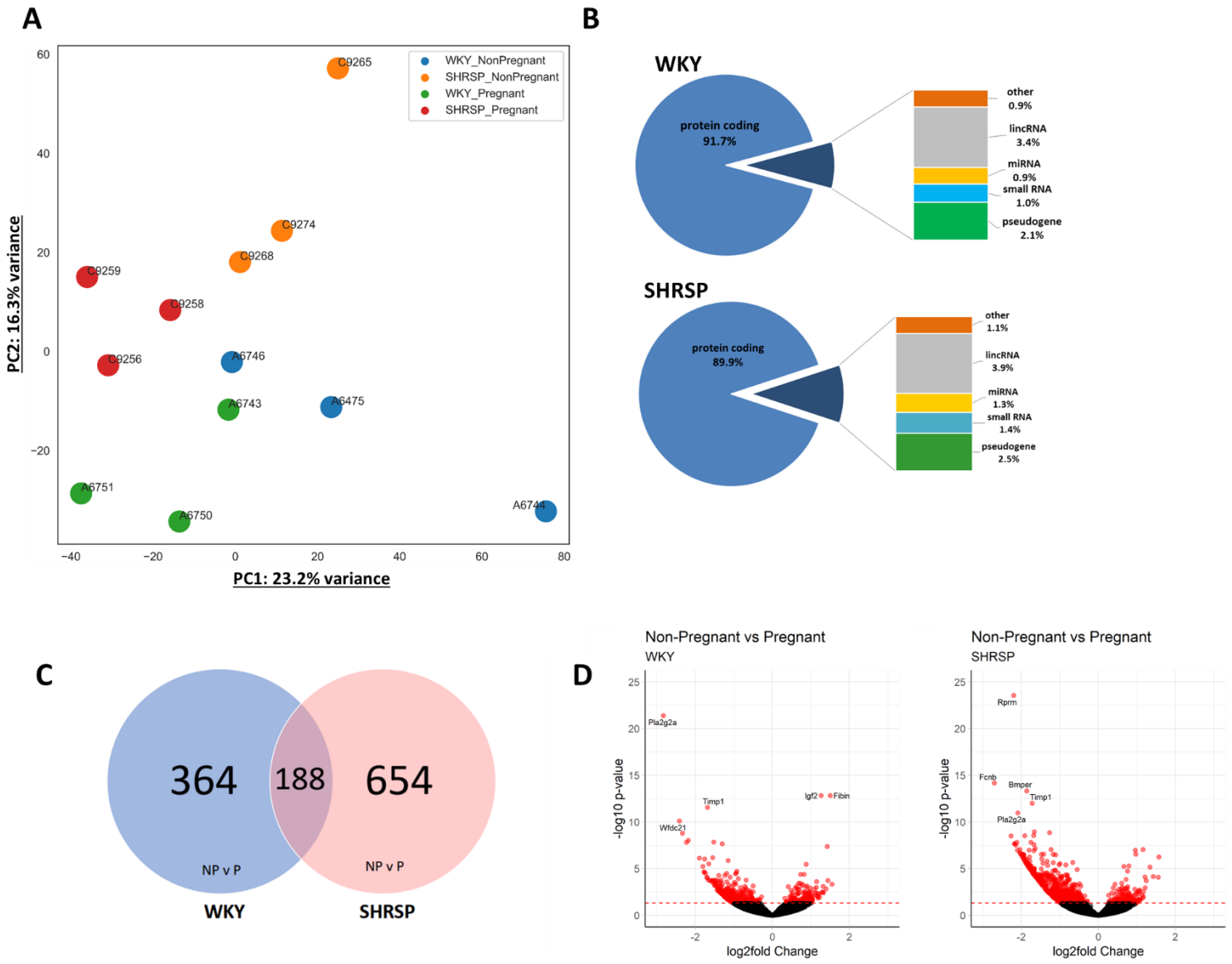
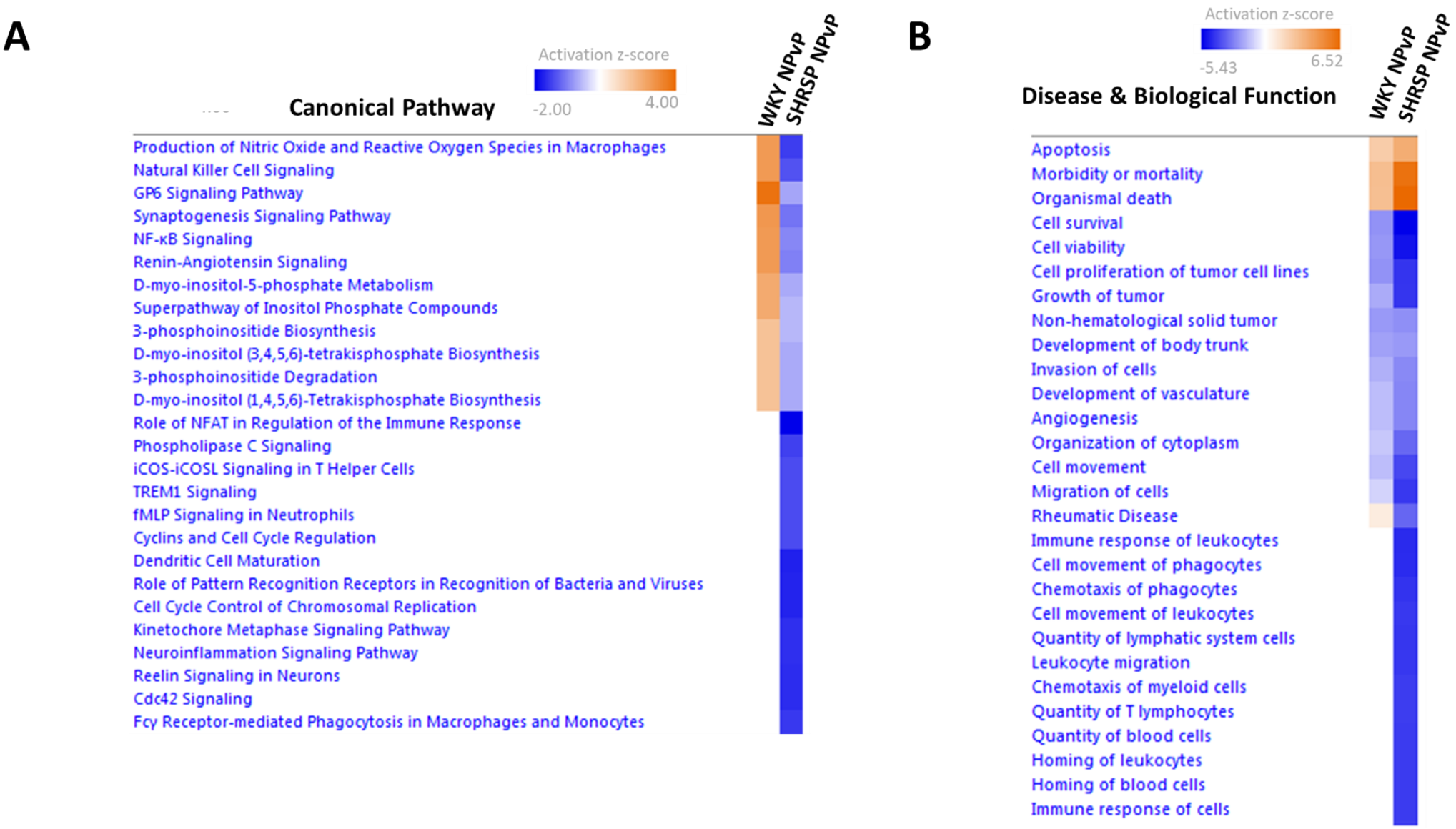


