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RESEARCH PAPER

Synthetic gestagens exert differential effects on arterial thrombosis and aortic gene expression in ovariectomized apolipoprotein E-deficient mice

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BACKGROUND AND PURPOSE

Combined hormone replacement therapy with oestrogens plus the synthetic progestin medroxyprogesterone acetate (MPA) is associated with an increased risk of thrombosis. However, the mechanisms of this pro-thrombotic effect are largely unknown. The purpose of this study was to: (i) compare the pro-thrombotic effect of MPA with another synthetic progestin, norethisterone acetate (NET-A), (ii) determine if MPA's pro-thrombotic effect can be antagonized by the progesterone and glucocorticoid receptor antagonist mifepristone and (iii) elucidate underlying mechanisms by comparing aortic gene expression after chronic MPA with that after NET-A treatment.

EXPERIMENTAL APPROACH

Female apolipoprotein E-deficient mice were ovariectomized and treated with placebo, MPA, a combination of MPA + mifepristone or NET-A for 90 days on a Western-type diet. Arterial thrombosis was measured *in vivo* in a photothrombosis model. Aortic gene expression was analysed using microarrays; GeneOntology and KEGG pathway analyses were conducted.

KEY RESULTS

MPA's pro-thrombotic effects were prevented by mifepristone, while NET-A did not affect arterial thrombosis. Aortic gene expression analysis showed, for the first time, that gestagens induce similar effects on a set of genes potentially promoting thrombosis. However, in NET-A-treated mice other genes with potentially anti-thrombotic effects were also affected, which might counterbalance the effects of the pro-thrombotic genes.

CONCLUSIONS AND IMPLICATIONS

The pro-thrombotic effects of synthetic progestins appear to be compound-specific, rather than representing a class effect of gestagens. Furthermore, the different thrombotic responses elicited by MPA and NET-A might be attributed to a more balanced, 'homeostatic' gene expression induced in NET-A- as compared with MPA-treated mice.

Abbreviations

ApoE, apolipoprotein E; BP, biological process; CC, cellular compartment; CEE, conjugated equine oestrogens; HCAEC, human coronary artery endothelial cells; HCASMC, human coronary artery smooth muscle cells; HRT, hormone replacement therapy; MF, molecular function; MPA, medroxyprogesterone acetate; NET-A, norethisterone acetate; OVX, ovariectomized; qPCR, quantitative real-time; WHI, Women's Health Initiative

Table of Links

TARGETS	LIGANDS
Glucocorticoid receptor (NR3C1)	Medroxyprogesterone acetate (MPA)
Progesterone receptor (NR3C3)	Norethisterone acetate (NET-A)
	Mifepristone

This Table lists key protein targets and ligands in this document, which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Clinical studies have suggested that hormone replacement therapy (HRT) may be associated with a reduced risk for cardiovascular events (Folsom *et al.*, 1995; Tremollieres *et al.*, 2000) implying beneficial effects of HRT on the cardiovascular system. This assumption was however questioned by the results obtained from the Women's Health Initiative (WHI) trial: on the one hand, conjugated equine oestrogens (CEE) alone exerted beneficial effects on the cardiovascular system (Anderson *et al.*, 2004), on the other hand their combination with medroxyprogesterone acetate (MPA) increased the risk of cardiovascular events, including stroke (Rossouw *et al.*, 2002). The observation that HRT is associated with a higher risk for stroke (Grodstein *et al.*, 2003; Rossouw *et al.*, 2007; Vickers *et al.*, 2007) might therefore be ascribed to prothrombotic MPA effects. Indeed, this hypothesis was confirmed in animal experiments showing that MPA enhances the thrombotic response at least partially through increased thrombin generation (Freudenberger *et al.*, 2009). Besides MPA, another synthetic gestagen, norethisterone acetate (NET-A), is commonly used in postmenopausal HRT (Koubovec *et al.*, 2005) together with oestrogens. NET-A and MPA differ from each other with regard to agonism of other steroid receptors in addition to the progesterone receptor. Specifically, unlike MPA, which is known to possess partial glucocorticoid effects (Wiegratz and Kuhl, 2004), NET-A has been found to exert only minimal glucocorticoid actions (Koubovec *et al.*, 2005). Therefore, further research using animal models of atherothrombosis will help to clarify the atherothrombotic risk distribution of synthetic gestagens and to investigate the underlying mechanisms. Accordingly, the aims of the present work were (i) to compare the prothrombotic MPA effect with another synthetic progestin, NET-A, (ii) to determine if the effects of MPA can be antagonized with mifepristone and (iii) to search for underlying mechanisms by comparing aortic gene expression after chronic treatment with MPA versus NET-A to define genes, functional terms and pathways that may potentially be involved in thrombotic responses in ovariectomized apolipoprotein E (ApoE)-deficient mice treated with MPA compared to those treated with NET-A.

Methods

Where applicable, the drug/molecular target nomenclature complies with Alexander *et al.* (2013).

Animals

Animal experiments were performed according to the guidelines of the 'Deutsches Tierschutzgesetz' and were authorized by the 'Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen' under the reference number Az. 8.87–50.10.37.09.107. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Homozygous female ApoE-deficient mice (Jackson Laboratory, Bar Harbor, ME, USA) were maintained on a 12 h dark/light cycle with unrestricted access to food and water. Animals were fed a normal chow diet (Ssniff, Soest, Germany) until commencement of hormone substitution. From this point on, mice received a Western-type diet (Ssniff) as previously described (Freudenberger *et al.*, 2009). Where indicated, anaesthesia was induced using Ketanest/xylazine [100 $mg \cdot kg^{-1}$ Ketanest (Pfizer, Berlin, Germany), 5 $mg \cdot kg^{-1}$ xylazine (Bayer, Leverkusen, Germany)]. Anaesthetics were intraperitoneally injected and sufficient anaesthesia was assured by the absence of the blink reflex and the inter-toe reflex. The number (n) of animals used for the different experiments is given in the respective figure legends.

Ovariectomy and hormone substitution

At the age of 4 to 5 weeks, mice were bilaterally ovariectomized (OVX) under anaesthesia. Post-operative analgesia was ensured by s.c. application of Carprofen (5 $mg \cdot kg^{-1}$; Pfizer). Roughly 14 days after OVX, mice were randomly assigned to six different treatment groups, namely placebo for

Table 1

Dose and release parameters of the different pellets implanted s.c.

Chemical compound	Total dose (mg)/pellet	Total time of release (days)	Release (μg)/day
Medroxyprogesterone acetate (MPA)	2.5	90	27.7
Norethisterone acetate (NET-A)	1.2	90	13.3
Mifepristone	90.0	90	1000.0

mifepristone, mifepristone, placebo for MPA/NET-A, MPA, MPA + mifepristone and NET-A. After anaesthesia, mice were s.c. implanted with slow-release hormone pellets (Innovative Research of America, Sarasota, FL, USA), designed to release hormone dosages as summarized in Table 1. The duration of hormone substitution (90 days) and the dose of MPA (27.7 μg·day^{-1}) were based on previous experiments (Freudenberger *et al.*, 2009). The dose of NET-A (13.3 μg·day^{-1}) was chosen according to experiments performed by Škarda *et al.* who described an increase of mammary growth at NET-A dosages between 12.5 μg·day^{-1} and 25 μg·day^{-1} (Skarda and Kohlerova, 2006), validating the efficacy of this dose range. Furthermore, Schindler *et al.* compared the efficacy of MPA and NET-A and reported that the dose of MPA needed for endometrium transformation was 1.3-fold–2.7-fold higher than the dose of NET-A (Schindler *et al.*, 2003). Accordingly, in the present study a roughly 2.1-fold higher dose of MPA than of NET-A was used for animal experiments, and thus a dose that nearly exactly mirrors the mid-fold dose of MPA as compared with NET-A needed to achieve a comparable progestogenic activity, as described by (Schindler *et al.* 2003). The dosage of mifepristone (1 mg·day^{-1}) was chosen on the basis of experiments described by (Goyeneche *et al.* 2007) indicating substantial pharmacological effects of this mifepristone dose without compromising animals' well-being. Experiments were terminated between days 78 and 90 after initiation of hormone substitution, assuring an approximately equal distribution of the differently treated animals in terms of the time point of final experiments. The experimental design is schematically depicted in Figure 1A.

Thrombosis measurement

Induction of thrombosis in the right carotid artery was performed according to the procedure described by (Wilson *et al.* 2003). In brief, an ultrasonic flow-probe (Transonic, Ithaca, NY, USA) was placed under the right carotid artery. Subsequently, a green-light laser (Melles Griot Carlsbad, CA, USA) was placed on the vessel in direct proximity to the flow probe. Development of an occlusive thrombus was induced by injection of Rose Bengal (Acros Organics, Geel, Belgium) at a dose of 50 mg·kg^{-1} through a 'catheter' placed in the left jugular vein. Determination of time to first and stable occlusion was conducted as previously defined (Freudenberger *et al.*, 2010). Animals that did not develop a thrombus within 120 min after Rose Bengal injection were assigned a time to first and stable occlusion of 120 min for statistical reasons.

Cell culture

Human coronary artery smooth muscle cells (HCASMC) and human coronary artery endothelial cells (HCAEC; both purchased from PromoCell, Heidelberg, Germany), were cultured in cell-specific medium according to the supplier's instructions. Cells were grown at 37°C and 5% CO_2. For experiments, 1×10^4 cells cm^{-2} were seeded into wells of 6-well plates; 24 h after seeding, HCASMC were synchronized in serum-free DMEM (Life Technologies, Paisley, UK; ordering-number: 41966-029) supplemented with 100 U·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin (Life Technologies, UK) and 1% non-essential amino acids (Life Technologies, Grand Island, NY, USA; DMEM 0) for 24 h. Subsquently, HCASMC were stimulated with MPA or NET-A (both Sigma-Aldrich, Steinheim, Germany) at concentrations of 10^{-8} M and 10^{-5} M in DMEM 0 supplemented with 5% FCS for 18 h. HCAEC received new cell-specific medium 24 h after seeding and after another 24 h were stimulated with MPA or NET-A in their cell-specific medium as described for HCASMC.

