1. TITLE PAGE

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Full title

Placental syncytiotrophoblast-derived extracellular vesicles carry active neprilysin and are increased in preeclampsia

Authors' names and affiliations

Manjot Gill[1], Carolina Motta-Mejia[1], Neva Kandzija[1], William Cooke[1], Wei Zhang[1], Ana Sofia Cerdeira[1], Claire Bastie[2], Christopher Redman[1], Manu Vatish[1]

[1] Nuffield Department of Women's & Reproductive Health, University of Oxford, Women's Centre, John Radcliffe Hospital, Oxford, UK

[2] Warwick Medical School, University of Warwick, Coventry, CV4 7AL

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Corresponding author

Name: Dr. Manu Vatish, MBBCh BA (Hons) DPhil MA MRCOG

Email: manu.vatish@wrh.ox.ac.uk

Telephone: 01865 221009

Fax: 01865 769141

Address: Nuffield Department of Women's & Reproductive Health, University of Oxford, Women's Centre, John Radcliffe Hospital, Oxford, UK

2. ABSTRACT

Neprilysin is a widely-expressed membrane-bound metalloprotease which binds and cleaves a variety of peptides including vasodilators, natriuretics and diuretics. Higher levels of neprilysin result in hypertension, a cardinal feature of the placental disease preeclampsia. Syncytiotrophoblast-derived extracellular vesicles, comprising microvesicles and exosomes are released into the peripheral circulation in pregnancy and are postulated as a key mechanism coupling placental dysfunction and maternal phenotype in preeclampsia. We aimed to determine whether higher levels of active neprilysin are found in syncytiotrophoblast-derived extracellular vesicles in preeclampsia compared to normal pregnancy.

Using immunostaining and Western blotting, we first demonstrated that neprilysin levels are greater not only in preeclampsia placental tissue, but also in syncytiotrophoblast-derived microvesicles and exosomes isolated from preeclampsia placentas ($p<0.05$, $n=5$). We confirmed placental origin using antibody-coated magnetic beads to isolate neprilysin-bound vesicles, finding that they stain for placental alkaline phosphatase. Neprilysin on syncytiotrophoblast-derived extracellular vesicles is active and inhibited by thiorphan ($p<0.01$, $n=3$; specific inhibitor). Syncytiotrophoblast-derived microvesicles, isolated from peripheral plasma, demonstrated higher neprilysin expression in preeclampsia using flow cytometry ($n=8$, $p<0.05$). We isolated plasma exosomes using size-exclusion chromatography and showed greater neprilysin activity in preeclampsia ($n=8$, $p<0.05$).

These findings show that the placenta releases active neprilysin into the maternal circulation on syncytiotrophoblast-derived extracellular vesicles, at significantly greater levels in preeclampsia. Neprilysin has pathological roles in hypertension, heart failure and amyloid deposition, all of which are features of preeclampsia. Circulating syncytiotrophoblast-derived extracellular vesicle-bound neprilysin thus may contribute to the pathogenesis of this disease.

Key words: pre-eclampsia, neprilysin, extracellular vesicles, placenta, hypertension

3. TEXT

A) INTRODUCTION

Preeclampsia (PE) is a pregnancy-specific syndrome characterised by new onset hypertension and proteinuria in the second half of pregnancy, but often associated with more widespread organ dysfunction[1,2]. Worldwide PE occurs in 2-8% of pregnancies and it is one of the major global causes of maternal and fetal mortality[3]. A pathological process of systemic vascular inflammation and endothelial activation in PE can affect multiple organs, including: the peripheral vasculature (hypertension), liver (hepatocellular necrosis), kidneys (acute glomerular dysfunction or, more rarely, tubular or renal cortical necrosis), central nervous system (seizures and intracerebral haemorrhage) and the heart (left ventricular hypertrophy with diastolic dysfunction)[4–7]. The proximate cause of PE is the placenta since the disease is pregnancy specific, can occur in molar pregnancies or trophoblast tumours (where no fetal tissue is present) and resolves only following delivery of the placenta[8]. Poor placentation in early pregnancy, specifically with inadequate spiral artery remodelling appears to predate the clinical syndrome of PE[9]. The mechanism by which a chronically hypoxic placenta causes such diffuse multi-organ dysfunction in the mother remains elusive.