FIGURE 3



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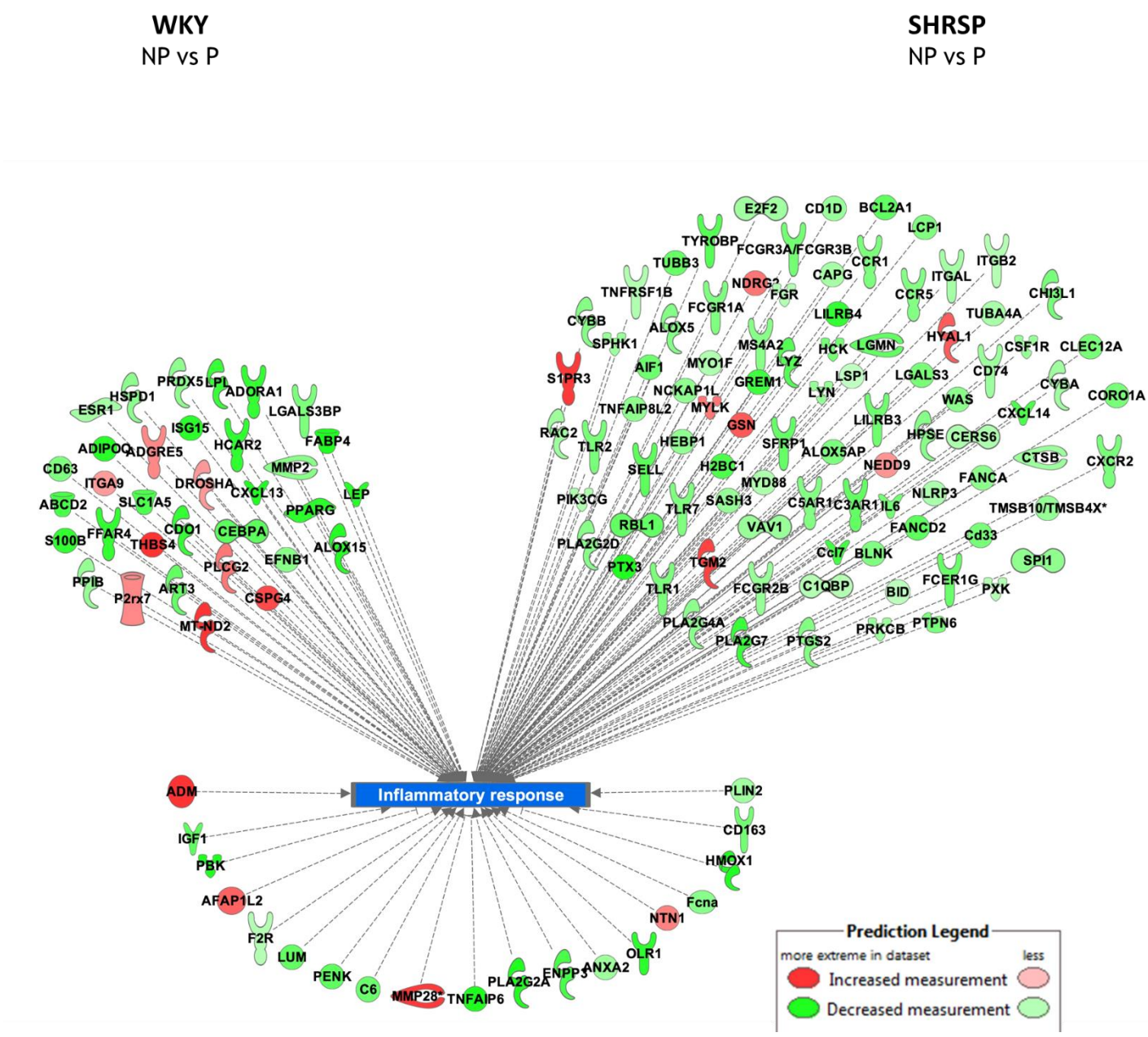