RNA isolation

Mice were killed and aortas rapidly removed from the region just behind the aortic arch curvature to approximately 1 mm before the kidney vessels branch, and immediately snap-frozen in liquid nitrogen. Tissue samples were pulverized, transferred into 1 mL of peqGOLD TriFast™ (Peqlab, Erlangen, Germany), incubated at room temperature for 1 h and vortexed every 10 min. HCASMC and HCAEC were harvested in 1 mL of peqGOLD TriFast (Peqlab). Extraction of RNA was performed according to the manufacturer's instructions. For microarray analyses, final purification of total RNA was performed according to the RNeasy Mini Kit RNA cleanup protocol (Qiagen, Hilden, Germany). RNA concentration and purity were determined at 260 nm/280 nm by spectrophotometry (Nanodrop, Thermo Scientific, Wilmington, DE, USA).

Microarray gene expression analyses

Total RNA preparations were checked for RNA integrity using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). All samples obtained in this study showed good quality RNA Integrity Numbers (median 7.3). Synthesis of cDNA and subsequent fluorescent labelling of cRNA was performed according to the manufacturer's protocol (One-Color Microarray-Based Gene Expression Analysis/Low Input Quick Amp Labeling; Agilent Technologies). Briefly, 100 ng of total RNA were converted to cDNA, followed by *in vitro* transcription and incorporation of Cy3-CTP into nascent cRNA.

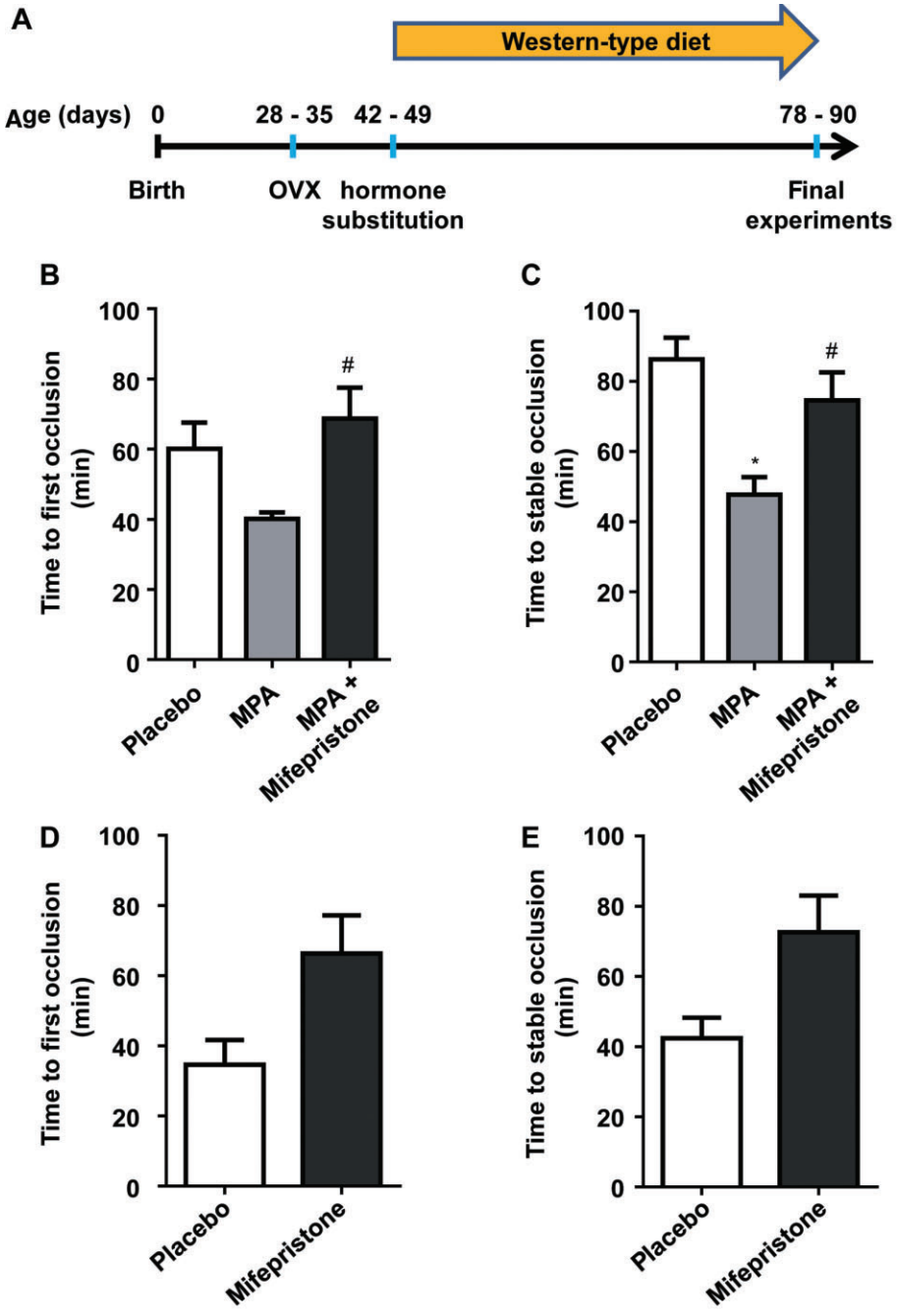

Figure 1

Combined substitution of MPA + mifepristone prevents the pro-thrombotic effects exerted by MPA alone in ovariectomized ApoE-deficient mice. (A) Experimental design. (B) Time to first occlusion after substitution of placebo, MPA (27.7 μg·day^{-1}) or a combination of MPA + mifepristone (1 mg·day^{-1}). (C) Time to stable occlusion after substitution of placebo, MPA (27.7 μg·day^{-1}) or a combination of MPA + mifepristone (1 mg·day^{-1}). (D) Time to first occlusion after substitution of placebo or mifepristone (1 mg·day^{-1}). (E) Time to stable occlusion after substitution of placebo or mifepristone (1 mg·day^{-1}). Data are presented as mean ± SEM; n = 9 – 11 in B, n = 8 – 11 in C and n = 5 – 9 in D + E; *P < 0.05 versus placebo; #P < 0.05 versus MPA.

After fragmentation, labelled cRNA was hybridized to Agilent 4x44k Whole Mouse Genome v1 Microarrays for 17 h at 65°C and scanned as described in the manufacturer's protocol. Signal intensities on 20 bit tiff images were calculated by Feature Extraction software (FE, Vers. 10.7.1.1/11.0.1.1; Agilent Technologies). Data analyses were conducted with GeneSpring GX software (Vers. 12.5; Agilent Technologies). Probe signal intensities were quantile normalized across all samples to reduce inter-array variability (Bolstad *et al.*, 2003). Input data pre-processing was concluded by baseline transformation to the median of all samples. Hierarchical cluster analysis was performed using Euclidian similarity measures

and Ward's linkage. After grouping of biological replicates according to their respective experimental condition, a given transcript had to be expressed above background (e.g. called 'detected' by FE) in at least three of four replicates in any one of two, or both conditions to be further analysed in pairwise comparisons of conditions. Differential gene expression was statistically determined by Welch's unpaired *t*-test ($P < 0.05$). Functional classification of differentially expressed genes was performed online using the DAVID Functional Annotation Tool (http://david.abcc.ncifcrf.gov/home.jsp; Huang da *et al.*, 2009a,b) for GeneOntology (GO) functional enrichment analyses and investigation of KEGG pathway enrichment. GO categories and KEGG pathways were regarded significantly enriched with differentially expressed genes at an EASE score < 0.1.

cDNA synthesis and quantitative real-time (qPCR)

cDNA synthesis was carried out using the QuantiTect® Reverse Transcription Kit (Qiagen). 300 ng of RNA (aortas) or 1 μg of RNA (cells) were used for cDNA synthesis. Platinum® SYBR® Green qPCR SuperMix-UDG (Life Technologies, USA) with ROX reference dye was used to perform qPCR experiments. qPCRs were performed using the Applied Biosystems 7300 Real-Time PCR System (aortas) and the StepOnePlus™ Real-Time PCR System (Life Technologies, Singapore, Singapore) (cells). Samples were measured in duplicate and analysed by the ΔΔCq method using GAPDH as reference gene. Primers as given in Table 2 were designed with Primer Express 3.0 software (Applied Biosystems), Primer3Plus software (Untergasser *et al.*, 2012) and Primer-BLAST (Ye *et al.*, 2012).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). A few outliers were identified using Grubb's test with regard to thrombosis measurements: a single one in Figure 1B (in the MPA group), two in Figure 1C (one in the placebo, one in the MPA group), a single one in the placebo groups of Figure 1D and E and a single one in the NET-A group in Figure 2A were excluded. Cleaned data were analysed using standard one-way ANOVA and Sidak's multiple comparison test in Figure 1B and C. In the case of two groups, Student's *t*-test was performed. Data groups statistically compared passed Shapiro–Wilk normality tests (except one group in Figure 1C). However, in this case also, non-parametric testing using Kruskal–Wallis test and Dunn's multiple comparison test led to the same significant differences as obtained by one-way ANOVA. The number of measurements in the placebo groups of Figures 1D and E and in the NET-A-group of Figure 2A were too small to perform Shapiro–Wilk normality test. However, Student's *t*-test and Mann–Whitney test gave similar results showing non-significance. With regard to qPCR results of aortas, the few outliers identified using Grubb's test were excluded and data were analysed using Mann–Whitney test. Gene expression in HCASMC and HCAEC was analysed using Kruskal–Wallis test and Dunn's multiple comparison test. All data are presented as mean ± SEM. *P*-values < 0.05 were regarded as statistically

Table 2

Primer pairs used for qPCR experiments

Gene symbol, murine	Forward (5′ – 3′)	Reverse (5′ – 3′)
Camta1	CTCAACACCGTGCCACCTAT	CGGTGCCTCTCTTTGGGTAA
Gapdh	TGGCAAAGTGGAGATTGTTGCC	AAGATGGTGATGGGCTTCCCG
Gp5	CCAGCTCACGTCTGTGGATT	CTACGGAGCGGAGGTGATTC
Gucy1a3	GACACCCATGCTGTCCAGAT	ACTCCGACAACTCCAGCAAA
Il18bp	AGACACCAGACTTGCTTGCA	AGTGGCAGTTGTCTGAGGTG
Mmp9	CCTGAAAACCTCCAACCTCA	GCTTCTCTCCCATCATCTGG
Plg	TCTCACCAAGAAGCAGCTCG	TTGCTGTTCTCCGCCATGAT
Ppbp	GCCTGCCCACTTCATAACCT	ATTCGTACATCTGCAGCGCA
Retnlg	CAGCTGATGGTCCCAGTGAA	TCTGCCTGAAGCCGTGATAC
S100a8	CCTTTGTCAGCTCCGTCTTCA	TCCAGTTCAGACGGCATTGT
S100a9	GCTCTTACCAACATCTGTGACTC	TTCTTGCTCAGGGTGTCAGG
Serpina3k	GCAAGCCAACAACCCTGAAC	TTGTGCCATCTGGGAAGCAT
Thbs1	AGGGAAGCAACAAGTGGTGT	AAGAAGGACGTTGGTAGCTGA
Gene symbol, human	**Forward (5′ – 3′)**	**Reverse (5′ – 3′)**
CAMTA1	CTCAACACCGTGCCACCTAT	GCGGTGCCTCTCTTTTGGTA
GAPDH	GTGAAGGTCGGAGTCAACG	TGAGGTCAATGAAGGGGTC
IL18BP	TCCTGACGCATGCATCATGA	TCTGACCAGGAGAGTGACGA
THBS1	CGGCGTGAAGTGTACTAGCT	TGTGGTTGAAGCAGGCATCA