The syncytiotrophoblast (STB) is the layer of placenta in direct contact with maternal blood, with a total surface area of up to 10 m^2[10]. Up to 3 grams per day of extracellular vesicles (EV) are released from STB into the maternal circulation at term. Significantly higher levels of STBEV are found in the peripheral blood of women with PE than those with normal pregnancies (NP)[11]. It is consequently postulated that STBEV could be the missing link between the placental dysfunction

and later clinical associated maternal syndrome in PE[12]. To explore this hypothesis we performed a proteomic analysis of STBEV released during dual-lobe placental perfusion from 8 women with PE and 7 women with NP (unpublished data). This showed that neprilysin was upregulated in STBEV from women with PE.

Neprilysin (NEP), also known as CD10, CALLA (Common Acute Lymphocytic Leukemia Antigen) or MME (membrane metallo-endopeptidase) is a zinc metalloendopeptidase with a wide tissue distribution. It cleaves and inactivates a variety of bioactive peptides[13]. NEP inhibitors have recently attracted attention in cardiovascular medicine as a novel treatment for heart failure[14]. By inhibiting the degradation of endogenous vasoactive peptides, NEP inhibitors promote vasodilatation and prevent sodium retention and consequently counter pathological cardiac remodelling[15].

In previous studies Li and colleagues reported greater expression of placental NEP in PE pregnancies, localised to the surface and cytoplasm of trophoblast cells[16]. Buhimschi et al found increased NEP in the urine of women with severe PE[17]. No group has previously reported on NEP expression and activity in STB-EV.

We hypothesised that NEP expression and activity are upregulated in STB-EV from women with PE compared to NP and might contribute to the clinical syndrome of PE.

B) METHODS

The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Human subjects

Approval for this project was granted by the Central Oxfordshire Research Ethics Committee (07/H0607/74 and 07/H0606/148). Women recruited for this study provided written informed consent; clinical characteristics are described in Table 1. According to the criteria defined by the International Society for the Study of Hypertension in Pregnancy (ISSHP), *de novo* hypertension (blood pressure >140/90 mmHg) and proteinuria (>300 mg/day) after gestational week 20 was used to diagnose PE. Women with NP had singleton pregnancies and no evidence of PE or previous hypertensive disorders.

Peripheral blood was taken from the left antecubital fossa and collected in 4.5 mL citrate tubes using a 21-gauge needle. Samples were centrifuged at 1,500 x g for 15 minutes and the platelet poor plasma (PPP) was collected. Aliquots of 1 mL PPP were stored at –80°C in preparation for flow cytometry analysis and EV isolation. PPP samples collected from women with PE and NP were matched according to gestational age. Placentae were collected at the time of elective caesarean section (a different cohort of patients)**.**

Immunohistochemistry

Paraffin-embedded placental tissue sections from NP and PE placentas were cut at 8 μm and attached onto slides. Tissues were stored at room temperature until use. Sections were heated at 62°C for 30 minutes, immersed in Histo-Clear (National Diagnostics, Atlanta, USA) and rehydrated using ethanol. Antigens were retrieved

using 10 mM sodium citrate buffer and endogenous peroxidase activity was blocked with 3% v/v hydrogen peroxide. The sections were treated with blocking agent 10% v/v fetal calf serum in PBS for 1 hour and incubated overnight at 4°C with mouse monoclonal antibodies: anti-NEP (1:25, ab951; Abcam, Cambridge, UK) and anti-IgG1 (1:25, 400153; BioLegend, California, USA). VECTASTAIN® Elite® ABC Kit (Vector Laboratories, Peterborough, UK) reagents were used to incubate sections at room temperature in biotinylated horse anti-mouse IgG secondary antibody for 1 hour and enhancer reagent for 30 minutes. Exposure to DAB substrate kit (Vector Laboratories) was used to detect specific antibody binding and nuclei were stained with Shandon™ Harris Hematoxylin (Thermo Fisher Scientific, Massachusetts, USA). Sections were dehydrated using ethanol, mounted with Depex (VWR, Philadelphia, USA) and imaged using a Leica DM2500 optical microscope (Leica Microsystems, Wetzlar, Germany) with a Micropublisher 5.0 RTV camera system (QImaging, Surrey, Canada).