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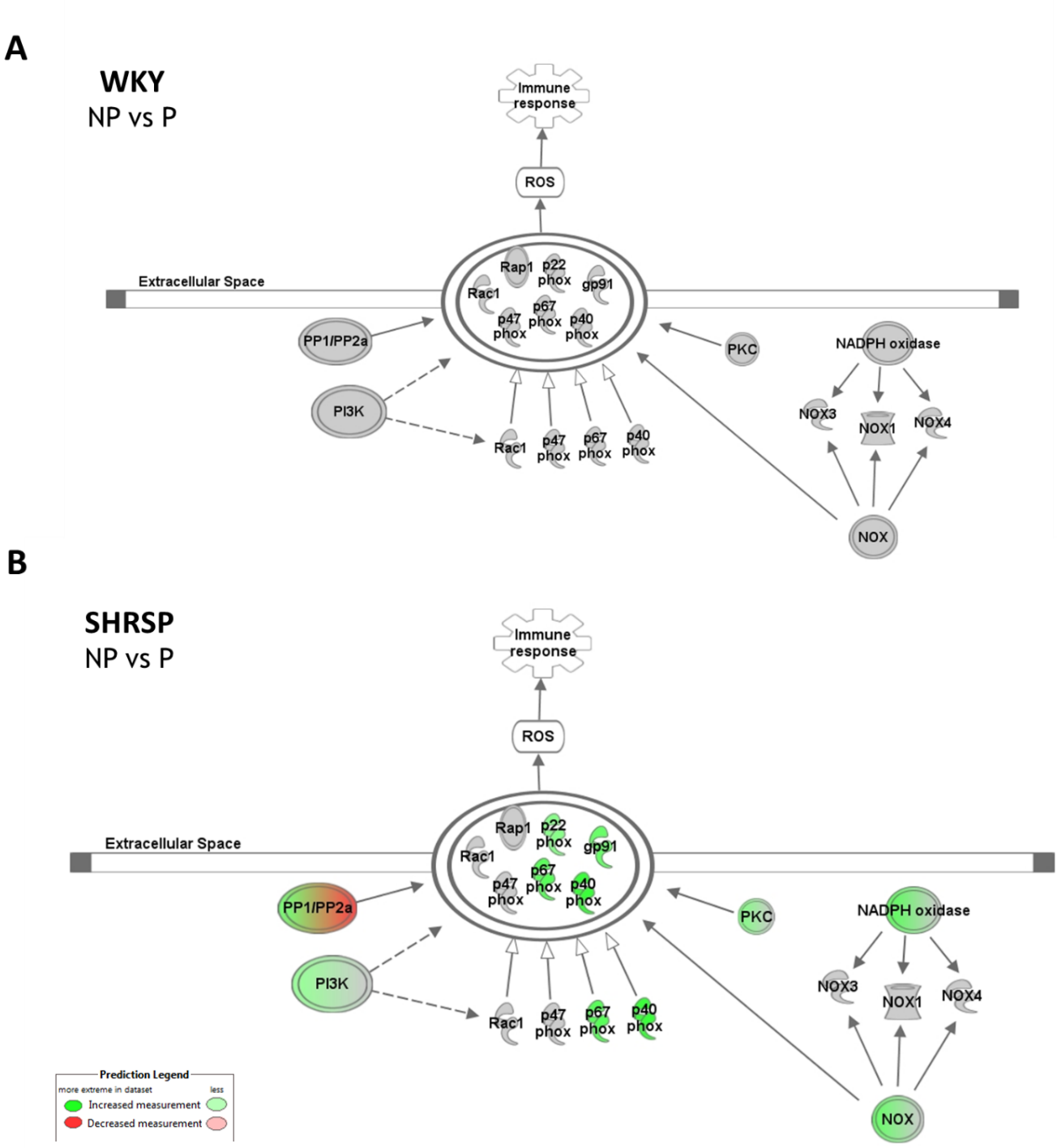