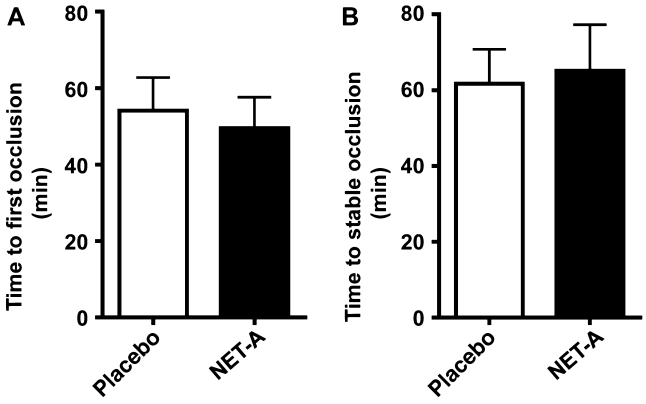

Figure 2

NET-A has no effect on the arterial thrombotic response in ovariectomized ApoE-deficient mice. (A) Time to first occlusion after substitution of placebo or NET-A (13.3 μg·day^{-1}). (B) Time to stable occlusion after substitution of placebo or NET-A (13.3 μg·day^{-1}). Data are presented as mean ± SEM; $n = 6 – 9$ in A and $n = 7 – 9$ in B.

significant. Microarray data were statistically analysed as described in the 'Microarray gene expression analyses' passage above.

Results

Arterial thrombosis

The experimental design is schematically depicted in Figure 1A. MPA reduced the 'time to first occlusion' and significantly shortened the 'time to stable occlusion' as compared with placebo controls (Figure 1B and C), the latter result mirroring our previous report (Freudenberger *et al.*, 2009). Importantly, mifepristone effectively antagonized the pro-thrombotic effects of MPA (Figure 1B and C) and mice substituted with mifepristone alone showed a trend towards a prolonged 'time to first occlusion' and a prolonged 'time to stable occlusion' (Figure 1D and E).

To address the question if the pro-thrombotic action is specific for MPA, the thrombotic response was also determined in NET-A-treated mice. However, in contrast to MPA, NET-A substitution did not alter the thrombotic response as compared with its placebo controls (Figure 2A and B). Absolute values among the placebo groups differ due to the fact that MPA- and NET-A-treated groups were each assigned an own placebo group because measurements were performed in different groups over some time. Mifepristone-treated animals were compared with their own placebos due to a different release profile of mifepristone.

Aortic gene expression in MPA- and NET-A-treated animals

To investigate potential differences in gene expression profiles, DNA microarray based global gene expression analyses were performed on aortas from differentially treated mice. For each hormone and its corresponding placebo treatment, four biological replicates were analysed in pairwise comparisons allowing statistical analysis of differential gene expression (Figure 3). Microarray results revealed that 1175 genes were regulated in aortas of MPA-treated animals while 1365 genes were regulated in aortas of NET-A-treated mice ($P < 0.05$; Figure 3).

Out of the 1175 differentially expressed genes in MPA-treated animals, 704 genes were up-regulated while 471 genes were down-regulated. Fold change reached up to +6.39-fold and down to –8.57-fold in MPA-treated animals.

In aortas of NET-A-treated mice, expression of 782 genes was induced while expression of 583 genes was reduced. Changes in expression reached from +7.26-fold to –8.04-fold.

In MPA-treated animals, expression of 38 genes was induced by ≥ 2-fold, while seven genes showed a more than threefold induction and expression of 42 genes showed a more than twofold decrease while expression of eight genes was reduced by more than threefold. Among the up-regulated genes were for example, S100 calcium-binding proteins A8 and A9 [*S100a8* (6.39-fold induction) and *S100a9* (6.09-fold induction)], resistin-like γ (*Retnlg*, 4.52-fold induction), matrix metallopeptidase 9 (*Mmp9*, 2.57-fold induction), α3-subunit of soluble guanylate cyclase 1 (*Gucy1a3*, 2.57-fold induction) and pro-platelet basic protein (*Ppbp*, 1.92-fold induction). With regard to genes whose expression was reduced, expression of IL18-binding protein (*Il18bp*) (2.14-fold inhibition) and the serine (or cysteine) peptidase inhibitor, clade A, member 3 K (*Serpina3k*, 2.7-fold inhibition) was found to be significantly decreased. Also, expression of calmodulin-binding transcription activator 1 (*Camta1*) was reduced (2.48-fold inhibition) in MPA-treated mice.

In NET-A-treated animals, results revealed 168 genes whose expression was induced above twofold and 54 genes showing a more than threefold induced expression. A more than twofold reduced expression was found for 45 genes; 11 genes showed a more than threefold decreased expression. Among the up-regulated genes in NET-A-treated mice, *Ppbp* (4.77-fold induction), glycoprotein 5 (*Gp5*, 4.38-fold induction), *Mmp9* (2.57-fold induction), *Retnlg* (2.42-fold induction) and *S100a9* (2.35-fold induction) were identified. Also, expression of *Camta1* was found to be increased (1.93-fold induction). Furthermore, plasminogen (*Plg*, 2.44-fold induction) and thrombospondin1 (*Thbs1*, 2.19-fold inhibition) were regulated in animals substituted with NET-A.

Some of these genes show up among the most prominently regulated genes while others do not: Tables 3 and 4 show the 15 most up-regulated genes while Tables 5 and 6 sum up the 15 most down-regulated genes either in MPA-treated mice (Tables 3 and 5) or in NET-A-treated animals (Tables 4 and 6).

Expression of the aforementioned genes was subsequently investigated using qPCR. In comparison of MPA with placebo-treated animals, 8 out of the 9 genes could be detected in qPCR experiments, with 7 of these 8 genes being regulated in the same direction as in microarray experiments and 2 of these 7 genes being significantly regulated (Figure 4A–H). Comparing NET-A- and placebo-substituted animals, 7 out of 8 genes were detectable by qPCR, with all seven genes being regulated in the same direction as in microarray experiments and 5 of these 7 genes being significantly regulated (Figure 4J–P). Further substantiating comparability of microarray and qPCR results, Figure 4I and Q show the correlation of fold regulation (microarray) versus fold

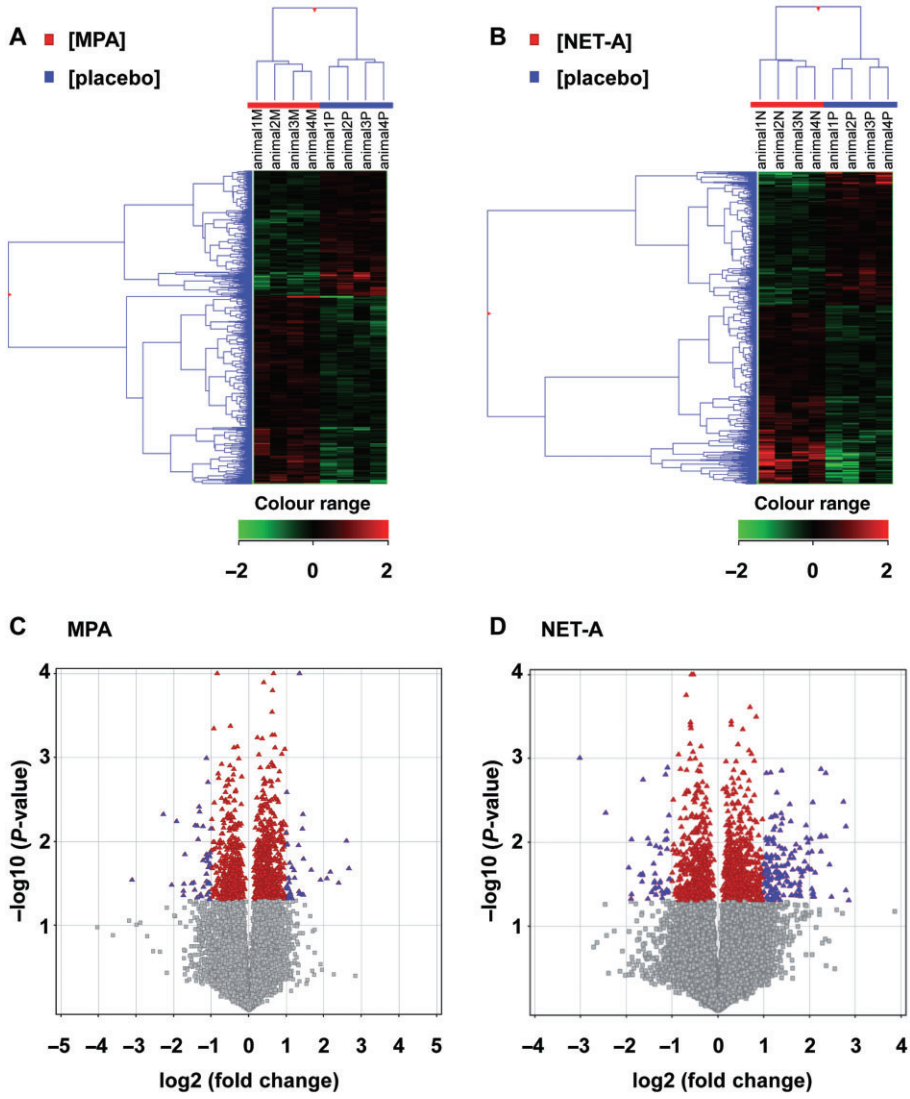

Figure 3

Hierarchical clustering and volcano plots of hormone-induced differentially expressed genes. Changes in aortic gene expression were analysed by microarrays comparing four hormone-treated samples to placebo controls. (A, B) Hierarchical clustering for (A) MPA and (B) NET-A shows grouping of the placebo- and the hormone-treated animals. Upon MPA treatment, 1175 genes were differentially regulated ($P < 0.05$; 704 genes were up- and 471 down-regulated respectively). NET-A treatment induced differential expression of 1365 genes ($P < 0.05$; 782 genes were up- and 583 down-regulated respectively). (C, D) Volcano plot distribution shows the mapping of gene expression fold change versus significance for (C) MPA-treated animals and (D) NET-A-treated mice. Significantly ($P < 0.05$) differentially expressed genes are shown in red. Genes found to be regulated > 2-fold are shown in blue. Fold changes range from –8.57-fold to +6.39-fold in MPA- and from –8.04-fold to +7.26-fold in NET-A-treated animals respectively.

regulation (qPCR) with eight (MPA) and seven (NET-A) XY pairs respectively. The correlation coefficients r of 0.66 (MPA) and 0.71 (NET-A) suggest a good correlation ($0.5 < r < 0.8$) between the data sets analysed.