Isolation and characterisation of STBEV

Placental perfusate from a dual placental lobe perfusion system was centrifuged to pellet fractions containing STBMV (10,000 x g) and STBEX (150,000 x g). The method has previously been described in detail by Dragovic et al[18]. A BD LSRII flow cytometer (BD Biosciences, California, USA) was used to phenotype STBMV and the NanoSight NS500 system (Malvern Instruments, Malvern, UK) was used to measure the size and concentration of STBEX using Nanoparticle Tracking Analysis (NTA). The protein concentration of isolated STBMV and STBEX was determined by the BCA protein assay and STBEV were stored at –80°C until further use.

Western blotting

Western blotting was performed using placental lysate (PL, 18 µg) and STBEV (6 µg) from NP and PE placentas. STBMV and STBEX were treated with HEPES lysis buffer 1:1 (50mM HEPES, pH 7.5; 2% SDS; 10% Glycerol). Western blots were incubated overnight at 4°C with mouse monoclonal anti-NEP antibody (1:1000, ab951; Abcam) and mouse monoclonal anti-placental alkaline phosphatase (PLAP) antibody (1:1000; NDOG2, in-house antibody). After incubation with HRP-conjugated anti-mouse IgG antibody (1:4000) for 1 hour at room temperature, Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific) was used for chemiluminescence detection of antigens on x-ray film. Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, California, USA) was used to stain total protein and bands were normalised to this using ImageJ version 1.51 (public domain).

Immunoprecipitation

Dynabeads™ Sheep-Anti Mouse IgG (50 µL; Life Technologies, California, USA) were incubated overnight at 4°C with either: anti-NEP antibody (6 µg/mL, MA1-19086; Thermo Fisher Scientific), anti-PLAP antibody (6 µg/mL; NDOG2, in-house antibody) or anti-IgG1 isotype control antibody (6 µg/mL, 400153; BioLegend). STBEV from NP placentas were incubated for 10 minutes at 4°C with 10x FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). Antibody-coated Dynabeads™ were then incubated overnight at 4°C with 25 µg of blocked STBMV or STBEX in PBS. Unbound sample was washed using PBS and STBEV separated from the Dynabeads™ by reducing agent, 1x Laemmli Sample Buffer (Bio-Rad Laboratories, California, USA) and centrifugation at 13,000 RPM. Western blotting was carried out on isolated STBEV and blots were probed

using anti-NEP antibody (1:1000, ab951; Abcam) and anti-PLAP antibody (1:1000, NDOG2, in-house antibody).

NEP activity of STBEV

NEP activity in STBEV derived from NP placentas was determined using the SensoLyte® 520 Neprilysin Activity Assay Kit (AnaSpec, California, USA). The provided substrate, 5-FAM/QXL® FRET, was incubated with different amounts of STBEX and STBMV (10, 25 and 50 µg) for 1 hour at 37°C. Specific cleavage of 5-FAM/QXL® FRET by NEP caused the release of fluorescent product, 5-FAM. Fluorescence readings were monitored at excitation/emission = 490/520 nm and accumulation of 5-FAM correlated to NEP activity. Measurements were also taken with the addition of a specific NEP inhibitor, thiorphan (0.1 µmol/L)[19]. Thiorphan dose was optimised by incubating 10 µg of STBEV with 0.025, 0.05 and 0.1 µmol/L of thiorphan prior to measuring NEP activity.