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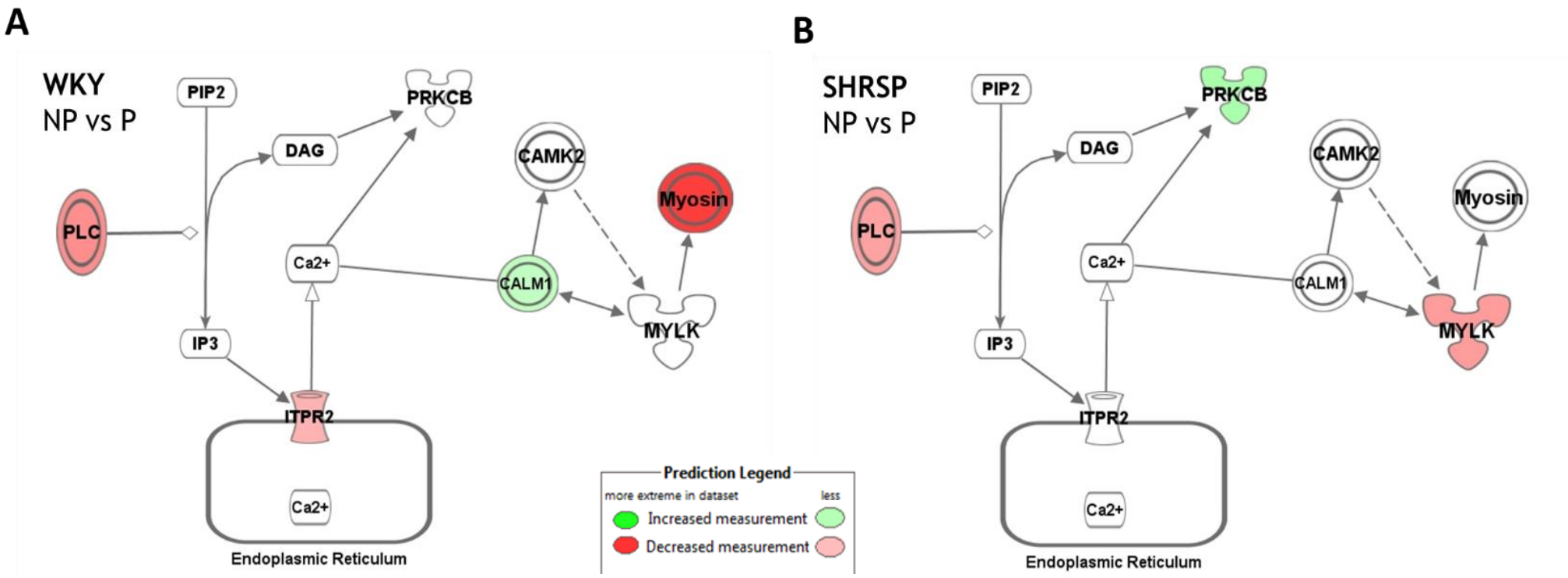

FIGURE 7

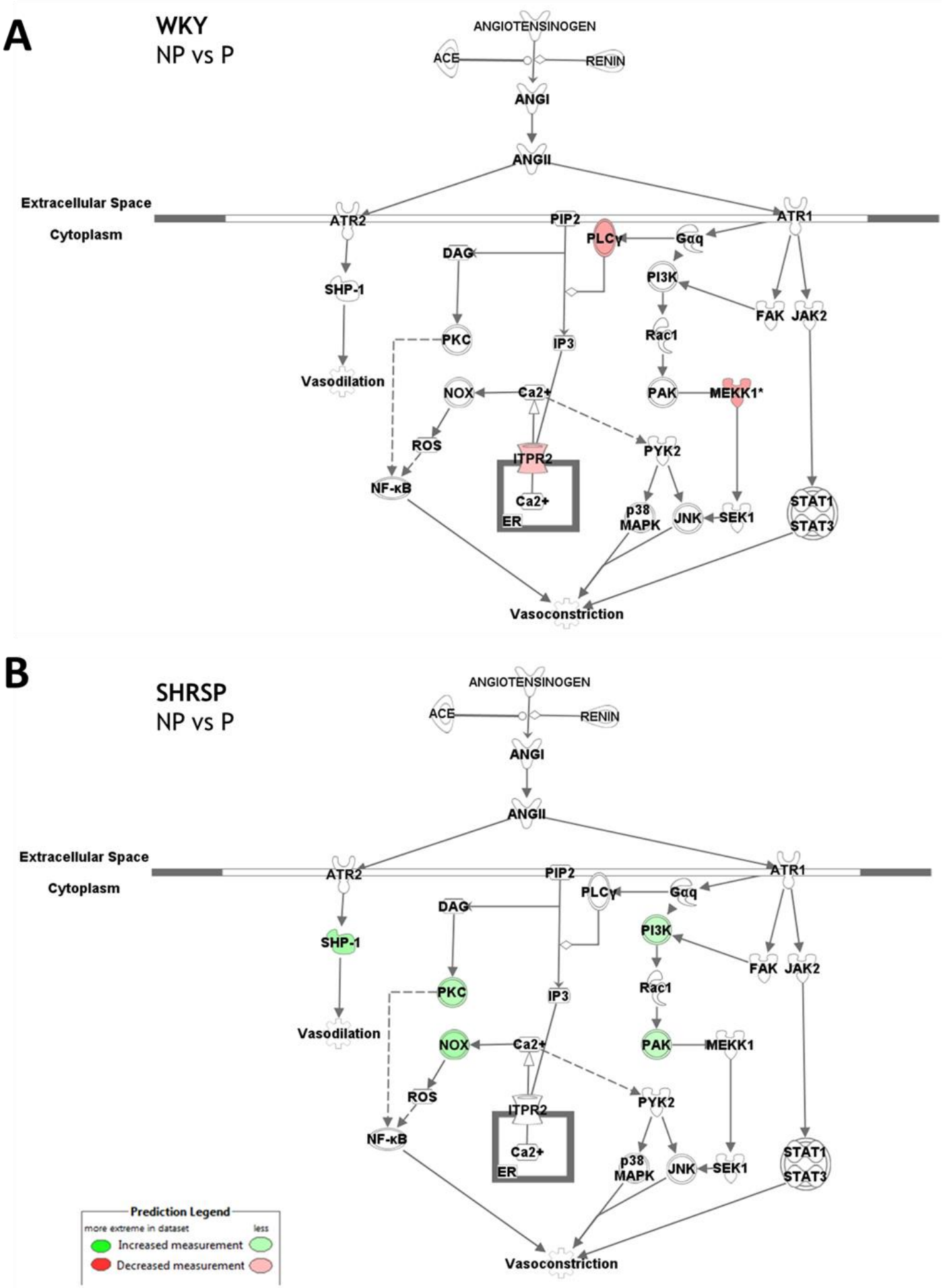

FIGURE 8