Importantly, reduced expression of IL18BP as seen in aortas of MPA-treated animals could be achieved by treatment of HCAEC with MPA *in vitro* (Figure 5A). Likewise, reduced expression of THBS1 and increased expression of CAMTA1 as observed upon NET-A substitution *in vivo*, could be accomplished after stimulation of HCASMC (THBS1) or HCAEC (CAMTA1) with NET-A *in vitro* (Figure 5B and C).

GO analysis of genes regulated in MPA- and NET-A-treated animals

GO analysis was performed online using the DAVID Functional Annotation Tool (Huang da *et al.*, 2009a,b). GO analysis was performed using the categories 'biological process' (BP), 'cellular compartment' (CC) and 'molecular function' (MF). In the following, description of the results refers to the top 25 GO terms in each of the GO categories that were significantly enriched with differentially expressed genes (Supporting Information Figs S1–3).

Table 3

List of the 15 most up-regulated genes in comparison of female ovariectomized ApoE-deficient mice treated with placebo or MPA*

Gene description	Gene symbol	UniGeneID	Fold change (MPA vs. placebo)	*P*-value
Mus musculus S100 calcium-binding protein A8 (calgranulin A) (S100a8), mRNA [NM_013650]	S100a8	Mm.21567	6.39	0.021
Mus musculus S100 calcium-binding protein A9 (calgranulin B) (S100a9), mRNA [NM_009114]	S100a9	Mm 2128	6.09	0.010
Mus musculus stefin A2 like 1 (Stfa2l1), mRNA [NM_173869]	Stfa2l1	Mm.187847	5.28	0.032
Mus musculus resistin-like γ (Retnlg), mRNA [NM_181596]	Retnlg	Mm.231288	4.52	0.023
Mus musculus stefin A1 (Stfa1), mRNA [NM_001082543]	Stfa1	Mm.327618	4.23	0.028
Mus musculus 10 days embryo whole-body cDNA, RIKEN full-length enriched library, clone: 2610024D04 product: unclassifiable, full insert sequence. [AK011526]	2310030G06Rik		3.77	0.029
Mus musculus leukaemia inhibitory factor receptor (Lifr), transcript variant 1, mRNA [NM_013584]	Lifr	Mm.149720	3.17	0.022
Mus musculus RIKEN cDNA A530023O14 gene (A530023O14Rik), mRNA [NM_175648]	A530023O14Rik	Mm.347647	2.76	0.016
Mus musculus adult male testis cDNA, RIKEN full-length enriched library, clone: 4930539J07 product: mitochondrial carrier homologue 2, full insert sequence. [AK016000]	Mtch2	Mm.28023	2.74	0.044
Mus musculus 2 days pregnant adult female ovary cDNA, RIKEN full-length enriched library, clone: E330037N20 product: hypothetical protein, full insert sequence. [AK087894]			2.73	0.005
Mus musculus predicted gene, ENSMUSG00000050599, mRNA (cDNA clone IMAGE: 3487972), partial cds. [BC001981]	BC001981	Mm.410874	2.70	0.007
Mus musculus ATPase, Ca++ transporting, plasma membrane 2 (Atp2b2), transcript variant 1, mRNA [NM_009723]	Atp2b2	Mm.321755	2.69	0.043
Mus musculus guanylate cyclase 1, soluble, α3 (Gucy1a3), mRNA [NM_021896]	Gucy1a3	Mm.143831	2.57	0.0001
Mus musculus matrix metallopeptidase 9 (Mmp9), mRNA [NM_013599]	Mmp9	Mm.4406	2.57	0.043
Mus musculus peptidoglycan recognition protein 1 (Pglyrp1), mRNA [NM_009402]	Pglyrp1	Mm.21855	2.53	0.040

*One gene was not attributed with a gene symbol (marked in light grey) and two genes did not receive a UniGeneID (marked in mid-grey).

Specifically, in MPA-treated animals, out of the aforementioned genes *S100a8*, *S100a9* and *Mmp9*, only the gene encoding for *Mmp9* matched a BP term, namely 'proteolysis', while all three genes matched for example, the most prominently regulated term within the MF category being enriched with more than 200 genes, namely 'ion binding'.

In mice substituted with NET-A, the genes encoding for *Mmp9* and *S100a9* were also mappable for example, the most considerably regulated term within the MF category, namely 'ion binding'. With regard to the category 'CC' the gene encoding for *Mmp9* matched the 'extracellular matrix' term. In the BP category, the genes encoding for *Mmp9* and *Plg* matched the term 'proteolysis'.

Analysis of KEGG pathways regulated in MPA- and NET-A-treated animals

Seventeen KEGG pathways were significantly regulated in MPA-treated animals while 14 KEGG pathways were regulated in NET-A-treated animals, with only three overlapping KEGG pathways being regulated in both treatment groups (Supporting Information Fig. S4).

Interestingly, the 'ECM–receptor interaction' pathway was regulated in NET-A-treated mice only (Supporting Information Fig. S4B). This KEGG pathway might have an impact on the different atherothrombotic responses because it is exclusively regulated in NET-A-treated animals and genes within this pathway (e.g. *Gp5* and *Thbs1*) may influence atherothrombosis.

Aortic gene expression in MPA- versus NET-A-treated animals

Finally, aortic gene expression profiles in MPA- and NET-A-treated mice were compared after normalization to their respective placebo controls. Of the genes regulated in aortas of mice substituted with MPA (1175 genes) and NET-A (1365 genes), an overlapping set of 56 genes was found in both

Table 4

List of the 15 most up-regulated genes in comparison of female ovariectomized ApoE-deficient mice treated with placebo or NET-A

Gene description	Gene symbol	UniGeneID	Fold change (NET-A vs. placebo)	*P*-value
Mus musculus myosin, heavy polypeptide 4, skeletal muscle (Myh4), mRNA [NM_010855]	Myh4	Mm.297382	7.26	0.049
Mus musculus myosin, heavy polypeptide 8, skeletal muscle, perinatal (Myh8), mRNA [NM_177369]	Myh8	Mm.340163	6.98	0.038
Mus musculus maestro (Mro), mRNA [NM_027741]	Mro	Mm.126220	6.95	0.007
Mus musculus hemogen (Hemgn), mRNA [NM_053149]	Hemgn	Mm.25793	6.73	0.003
Mus musculus myosin, heavy polypeptide 2, skeletal muscle, adult (Myh2), mRNA [NM_001039545]	Myh2	Mm.422801	5.61	0.045
Mus musculus erythrocyte protein band 4.2 (Epb4.2), mRNA [NM_013513]	Epb4.2	Mm.240051	5.44	0.019
Mus musculus anoctamin 4 (Ano4), mRNA [NM_178773]	Ano4	Mm.236464	5.15	0.002
Mus musculus potassium voltage gated channel, Shaw-related subfamily, member 1 (Kcnc1), transcript variant B, mRNA [NM_008421]	Kcnc1	Mm.249386	5.13	0.009
Mus musculus α-haemoglobin stabilizing protein (Ahsp), mRNA [NM_133245]	Ahsp	Mm.423023	4.79	0.008
Mus musculus potassium channel, subfamily K, member 1 (Kcnk1), mRNA [NM_008430]	Kcnk1	Mm.10800	4.79	0.001
Mus musculus pro-platelet basic protein (Ppbp), mRNA [NM_023785]	Ppbp	Mm.293614	4.77	0.013
Mus musculus Rh blood group, D antigen (Rhd), mRNA [NM_011270]	Rhd	Mm.195461	4.72	0.009
Mus musculus glycoprotein 5 (platelet) (Gp5), mRNA [NM_008148]	Gp5	Mm.3519	4.38	0.023
Mus musculus apolipoprotein L 11b (Apol11b), mRNA [NM_001143686]	Apol11b	Mm.17957	4.31	0.045
Mus musculus olfactory receptor 206 (Olfr206), mRNA [NM_146991]	Olfr206	Mm.377781	4.26	0.040

treatment groups (Figure 6A). Of these, 50 genes that are shown in Figure 6B, could be assigned a gene symbol and a UniGeneID. Figure 6B shows that for example, expression of *Mmp9* was induced comparably in both MPA- and NET-A-treated mice. Likewise, expression of *S100a9* and *Ppbp* was induced in both treatment groups, however, to a different extent. This points towards a set of genes likely regulated in the same direction in response to progestin treatment, maybe representing a 'class effect' of synthetic progestins.

Contrasting this concordant regulation of expression, 20 of these 50 genes were regulated in an opposite direction (induction vs. inhibition) in the two treatment groups (marked with arrows in Figure 6B). Only one of these 20 differentially regulated genes, namely *Camta1*, showed an approximately twofold inhibition or induction, making *Camta1* a potentially interesting target gene in terms of the different atherothrombotic effects of MPA versus NET-A.