Flow cytometry analysis

PPP samples from women with PE and NP were matched according to gestational age. Flow cytometry was used to specifically interrogate STBMV for NEP (the protocol used has been previously described[20,21]). We have previously described in detail the measures to remove contaminants, the use of PPP for labelling with target antibody and data acquisition using appropriate control gates [22]. Alterations to our previously published method were as follows: (a) samples were stained using PLAP-PE (0.05 µg/mL; NDOG2, in-house antibody) and NEP-APC antibody (1:10, 130-093-450; Miltenyi Biotec) for co-expression analysis. (b) Double positive (PLAP+ NEP+) events per mL on STBMV present in PPP were calculated. BD FACSDiva™

8.0 (BD Biosciences, New Jersey, USA) and FlowJo® 10 (FlowJo LLC, Oregon, USA) were used for flow cytometer data acquisition and analysis.

Plasma EV isolation and activity assay

PPP samples from women with PE and NP were analysed together with non-pregnant samples (included as an additional control). Samples were centrifuged at 13,000 x g for 2 minutes to make platelet free plasma (PFP), then 300 µL of each sample was processed using Izon qEVsingle columns (Izon Science, Christchurch, New Zealand). Columns were used according to manufacturer's guidelines and enabled EV to be purified from PFP using size exclusion chromatography. Isolated exosomes in fractions 6-8 (600 µL) were collected in PBS buffer and concentrated using Amicon Ultra-4 Centrifugal Filter Units (Merck Millipore, Massachusetts, USA). A final volume of 110 µL was then measured for NEP activity and analysed using Nanoparticle Tracking Analysis. Following the same protocol as the STBEV activity assay, duplicate readings were recorded for NEP activity in 50 µL of isolated EV.

Statistical analysis

Data was analysed using GraphPad Prism 7 (GraphPad Software, California, USA). Either paired Student *t*-test or unpaired Welch's *t*-test was applied, assuming Gaussian distribution, and values were expressed as mean ± SEM.

C) RESULTS

Upregulated NEP expression in PE placenta and STBEV

Strong NEP staining was observed on PE placental tissue (Figure 1A), compared to weak NEP staining on NP placental tissue (Figure 1B) and absent staining with control IgG1 antibody on PE placental tissue (Figure 1C). NEP expression was consistent, localised on the STB and confirmed previous results by Li et al[16]. Findings were corroborated by Western blot analysis of PE and NP placental lysate (PL); an 11-fold increase in NEP expression was detected ($n=3$; $p<0.05$, Figure 1D). Increased levels of NEP were also present in PE STBMV (2.3-fold; $n=5$; $p<0.05$; Figure 1E) and PE STBEX (4.3-fold; $n=5$; $p<0.05$, Figure 1F) derived by placental perfusion. An enrichment of NEP expression in STBEV relative to PL was also observed and all samples were positive for PLAP, an STB marker.

Active NEP is co-expressed with PLAP in STBEV

STBEV were isolated using either anti-PLAP or anti-NEP antibody on magnetic Dynabeads (Figure 2A). Western blot analysis identified that STBEV precipitated using anti-NEP antibody were positive for PLAP and conversely, STBEV precipitated using anti-PLAP antibody were positive for NEP (Figure 2B). This confirmed that NEP was co-expressed with PLAP on the same population of STBEV ($n=3$). NEP activity was quantified by measuring rate of accumulation of a substrate (5-FAM); thiorphan was used as a specific NEP inhibitor. A dose response of thiorphan on NEP activity revealed an optimal concentration of 0.1umol/L (data not shown). Increasing the protein concentration of STBMV and STBEX increased NEP activity, which could be blocked with almost 100% efficacy by thiorphan (Figures 3A and 3B).

NEP positive STBMV events are significantly elevated in PE plasma

PPP from women with PE and gestational age-matched NP was analysed using flow-cytometry to determine PLAP+ and NEP+ events (a gating strategy previously published by us was used to exclude EV originating from non-STB cell types [20]). We first confirmed the previously well described [23] increase in STBMV events positive for PLAP in plasma derived from PE (4,188 ± 1,157) compared to NP (1,398 ± 641; Figure 4A). We found a significant increase in PLAP and NEP double positive events per mL in STBMV from PE (2,730 ± 731) compared to NP plasma (805 ± 350; $p<0.05$; Figure 4B).