Discussion

Different synthetic progestins are used in combination with oestrogens in HRT to lower the risk of endometrial carcinogenesis (Langer, 2009) as compared with oestrogen substitution alone. However, combined application of CEE together with MPA increased the risk of thromboembolic events in the WHI trial as compared with CEE alone (Rossouw *et al.*, 2002). When analysing the potential detrimental side effects of synthetic gestagens on the cardiovascular system, one has to consider that these gestagens also exert agonistic or antagonistic effects on steroid receptors in addition to the progesterone receptor. In this regard, it has been demonstrated that MPA among others exerts partial effects on glucocorticoid receptors (Sitruk-Ware, 2002), while another progestin, NET-A, possesses only very little glucocorticoid receptor-binding affinity relative to MPA (Koubovec *et al.*, 2005). Therefore, we first sought to analyse if the pro-thrombotic MPA effect can be blocked by mifepristone, a strong glucocorticoid receptor antagonist in addition to being a progesterone receptor antagonist (Check *et al.*, 2010). Results showed that the combined application of MPA and mifepristone abolished the pro-thrombotic MPA effect. These results suggest that the pro-thrombotic actions of MPA occur in a steroid receptor-dependent manner. Subsequent analysis of the effect of NET-A on arterial thrombosis provides evidence that NET-A – unlike MPA – does not enhance the thrombotic response in a murine model of arterial thrombosis. This is in line with experiments performed in rats showing a comparable wet weight of thrombi from control versus NET-A-treated animals (Emms and Lewis, 1985).

The present findings clearly show that the pro-thrombotic effect of MPA (27.7 $\mu g \cdot day^{-1}$) on arterial thrombus formation

Table 5

List of the 15 most down-regulated genes in comparison of female ovariectomized ApoE-deficient mice treated with placebo or MPA*

Gene description	Gene symbol	UniGeneID	Fold change (MPA versus placebo)	*P*-value
Mus musculus IL6, mRNA [NM_031168]	Il6	Mm.1019	8.57	0.029
Mus musculus glycosyltransferase 25 domain containing 2 (Glt25d2), mRNA [NM_177756]	Glt25d2	Mm.23782	4.81	0.005
Mus musculus oxidized low-density lipoprotein (lectin-like) receptor 1 (Olr1), mRNA [NM_138648]	Olr1	Mm.293626	4.15	0.033
Mus musculus aldolase B, fructose-bisphosphate (Aldob), mRNA [NM_144903]	Aldob	Mm.479534	3.33	0.039
Mus musculus 6 days neonate head cDNA, RIKEN full-length enriched library, clone: 5430437H21 product: unclassifiable, full insert sequence. [AK019950]	Rrp15	Mm.452752	3.33	0.045
Mus musculus FK506 binding protein 5 (Fkbp5), mRNA [NM_010220]	Fkbp5	Mm.276405	3.31	0.032
Mus musculus aquaporin 8 (Aqp8), transcript variant 1, mRNA [NM_007474]	Aqp8	Mm.273175	3.22	0.014
Mus musculus retinol dehydrogenase 7 (Rdh7). transcript variant 2, mRNA [NM_017473]	Rdh7	Mm.6696	2.85	0.032
Mus musculus arylacetamide deacetylase (esterase) (Aadac), mRNA [NM_023383]	Aadac	Mm.24547	2.75	0.031
Mus musculus serine (or cysteine) peptidase inhibitor, clade A, member 3K (Serpina3k), mRNA [NM_011458]	Serpina3k	Mm.291569	2.70	0.038
Mus musculus lipoma HMGIC fusion partner-like 2 (Lhfpl2), mRNA [NM_172589]	Lhfpl2	Mm.316553	2.59	0.007
Mus musculus apolipoprotein B (Apob), mRNA [NM_009693]	Apob	Mm.221239	2.58	0.047
Mus musculus angiotensinogen (serpin peptidase inhibitor, clade A, member 8) (Agt), mRNA [NM_007428]	Agt	Mm.301626	2.53	0.009
Mus musculus apolipoprotein C-IV (Apoc4), mRNA [NM_007385]	Apoc4	Mm.477720	2.49	0.004
Mus musculus calmodulin-binding transcription activator 1 (Camta1), transcript variant 1, mRNA [NM_001081557]	Camta1	Mm.318846	2.48	0.004

*Two genes without gene symbol and gene description were excluded.

does not represent a 'class effect' of synthetic gestagens but, at least in comparison with NET-A (13.3 $\mu g \cdot day^{-1}$), appears to be specific for MPA, considering that the ratio of hormone dosages used likely lead to a comparable progestogenic efficacy as described in detail in the Methods section. This prothrombotic effect may be due to MPA's partial glucocorticoid effects because MPA and NET-A bind to progesterone and androgen receptors (even with similar affinity), while substantially differing with regard to their glucocorticoid receptor affinity (Hapgood *et al.*, 2004). Furthermore, MPA was shown to increase expression of the PAR-1 receptor in smooth muscle cells which might be attributable to the glucocorticoid actions of MPA (Herkert *et al.*, 2001).

To evaluate if this difference in the thrombotic response between MPA- and NET-A-treated animals might also be due to differential arterial gene expression, the aortic gene expression profile was analysed. A limitation of this approach is that thrombotic events are not occurring in the aorta. However, the aortic gene expression was chosen in order to obtain sufficient high quality mRNA for analysis of the 'arterial transcriptome' in the mouse model. Interestingly, functional GO analysis revealed that for example, 'proteolysis' was a prominent BP term, which showed significant regulation in both treatment groups. Additionally, KEGG pathway analyses showed regulation of the 'ECM–receptor interaction' pathway in NET-A-treated animals only and genes mapping this pathway may influence atherothrombosis. However, the most profound results in the context of the atherothrombotic question of this work were obtained on the level of gene expression changes. Separate comparison of the groups 'MPA versus placebo' and 'NET-A versus placebo' revealed genes significantly regulated after hormone substitution, while comparison of 'MPA versus NET-A' after normalization of each of the hormone groups to their respective placebo group, allowed us to identify genes concordantly and divergently regulated by the two progestins.

Interestingly, a set of genes was regulated in the same direction in both treatment groups: Expression of *Mmp9* was up-regulated in MPA- and NET-A-treated animals, even to the

Table 6

List of the 15 most down-regulated genes in comparison of female ovariectomized ApoE-deficient mice treated with placebo or NET-A*

Gene description	Gene symbol	UniGeneID	Fold change (NET-A vs. placebo)	*P*-value
Mus musculus RIKEN cDNA 9930013L23 gene (9930013L23Rik), mRNA [NM_030728]	9930013L23Rik	Mm.160389	8.04	0.001
Mus musculus adult male medulla oblongata cDNA, RIKEN full-length enriched library, clone: 6330404C01 product: hypothetical protein, full insert sequence. [AK018112]	9930013L23Rik	Mm.160389	5.43	0.005
Mus musculus glycosylation-dependent cell adhesion molecule 1 (Glycam1), mRNA [NM_008134]	Glycam1	Mm.219621	3.85	0.020
Mus musculus 0 day neonate thymus cDNA, RIKEN full-length enriched library, clone: A430085B12 product: unclassifiable, full insert sequence. [AK040303]	B930042K01Rik	Mm.446882	3.71	0.048
Mus musculus oxidized low-density lipoprotein (lectin-like) receptor 1 (Olr1), mRNA [NM_138648]	Olr1	Mm.293626	3.69	0.009
Mus musculus collagen triple helix repeat containing 1 (Cthrc1), mRNA [NM_026778]	Cthrc1	Mm.41556	3.69	0.042
Mus musculus adult male testis cDNA, RIKEN full-length enriched library, clone: 1700018G05 product: unclassifiable, full insert sequence. [AK006087]	1700018G05Rik	Mm.138009	3.22	0.024
RIKEN cDNA 4932438A13 gene [Source: MGI Symbol; Acc: MGI: 2444631] [ENSMUST00000148698]	4932438A13Rik	Mm.207907	3.14	0.030
Mus musculus cell adhesion molecule with homology to L1CAM (Chl1), mRNA [NM_007697]	Chl1	Mm.251288	3.12	0.025
Mus musculus CD72 antigen (Cd72), transcript variant 2, mRNA [NM_007654]	Cd72	Mm.188157	3.11	0.024
Mus musculus secreted Ly6/Plaur domain containing 1 (Slurp1), mRNA [NM_020519]	Slurp1	Mm.27630	3.09	0.002
Mus musculus 13 days embryo forelimb cDNA, RIKEN full-length enriched library, clone: 5930400C17 product: unclassifiable, full insert sequence. [AK031058]			2.88	0.009
Mus musculus tetratricopeptide repeat domain 25 (Ttc25), mRNA [NM_028918]	Ttc25	Mm.31590	2.87	0.048
Mus musculus plakophilin 1 (Pkp1), mRNA [NM_019645]	Pkp1	Mm.4494	2.80	0.011
Mus musculus 3 days neonate thymus cDNA, RIKEN full-length enriched library, clone: A630081D01 product: unclassifiable, full insert sequence. [AK042310]	A630081D01Rik	Mm.404411	2.72	0.039

*One gene was not attributed with a gene symbol (marked in light grey) nor did it receive a UniGeneID (marked in mid-grey).

same extent. MMPs are known to be involved in proteolytic degradation of extracellular matrix and MMP-9 levels are increased in unstable atherosclerotic plaques (Sigala *et al.*, 2010). Furthermore, overexpression of activated MMP-9 in macrophages was shown to increase the incidence of plaque rupture in ApoE-deficient mice (Gough *et al.*, 2006). Therefore, the higher expression of *Mmp9* might lead to enhanced degradation of extracellular matrix and destabilization of the fibrous cap of atherosclerotic plaques. A limitation of this conclusion is that spontaneous plaque rupture, as seen in humans, does not occur in mice. However, the up-regulation of *Mmp9* might nevertheless imply increased destabilization of atherosclerotic plaques in general. Furthermore, *S100a9* was up-regulated in both progestin treatment groups. It is known that S100A8/A9 form heterodimers (Kerkhoff *et al.*, 1999) and S100A8 and S100A9 proteins were detected in plaque-derived material (McCormick *et al.*, 2005). Given this observation and their potential to increase macrophage LDL uptake (Lau *et al.*, 1995) and to promote monocyte-infiltration at sites of inflammation (Eue *et al.*, 2000) these proteins might also be involved in regulation of atherothrombosis. Especially, the heterodimeric form of S100A8/A9 may be involved in thrombosis because expression of both genes was induced by more than sixfold in thrombosis-prone mice substituted with MPA, while in NET-A-treated animals only *S100a9* was up-regulated. Expression of *Ppbp* was increased in MPA- and NET-A-treated animals. Morrell described that pro-platelet basic protein (Ppbp) as well as its

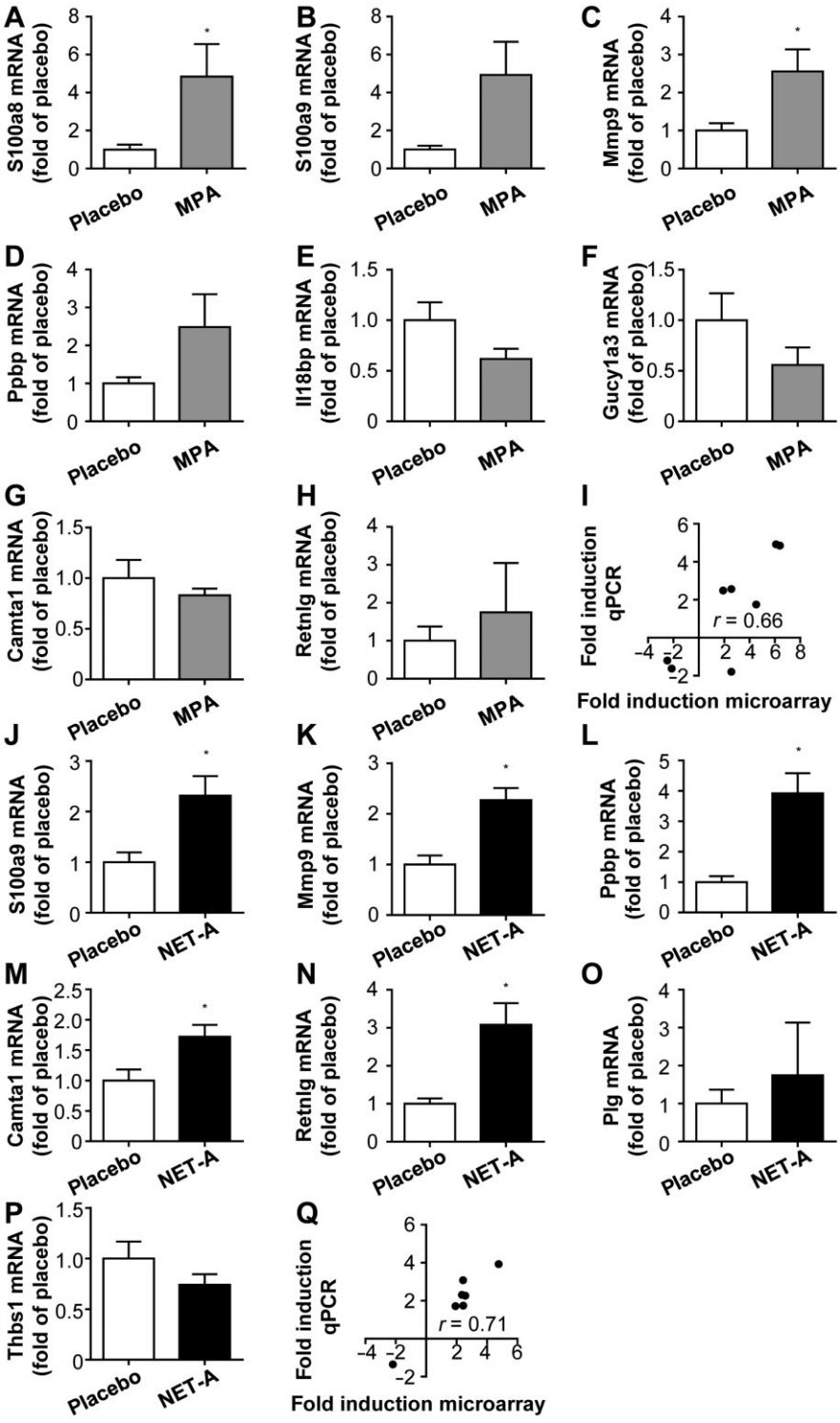

Figure 4

qPCR verification of expression of genes found to be significantly regulated in microarray experiments. Expression of genes found to be regulated in microarray analyses was verified by qPCR. Expression of genes regulated in (A–H) MPA- versus placebo-treated animals and (J– P) NET-A- versus placebo-treated mice. Data are expressed as fold of placebo and presented as mean ± SEM; $n = 8 – 9$ in A, $n = 7$ in B, $n = 7 – 8$ in C, $n = 8 – 9$ in D, $n = 7 – 9$ in E, $n = 3 – 5$ in F, $n = 7 – 10$ in G, $n = 3 – 5$ in H, $n = 7 – 8$ in J, $n = 8$ in K, $n = 7 – 9$ in L, $n = 9$ in M, $n = 8$ in N, $n = 3 – 7$ in O and $n = 8 – 10$ in P, $^*P < 0.05$ versus placebo. (I, Q) Correlation graphs showing fold regulation as evidenced by qPCR as compared with fold regulation according to microarray results for (I) MPA versus placebo and (Q) NET-A versus placebo. Correlation coefficients r of 0.66 (MPA) and 0.71 (NET-A) suggest a good correlation ($0.5 < r < 0.8$) of results obtained by qPCR and microarray experiments with eight XY pairs for MPA and seven XY pairs for NET-A respectively.

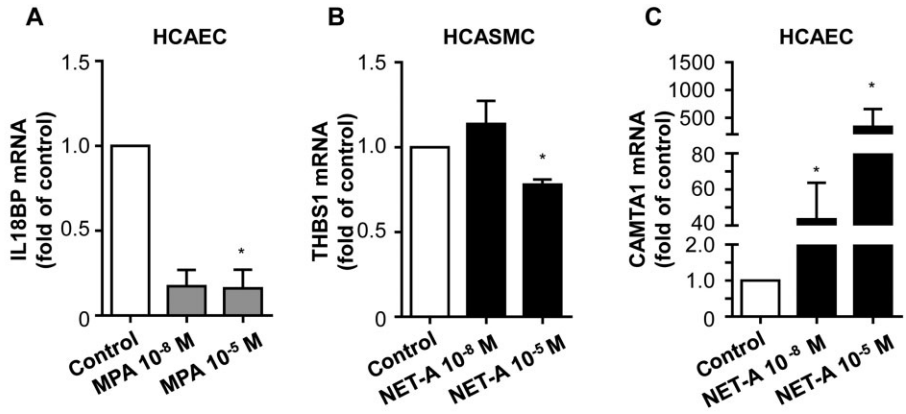

Figure 5

Expression of *IL18BP*, *THBS1* and *CAMTA1* is regulated in HCASMC or HCAEC upon hormone treatment. qPCR experiments showing expression of *IL18BP*, *THBS1* and *CAMTA1 in vitro*. Cells were stimulated with (A) MPA or (B, C) NET-A for 18 h. (A) *IL18BP* expression was reduced in HCAEC upon MPA stimulation while (B) *THBS1* expression was reduced after stimulation of HCASMC with NET-A. (C) Increased *CAMTA1* expression was observed in HCAEC upon NET-A stimulation. Data are expressed as fold of control and presented as mean ± SEM; $n = 4$ in A–C, $^*P < 0.05$ versus control.

'breakdown product CXCL7/NAP-2' have the capacity to activate leucocytes as well as endothelial cells (Morrell, 2011), which subsequently might play a role in promoting a pro-thrombogenic phenotype. Also, expression of *Retnlg* was increased in both MPA- and NET-A-treated animals (however, according to microarray data, to a lesser extent in NET-A-treated mice). *Retnlg* has been described to be a resistin family member (Nagaev *et al.*, 2006) and stimulation of endothelial cells with resistin results in increased tissue factor expression. Furthermore, resistin led to a decrease of eNOS and reduction of cellular NO (Jamaluddin *et al.*, 2012). Due to its nature to be a resistin family member, *Retnlg* might exert similar effects and thereby contribute to a pro-thrombotic phenotype.

In conclusion, increased arterial expression of *Mmp9*, *S100a9*, *Ppbp* and *Retnlg* in MPA- and NET-A-treated animals might represent a 'class effect' of synthetic progestins implying that synthetic progestins carry the potential to direct aortic gene expression towards a more pro-thrombogenic expression profile.

Paradoxically, arterial thrombosis was not changed in NET-A-treated animals raising the question if regulation of genes, exclusively in either MPA- or NET-A-treated mice, might partially explain the observed difference in the arterial thrombotic response. Therefore, it is interesting to consider genes specifically changed only by MPA or NET-A. In this context, *Serpina3k* was found to be down-regulated exclusively in MPA-treated animals according to microarray results. Serpina3 might, among others, act anti-coagulatory through inhibition of cathepsin G, which itself is known to promote platelet aggregation (Chelbi *et al.*, 2012). Therefore, it should be considered that inhibition of *Serpina3k* expression might contribute to MPA's pro-thrombotic effect. Furthermore, expression of *Il18bp* was found to be reduced in MPA-treated animals both, in microarray as well as qPCR experiments. Il18bp has been shown to be likely involved in plaque stabilization (Mallat *et al.*, 2001). Therefore, reduced expression of *Il18bp* might lead to plaque destabilization and enhancement of the thrombotic response. HCAEC stimulated with MPA *in vitro* showed a markedly reduced expression of *IL18BP* suggesting that endothelial cells may be the arterial cell type responsible for reduced *Il18bp* expression observed in aortas of MPA-treated mice. Taken together, the unique gene expression profile in MPA-treated mice may partially contribute to the pro-thrombotic effect of MPA. Interestingly, also expression of *Gucy1a3* was increased in MPA-treated animals according to microarray results. However, sGC is associated with anti-thrombotic effects. Therefore, it may well be considerable that increased expression of *Gucy1a3* occurs as a compensatory 'defence' mechanism to counteract MPA's pro-thrombotic actions. However, because qPCR results rather suggested an inhibition of *Gucy1a3* expression, it is not possible to draw a resilient conclusion with regard to the impact of *Gucy1a3* in the context of the present experiments.

Also in NET-A-treated animals, several genes potentially relevant for the atherothrombotic response were exclusively regulated in these mice. In this context, the gene encoding for *Gp5*, which is part of the glycoprotein Ib-IX-V (GPIb-IX-V)-complex that has been described to initiate platelet aggregation (Andrews *et al.*, 2003) was markedly up-regulated in microarray experiments, even more so raising an obvious discrepancy between the gene expression profile and the unaltered thrombotic response in these mice. However, *Gp5* was below the detection limit in qPCR experiments.