NEP activity is higher in EV isolated from PE plasma

EV were isolated from PE and gestational age matched NP peripheral venous plasma (n=8) using size exclusion chromatography. NEP activity was higher in EV from PE plasma than NP ($p<0.05$; Figure 5A). EV purified from PFP samples of healthy, non-pregnant controls (n=3) had significantly less NEP activity than EV NP ($p<0.05$; Figure 5B) consistent with the latter being of placental origin.

D) DISCUSSION

This study is the first to investigate peripherally circulating NEP expression and activity in PE. It demonstrates that NEP is expressed in the placenta (at the STB) and in the peripheral circulation on STBEV, where it is co-expressed with PLAP. It reports that NEP expression is upregulated in PE STBEV isolated by placental perfusion (both in STBMV and STBEX) compared to normal placentae. Moreover, it shows that circulating levels of STBMV and circulating exosomes have more NEP activity in PE. This NEP is biologically active and can be inhibited by a specific inhibitor, thiorphan. These findings are novel. Our findings corroborate an existing published report of increased NEP expression in PE syncytiotrophoblast[16].
NEP has many potential signalling functions. The most relevant to PE is enhanced degradation of multiple types of vasopeptides (including ANP, BNP, oxytocin, endothelin and bradykinin),[13,15] whose roles are normally to maintain vasodilatation, natriuresis and diuresis. As such, NEP might contribute to the defining hallmark of PE, namely hypertension, although its actions are difficult to predict given their pleotropicity.

NEP inhibitors have recently been successfully trialled in combination with angiotensin inhibitors in the treatment of heart failure in non-pregnant individuals [15]. A double-blind randomised trial of a combined NEP-angiotensin inhibitor compared to angiotensin inhibitor alone was stopped early because of substantial reduction in all-cause mortality, despite the trial being powered only for cardiovascular mortality [14]. Authors hypothesised that inhibiting the broader effects of NEP in heart failure (affecting multiple vasoactive peptides) underlies its likely effect on neurohormonal systems, sodium retention and myocardial remodelling.

During normal pregnancy women also undergo significant cardiac remodelling[24]. In PE the cardiovascular burden associated with hypertension and increased peripheral resistance, imposes extra changes such that 50% of these women develop left ventricular diastolic dysfunction and a further 20% biventricular systolic dysfunction and severe left ventricular hypertrophy [7]. Some researchers believe that this cardiac dysfunction induces changes in the placenta resulting in the PE syndrome[25]. Our data convincingly show that NEP signalling via the placenta is upregulated in PE; we hypothesise that increased NEP might contribute to the cardiac remodelling seen in PE, as has been suggested by NEP inhibition outside pregnancy[15]. Further studies are required.

Strengths of this study include the use of multiple experimental approaches to corroborate findings. STBEV were isolated by placental perfusion and from peripheral plasma, using differential ultracentrifugation, immunoprecipitation, size-exclusion chromatography and flow cytometry. STBEV were identified at every stage using PLAP, a unique marker of trophoblast-derived material, and double-positivity for PLAP and NEP was shown bi-directionally. Moreover, biological activity of STBEV-bound NEP was confirmed in samples sourced in different ways, providing strong evidence that NEP could play a signalling role. Our study has some limitations. Our placental perfusion data show greater upregulation of NEP in PE on STBEX than STBMV. However size limitations prevent STBEX from being analysed by flow cytometry. Our size-exclusion chromatography data did not exclude non-placental exosomes; thus, whilst all our data point towards STB-EX being the source of circulating NEP it is not technically possible to demonstrate this in peripheral plasma samples.