Of considerable interest, in NET-A-treated animals, *Plg* was up-regulated in microarray analyses and was also detectable in at least three animals per group, although not in all samples investigated, in qPCR experiments, with a regulation concordant to that one seen in microarray experiments. Bugge *et al.* showed that plasminogen-deficient mice developed thrombosis in different organs (Bugge *et al.*, 1995) emphasizing the importance of plasminogen for maintaining

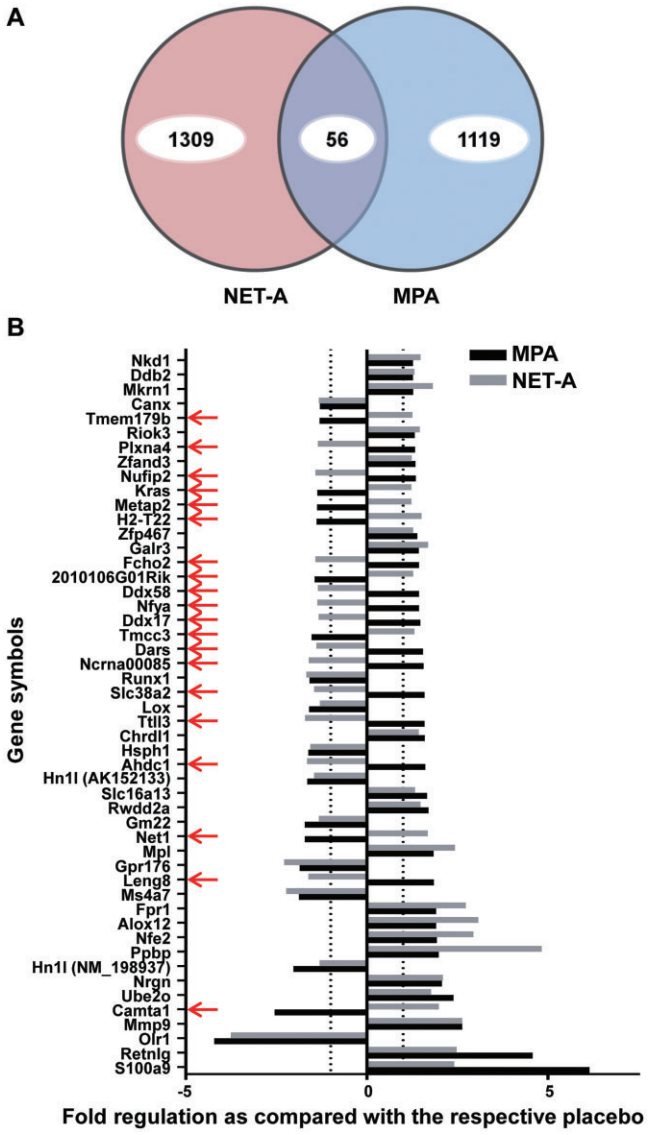

Figure 6

Comparison of aortic gene expression in MPA- versus NET-A-treated mice reveals differential expression of several genes. (A) Depiction of the number of genes with overlapping and distinct regulation in MPA- and NET-A-treated mice. (B) Genes (only those ones that could be assigned a gene symbol and a UniGeneID) regulated in both MPA- and NET-A-treated animals. Data were obtained and statistically analysed comparing quadrupletts in each of the groups after normalization of each hormone-treated group to its placebo controls. Arrows mark the genes that were differentially regulated (induction vs. inhibition) in MPA-treated mice as compared with animals substituted with NET-A.

a homeostatic balance. Furthermore, expression of *Thbs1* was found to be markedly decreased in aortas of NET-A-treated mice. Bonnefoy *et al*. showed that thrombospondin-1 likely plays a role in 'recruitment of platelets' to sites of activated endothelium and in stabilization of thrombi (Bonnefoy *et al*., 2006). Additionally, thrombospondin-1 has been proposed to counteract the anti-thrombotic actions of NO (Isenberg *et al*., 2008). Therefore, down-regulation of *Thbs1* might exert anti-thrombotic effects as might the up-regulation of *Plg* do as well. *In vitro*, HCASMC showed decreased *Thbs1* expression upon NET-A-treatment, suggesting that down-regulation of *Thbs1* might be attributable to the smooth muscle cell moiety in arteries. Taken together, these results suggest that increased expression of genes such as *Ppbp*, *S100a9*, *Mmp9* and *Retnlg*, likely associated with a pro-thrombotic phenotype, might well be counterbalanced by increased expression of genes involved in fibrinolysis, namely *Plg*, and down-regulation of genes with a potential pro-thrombotic effect, namely *Thbs1*. This might, at least partially, account for the fact that NET-A does not aggravate arterial thrombosis.

Importantly, *Camta1* was the most markedly differentially regulated gene in MPA- versus NET-A-treated mice. Camtas belong to the 'family of calmodulin-binding transcriptional activators (CAMTAs)' and Camta1 possesses the ability to interact with DNA, to act as a transcription factor and to interact with calmodulin (Bouche *et al*., 2002). It has been suggested that calmodulin associates with the GPIb-IX-V complex in platelets (Andrews *et al*., 2001). Although the functional impact of Camta1 on the GPIb-IX-V–calmodulin interaction is unknown to date, Camta1 may be involved in thrombotic events through its selective binding to calmodulin or via as yet unresolved regulatory control of transcriptional processes. Importantly, qPCR results suggest that endothelial cells likely represent the arterial cell type being involved in increased *Camta1* expression upon NET-A treatment. However, further studies are required to clarify the potential importance of Camta1 in arterial thrombosis.

To summarize the present findings, Figure 7 schematically depicts the results discussed above.

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Author contributions

T. F., R. D., I. K., P. M., H.-K. H., K. K. and J. W. F. designed and conceived the experiments; T. F., R. D., I. K., A. Z. and L. F. S. performed the experiments; T. F., R. D. and I. K. analysed the data; T. F. and J. W. F. wrote the manuscript.

Conflict of interest

None.

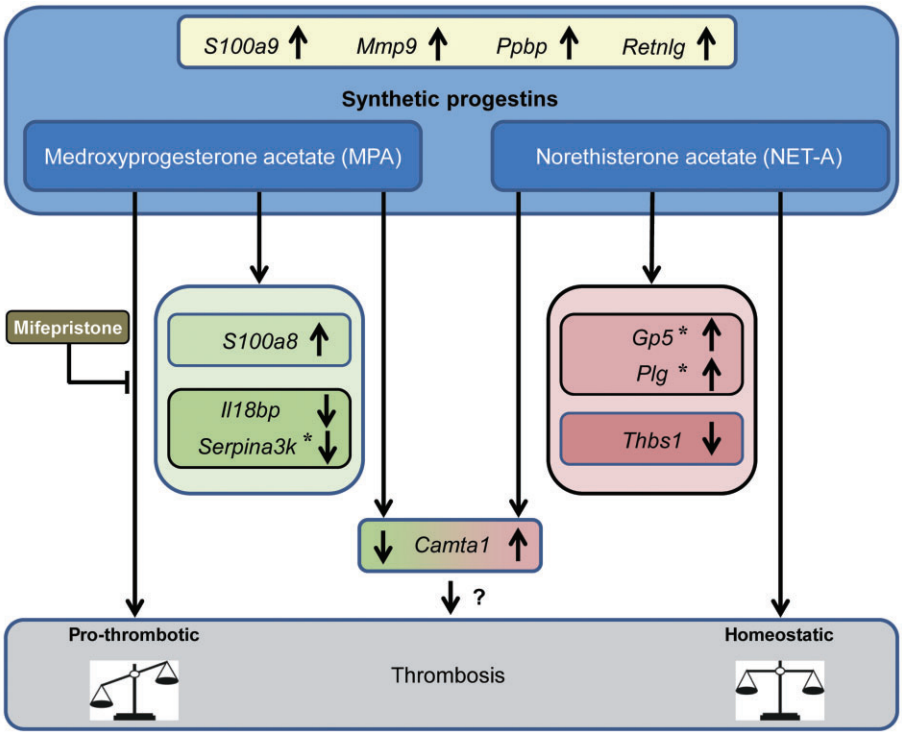

Figure 7

Scheme showing the working hypothesis as drawn from the present results. MPA elicits pro-thrombotic effects that can be antagonized by mifepristone while NET-A does not affect arterial thrombus formation. Expression of the genes encoding for *S100a9*, *Mmp9*, *Ppbp* and *Retnlg*, which are potentially associated with a pro-thrombotic phenotype, is increased after chronic treatment with the synthetic progestins MPA and NET-A possibly pointing towards a 'class effect' of synthetic progestins with regard to regulation of these genes. Furthermore, some genes possibly affecting atherothrombosis, such as *S100a8*, *Il18bp* and *Serpina3k* in MPA-treated mice or *Thbs1*, *Plg* and *Gp5* in NET-A-treated animals are specifically regulated in only one treatment group. Of note, the direction of regulation of the genes encoding for *S100a8*, *Il18bp* and *Serpina3k* in MPA-treated mice may be associated with pro-thrombotic effects. In contrast, the direction of regulation of the genes encoding for *Plg* and *Thbs1* in mice substituted with NET-A is probably associated with anti-thrombotic effects. These findings in turn suggest that the aortic gene expression in MPA-treated mice is pro-thrombotic, while the expression of genes associated with a pro-thrombotic phenotype in NET-A-treated mice might be counterbalanced by distinct regulation of genes such as *Plg* and *Thbs1*, resulting in a more 'homeostatic' arterial gene expression profile. Finally, the gene encoding for *Camta1* is regulated antidromic in MPA- versus NET-A-treated mice, maybe making it a potentially interesting target gene in terms of arterial thrombus formation. Genes marked with an asterisk were detected to be significantly regulated in microarray experiments, but could not or not in all samples be detected by qPCR.

References

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013). The Concise Guide to PHARMACOLOGY 2013/14: Nuclear hormone receptors. Br J Pharmacol. 170: 1652–1675.

Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H *et al.* (2004). Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. JAMA 291: 1701–1712.

Andrews RK, Munday AD, Mitchell CA, Berndt MC (2001). Interaction of calmodulin with the cytoplasmic domain of the platelet membrane glycoprotein Ib-IX-V complex. Blood 98: 681–687.

Andrews RK, Gardiner EE, Shen Y, Whisstock JC, Berndt MC (2003). Glycoprotein Ib-IX-V. Int J Biochem Cell Biol 35: 1170–1174.

Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19: 185–193.

Bonnefoy A, Daenens K, Feys HB, De Vos R, Vandervoort P, Vermylen J *et al.* (2006). Thrombospondin-1 controls vascular platelet recruitment and thrombus adherence in mice by protecting (sub)endothelial VWF from cleavage by ADAMTS13. Blood 107: 955–964.

Bouche N, Scharlat A, Snedden W, Bouchez D, Fromm H (2002). A novel family of calmodulin-binding transcription activators in multicellular organisms. J Biol Chem 277: 21851–21861.

Bugge TH, Flick MJ, Daugherty CC, Degen JL (1995). Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. Genes Dev 9: 794–807.

Check JH, Dix E, Cohen R, Check D, Wilson C (2010). Efficacy of the progesterone receptor antagonist mifepristone for palliative therapy of patients with a variety of advanced cancer types. Anticancer Res 30: 623–628.

Chelbi ST, Wilson ML, Veillard AC, Ingles SA, Zhang J, Mondon F *et al.* (2012). Genetic and epigenetic mechanisms collaborate to control SERPINA3 expression and its association with placental diseases. Hum Mol Genet 21: 1968–1978.

Emms H, Lewis GP (1985). The effect of synthetic ovarian hormones on an in vivo model of thrombosis in the rat. Br J Pharmacol 84: 243–248.

Eue I, Pietz B, Storck J, Klempt M, Sorg C (2000). Transendothelial migration of 27E10 + human monocytes. Int Immunol 12: 1593–1604.

Folsom AR, Mink PJ, Sellers TA, Hong CP, Zheng W, Potter JD (1995). Hormonal replacement therapy and morbidity and mortality in a prospective study of postmenopausal women. Am J Public Health 85 (8 Pt 1): 1128–1132.

Freudenberger T, Oppermann M, Marzoll A, Heim HK, Mayer P, Kojda G *et al.* (2009). Differential effects of medroxyprogesterone acetate on thrombosis and atherosclerosis in mice. Br J Pharmacol 158: 1951–1960.

Freudenberger T, Oppermann M, Heim HK, Mayer P, Kojda G, Schror K *et al.* (2010). Proatherogenic effects of estradiol in a model of accelerated atherosclerosis in ovariectomized ApoE-deficient mice. Basic Res Cardiol 105: 479–486.

Gough PJ, Gomez IG, Wille PT, Raines EW (2006). Macrophage expression of active MMP-9 induces acute plaque disruption in apoE-deficient mice. J Clin Invest 116: 59–69.

Goyeneche AA, Caron RW, Telleria CM (2007). Mifepristone inhibits ovarian cancer cell growth in vitro and in vivo. Clin Cancer Res 13: 3370–3379.

Grodstein F, Clarkson TB, Manson JE (2003). Understanding the divergent data on postmenopausal hormone therapy. N Engl J Med 348: 645–650.

Hapgood JP, Koubovec D, Louw A, Africander D (2004). Not all progestins are the same: implications for usage. Trends Pharmacol Sci 25: 554–557.

Herkert O, Kuhl H, Sandow J, Busse R, Schini-Kerth VB (2001). Sex steroids used in hormonal treatment increase vascular procoagulant activity by inducing thrombin receptor (PAR-1) expression: role of the glucocorticoid receptor. Circulation 104: 2826–2831.

Huang da W, Sherman BT, Lempicki RA (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37: 1–13.

Huang da W, Sherman BT, Lempicki RA (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4: 44–57.

Isenberg JS, Romeo MJ, Yu C, Yu CK, Nghiem K, Monsale J *et al.* (2008). Thrombospondin-1 stimulates platelet aggregation by blocking the antithrombotic activity of nitric oxide/cGMP signaling. Blood 111: 613–623.

Jamaluddin MS, Weakley SM, Yao Q, Chen C (2012). Resistin: functional roles and therapeutic considerations for cardiovascular disease. Br J Pharmacol 165: 622–632.

Kerkhoff C, Klempt M, Kaever V, Sorg C (1999). The two calcium-binding proteins, S100A8 and S100A9, are involved in the metabolism of arachidonic acid in human neutrophils. J Biol Chem 274: 32672–32679.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Koubovec D, Ronacher K, Stubsrud E, Louw A, Hapgood JP (2005). Synthetic progestins used in HRT have different glucocorticoid agonist properties. Mol Cell Endocrinol 242: 23–32.

Langer RD (2009). Efficacy, safety, and tolerability of low-dose hormone therapy in managing menopausal symptoms. J Am Board Fam Med 22: 563–573.

Lau W, Devery JM, Geczy CL (1995). A chemotactic S100 peptide enhances scavenger receptor and Mac-1 expression and cholesteryl ester accumulation in murine peritoneal macrophages in vivo. J Clin Invest 95: 1957–1965.

Mallat Z, Corbaz A, Scoazec A, Graber P, Alouani S, Esposito B *et al.* (2001). Interleukin-18/interleukin-18 binding protein signaling modulates atherosclerotic lesion development and stability. Circ Res 89: E41–E45.

McCormick MM, Rahimi F, Bobryshev YV, Gaus K, Zreiqat H, Cai H *et al.* (2005). S100A8 and S100A9 in human arterial wall. Implications for atherogenesis. J Biol Chem 280: 41521–41529.

McGrath JC, Drummond GB, McLachlan EM, Kilkenny C, Wainwright CL (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Morrell CN (2011). Immunomodulatory mediators in platelet transfusion reactions. Hematology Am Soc Hematol Educ Program 2011: 470–474.

Nagaev I, Bokarewa M, Tarkowski A, Smith U (2006). Human resistin is a systemic immune-derived proinflammatory cytokine targeting both leukocytes and adipocytes. PLoS ONE 1: e31.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP, Davenport AP, McGrath JC, Peters JA, Southan C, Spedding M, Yu W, Harmar AJ; NC-IUPHAR. (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledgebase of drug targets and their ligands. Nucl Acids Res 42 (Database Issue): D1098–106.

Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML *et al.* (2002). Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. JAMA 288: 321–333.

Rossouw JE, Prentice RL, Manson JE, Wu L, Barad D, Barnabei VM *et al.* (2007). Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. JAMA 297: 1465–1477.

Schindler AE, Campagnoli C, Druckmann R, Huber J, Pasqualini JR, Schweppe KW *et al.* (2003). Classification and pharmacology of progestins. Maturitas 46 (Suppl. 1): S7–S16.

Sigala F, Savvari P, Liontos M, Sigalas P, Pateras IS, Papalampros A *et al.* (2010). Increased expression of bFGF is associated with carotid atherosclerotic plaques instability engaging the NF-kappaB pathway. J Cell Mol Med 14: 2273–2280.

Sitruk-Ware R (2002). Progestogens in hormonal replacement therapy: new molecules, risks, and benefits. Menopause 9: 6–15.

Skarda J, Kohlerova E (2006). Mouse bioassay for in vivo screening of oestrogen and progesterone antagonists. J Vet Med A Physiol Pathol Clin Med 53: 145–153.

Tremollieres FA, Cigagna F, Alquier C, Cauneille C, Pouilles J, Ribot C (2000). Effect of hormone replacement therapy on age-related increase in carotid artery intima-media thickness in postmenopausal women. Atherosclerosis 153: 81–88.

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M *et al.* (2012). Primer3–new capabilities and interfaces. Nucleic Acids Res 40: e115.

Vickers MR, MacLennan AH, Lawton B, Ford D, Martin J, Meredith SK *et al.* (2007). Main morbidities recorded in the Women's International Study of long duration oestrogen after menopause (WISDOM): a randomised controlled trial of hormone replacement therapy in postmenopausal women. BMJ 335: 239.

Wiegratz I, Kuhl H (2004). Progestogen therapies: differences in clinical effects? Trends Endocrinol Metab 15: 277–285.

Wilson KM, Lynch CM, Faraci FM, Lentz SR (2003). Effect of mechanical ventilation on carotid artery thrombosis induced by photochemical injury in mice. J Thromb Haemost 1: 2669–2674.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13: 134.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 GO analysis reveals that approximately 2/3 of the top 25 regulated BP terms are exclusively regulated in either MPA- or NET-A-treated animals. Shown are the top 25 regulated terms associated with the BP category and the total number of genes that affiliate with each term (A) in MPA- versus placebo-treated mice and (B) in NET-A- versus placebo-treated mice. Data were obtained and statistically analysed comparing $n = 4$ mice in each group. Columns marked in blue represent terms that are regulated in both groups when comparing MPA- with placebo-treated mice as well as animals substituted with NET-A with their placebo controls. Columns filled in orange indicate terms that are exclusively regulated when comparing MPA- with placebo-treated mice, while columns shown in green define terms that are regulated only when comparing animals substituted with NET-A versus placebo.

Figure S2 GO analysis shows that more than 50% of the top 25 regulated CC terms are overlappingly regulated in MPA- and NET-A-treated animals as compared with their placebo controls. Shown are the top 25 regulated terms associated with the CC category and the total number of genes that affiliate with each term (A) in MPA- versus placebo-treated mice and (B) in NET-A- versus placebo-treated mice. Data were obtained and statistically analysed comparing $n = 4$ mice in each group. Columns marked in blue represent terms that are regulated in both groups when comparing MPA- with placebo-treated mice as well as animals substituted with NET-A with their placebo controls. Columns filled in orange indicate terms that are exclusively regulated when comparing MPA- with placebo-treated mice, while columns shown in green define terms that are regulated only when comparing animals substituted with NET-A versus placebo.

Figure S3 GO analysis indicates that more than half of the top 25 regulated MF terms are exclusively regulated in either MPA- or NET-A-treated animals as compared with their placebo controls. Shown are the top 25 regulated terms associated with the MF category and the total number of genes that affiliate with each term (A) in MPA- versus placebo-treated mice and (B) in NET-A- versus placebo-treated mice. Data were obtained and statistically analysed comparing $n = 4$ mice in each group. Columns marked in blue represent terms that are regulated in both groups when comparing MPA- with placebo-treated mice as well as animals substituted with NET-A with their placebo controls. Columns filled in orange indicate terms that are exclusively regulated when comparing MPA- with placebo-treated mice, while columns shown in green define terms that are regulated only when comparing animals substituted with NET-A versus placebo.

Figure S4 Analysis of the significantly regulated KEGG pathways shows only little overlap in comparison of MPA- versus placebo- and NET-A- versus placebo-treated animals. Shown are the significantly regulated KEGG pathways (A) in MPA- versus placebo-treated mice and (B) in NET-A- versus placebo-treated mice. Data were obtained and statistically analysed comparing $n = 4$ mice in each group. Columns marked in blue represent pathways that are regulated in both groups when comparing MPA- with placebo-treated mice as well as animals substituted with NET-A versus placebo. Columns filled in orange indicate terms that are exclusively regulated when comparing MPA- with placebo-treated mice while columns shown in green define terms that are regulated only when comparing animals substituted with NET-A with their placebo controls. The column filled in light green indicates a KEGG pathway exclusively regulated in NET-A-treated mice which might be relevant in terms of atherothrombosis.