Future work should focus on the expression and role of NEP in normal pregnancy, to determine whether NEP could form part of a diagnostic or prognostic test in PE. NEP also represents a potentially exciting therapeutic target in PE, though more work is needed to determine the biological function of STBEV-bound NEP in PE on both vasoactive peptides as well as myocardial tissue. Notably, oxytocin is a recognised substrate of NEP[13]; although no studies have evaluated oxytocin levels in PE compared to normal pregnancy. Regulation of the NEP gene by histone deacetylation has been reported in the context of diabetes[26]. These authors reported an increase in cardiac NEP as a result of this. Interestingly, histone deacetylation is reported to play a role in preeclampsia by altering trophoblast cell migration[27]. It is possible that such histone deacetylation might regulate placental NEP but further work is required to elucidate this.

E) PERSPECTIVES

We have demonstrated that enzymatically active NEP is released into the maternal circulation, bound to STBEV, and its expression is increased in PE. These findings raise the possibility that NEP is an important mediator between placental pathology and the maternal phenotype of PE. NEP is known to break down key vasoactive molecules such as ANP, BNP, endothelin and bradykinin, all of which have been shown to play a role in the pathogenesis of preeclampsia. These molecules are also involved in cardiovascular dysfunction, which is also a feature of preeclampsia. Clinical trials have been published demonstrating the efficacy of NEP inhibitors in both the treatment of heart failure and also of hypertension. Our findings raise the possibility of using NEP inhibitors in the treatment of preeclampsia. Additionally, identification of NEP STBEV could provide diagnostic applications.

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5) SOURCES OF FUNDING

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6) DISCLOSURES

None.

7) REFERENCES

1 Steegers EAP, Von Dadelszen P, Duvekot JJ, Pijnenborg R. Pre-eclampsia. *Lancet* 2010; 376:631–644.

2 Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. *Obstet Gynecol* 2013; 122:1122–1131.

3 Duley L. The Global Impact of Pre-eclampsia and Eclampsia. *Semin Perinatol* 2009; 33:130–137.

4 Hammoud GM, Ibdah JA. Preeclampsia-induced Liver Dysfunction, HELLP syndrome, and acute fatty liver of pregnancy. *Clin Liver Dis* 2014; 4:69–73.

5 Cornelis T, Odutayo A, Keunen J, Hladunewich M. The Kidney in Normal Pregnancy and Preeclampsia. *Semin Nephrol* 2011; 31:4–14.

6 Kane SC, Dennis A, da Silva Costa F, Kornman L, Brennecke S. Contemporary clinical management of the cerebral complications of preeclampsia. *Obstet Gynecol Int* 2013; 2013:985606.

7 Melchiorre K, Sharma R, Thilaganathan B. Cardiovascular implications in preeclampsia: An overview. *Circulation* 2014; 130:703–714.

8 Redman CW. Current topic: pre-eclampsia and the placenta. *Placenta* 1991; 12:301–8.

9 Redman CW. Latest Advances in Understanding Preeclampsia. *Science (80-)* 2005; 308:1592–1594.

10 Salomon C, Torres MJ, Kobayashi M, Scholz-Romero K, Sobrevia L, Dobierzewska A, *et al.* A gestational profile of placental exosomes in maternal plasma and their effects on endothelial cell migration. *PLoS One* 2014; 9. doi:10.1371/journal.pone.0098667

11 Knight M, Redman CWG, Linton EA, Sargent IL. Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. *BJOG An Int J Obstet Gynaecol* 1998; 105:632–640.

12 Tannetta D, Sargent I. Placental disease and the maternal syndrome of preeclampsia: Missing links? *Curr Hypertens Rep* 2013; 15:590–599.

13 Bayes-Genis A, Barallat J, Richards AM. A Test in Context: Neprilysin: Function, Inhibition, and Biomarker. *J Am Coll Cardiol* 2016; 68:639–653.

14 McMurray JJV, Packer M, Desai AS, Gong J, Lefkowitz MP, Rizkala AR, *et al.* Angiotensin–Neprilysin Inhibition versus Enalapril in Heart Failure. *N Engl J Med* 2014; 371:993–1004.

15 Bavishi C, Messerli FH, Kadosh B, Ruilope LM, Kario K. Role of neprilysin inhibitor combinations in hypertension: insights from hypertension and heart failure trials. *Eur Heart J* 2015; 36:1967–1973.

16 Li XM, Moutquin JM, Deschênes J, Bourque L, Marois M, Forest JC. Increased immunohistochemical expression of neutral metalloendopeptidase (enkephalinase; EC 3.4.24.11) in villi of the human placenta with pre-eclampsia. *Placenta* 1995; 16:435–45.

17 Buhimschi I, Zhao G, Labatte S, Jing H, Cackovic M, Buhimschi CS. 787:

Evidence for neprilysin (NEP) involvement in disturbance of the amyloid clearing protein pathway in preeclampsia (PE). *Am J Obstet Gynecol* 2016; 214:S411–S412.

18 Dragovic RA, Collett GP, Hole P, Ferguson DJP, Redman CW, Sargent IL, *et al.* Isolation of syncytiotrophoblast microvesicles and exosomes and their characterisation by multicolour flow cytometry and fluorescence Nanoparticle Tracking Analysis. *Methods* 2015; 87:64–74.

19 Spillantini MG, Geppetti P, Fanciullacci M, Michelacci S, Lecomte JM, Sicuteri F. In vivo "enkephalinase" inhibition by acetorphan in human plasma and CSF. *Eur J Pharmacol* 1986; 125:147–50.

20 Dragovic RA, Southcombe JH, Tannetta DS, Redman CWG, Sargent IL. Multicolor flow cytometry and nanoparticle tracking analysis of extracellular vesicles in the plasma of normal pregnant and pre-eclamptic women. *Biol Reprod* 2013; 89:151.

21 Inglis HC, Danesh A, Shah A, Lacroix J, Spinella PC, Norris PJ. Techniques to improve detection and analysis of extracellular vesicles using flow cytometry. *Cytom Part A* 2015; 87:1052–1063.

22 Motta-Mejia C, Kandzija N, Zhang W, Mhlomi V, Cerdeira AS, Burdujan A, *et al.* Placental Vesicles Carry Active Endothelial Nitric Oxide Synthase and Their Activity is Reduced in Preeclampsia. *Hypertension* 2017; 70:372–381.

23 Goswamia D, Tannetta DS, Magee LA, Fuchisawa A, Redman CWG, Sargent IL, *et al.* Excess syncytiotrophoblast microparticle shedding is a feature of early-onset pre-eclampsia, but not normotensive intrauterine growth restriction.

Placenta 2006; 27:56–61.

24 Melchiorre K, Sharma R, Khalil A, Thilaganathan B. Maternal cardiovascular function in normal pregnancy: Evidence of maladaptation to chronic volume overload. *Hypertension* 2016; 67:754–762.

25 Thilaganathan B. Pre-eclampsia and the cardiovascular-placental axis. *Ultrasound Obstet Gynecol* 2018; 51:714–717.

26 Malek V, Sharma N, Gaikwad AB. Histone Acetylation Regulates Natriuretic Peptides and Neprilysin Gene Expressions in Diabetic Cardiomyopathy and Nephropathy. *Curr Mol Pharmacol* 2019; 12:61–71.

27 Xie D, Zhu J, Liu Q, Li J, Song M, Wang K, *et al.* Dysregulation of HDAC9 represses trophoblast cell migration and invasion through TIMP3 activation in preeclampsia. *Am J Hypertens* Published Online First: 2 February 2019. doi:10.1093/ajh/hpz006

8) NOVELTY AND SIGNIFICANCE

1. What Is New?

Ex vivo and circulating placental extracellular vesicles express functional neprilysin.

Vesicle bound neprilysin is biologically active and can be inhibited by thiorphan (a specific neprilysin inhibitor).

Placentae, microvesicles and exosomes have higher neprilysin levels/activity in women with preeclampsia compared to women with normal pregnancy.

2. What Is Relevant?

Neprilysin is known to play significant roles in hypertension and cardiovascular remodelling in non-pregnant individuals by breaking down vasoactive peptides.

Hypertension and cardiovascular remodelling are key features of preeclampsia.

Placental extracellular vesicle bound neprilysin may play a role in the pathogenesis of preeclampsia.

3. Summary

Our data has shown convincingly that neprilysin signalling via placental extracellular vesicles is upregulated in preeclampsia.

9) FIGURE LEGENDS

Figure 1. Increased NEP expression in PE placenta shown by immunohistochemical staining of placental tissue and quantitative Western blot analysis of placental lysate (PL) and STBEV. **A**, Intense NEP staining localised on the surface of syncytiotrophoblast cells in PE placental tissue. **B**, Weak NEP staining of placental tissue from normal pregnancy (NP). **C**, Negative immunoreactivity of placental tissue with IgG1 control antibody. **D**, PL (n=3), **E**, STBMV (n=5), and **F**, STBEX (n=5) analysed for NEP (100 kDa) and PLAP (60 kDa) expression. Quantitative comparison of NEP expression in NP and PE placentas also shown (*$p<0.05$). Scale bar: 30 μm.

Figure 2. NEP is expressed with PLAP in STBMV and STBEX. **A**, Schematic representation of the immunoprecipitation protocol carried out using anti-NEP antibody. The same protocol was used, substituting anti-NEP for anti-PLAP and anti-IgG1 (control) to formulate the Western blot below. **B**, Representative immunoblot showing co-existence of NEP and PLAP in both NEP+ positive (anti-NEP) and PLAP+ (anti-PLAP) STBMV and STBEX derived from normal pregnancy (n=3). Mouse IgG1 was used as the control antibody (ctrl IgG1).

Figure 3. STBMV and STBEX express active NEP. **A**, STBMV and **B**, STBEX express functional NEP, demonstrated by a dose dependent accumulation of fluorescent product**,** 5-FAM. Incubation with 0.1 μmol/L thiorphan resulted in a significant reduction in NEP activity (*$p<0.05$, **$p<0.01$, n=3).

Figure 4. Flow cytometry analysis of circulating STBMV in PPP collected from patients with NP (n=8) and PE (n=8). Samples connected by lines were matched by gestational age to facilitate comparison. **A**, PLAP positive STBMV events were higher in plasma from PE compared to NP. **B**, PLAP and NEP double positive STBMV events were significantly higher in plasma from PE compared to NP (*p<0.05).

Figure 5. NEP activity and characterisation of EV isolated from PFP using size exclusion chromatography. **A**, Significantly higher NEP activity measured in exosomes from PE PFP compared to NP (n=8, *p<0.05). Samples connected by lines were matched by gestational age to facilitate comparison. **B**, EV from non-pregnant patients (n=3) showed a significant reduction in NEP activity (*p<0.05).

10) TABLES

Table 1. Clinical Characteristics of the Study Groups

	Placental EV		Plasma EV	
Characteristic	NP (n=5)	PE (n=5)	NP (n=9)	PE (n=9)
Age, y	33.0 ± 1.7	31.4 ± 2.0	29.7 ± 1.6	30.1 ± 2.3
GA, weeks	39.6 ± 0.8	34.8 ± 1.5*	35.9 ± 0.2	35.8 ± 0.2
Primiparous, n	0	1	5	6
BMI, kg/m^2	28.0 ± 3.4	31.2 ± 2.9	23.6 ± 0.6	28.7 ± 2.1‡
Proteinuria, mg/mmol	-	232.9 ± 66.8	-	234.1 ± 84.4
Systolic BP, mm Hg	128.4 ± 5.1	164.4 ± 7.3†	124.7 ± 1.3	173.0 ± 4.0‖
Diastolic BP, mm Hg	75.2 ± 0.5	102.6 ± 4.2†	79.0 ± 2.3	106.9 ± 2.7‖
Birth weight, g	3628 ± 279	2136 ± 400*	3544 ± 170	2618 ± 137‖
Smoking history, n	0	0	0	1

Data are expressed as mean ± SEM. BMI indicates body mass index; BP, blood pressure; EV, extracellular vesicles; GA, gestational age; NP, normal pregnancy; and PE, preeclampsia. *P<0.05, †P<0.01 between placental EV groups. ‡P<0.05, ‖P<0.01 between plasma EV groups.