Clinical & Experimental Immunology
The Journal of Translational Immunology

doi:10.1111/cei.13278

Clinical and Experimental Immunology REVIEW ARTICLE

REVIEW SERIES: IMMUNOLOGY OF PREGNANCY
Series Editor: Angelo A. Manfredi and Patrizia Rovere-Querini

The role of neutrophil activation in determining the outcome of pregnancy and modulation by hormones and/or cytokines

OTHER ARTICLES PUBLISHED IN THIS REVIEW SERIES
The immunology of the fetal–placental unit comes of age. Clinical and Experimental Immunology 2019, 198: 11-14.
Embryonic extracellular vesicles as informers to the immune cells at the maternal–fetal interface. Clinical and Experimental Immunology 2019, 198: 15-23.
Overview of procalcitonin in pregnancy and in pre-eclampsia. Clinical and Experimental Immunology 2019, 198: 37-46.
Influence of maternal microbiota during pregnancy on infant immunity. Clinical and Experimental Immunology 2019, 198: 47-56.

S. Hahn,* P. Hasler,† L. Vokalova,* S. V. van Breda,*† O. Lapaire,‡ N. G. Than,§ I. Hoesli‡ and S. W. Rossi *

**Department of Biomedicine, University and University Hospital Basel, Basel, †Department of Rheumatology, Kantonsspital Aarau, Aarau, ‡Department of Obstetrics, University Women's Hospital Basel, Basel, Switzerland and §Systems Biology of Reproduction Lendulet Research Group, Institute of Enzymology, Research Center for Natural Sciences, Hungarian Academy of Sciences, Budapest, Hungary.*

Accepted for publication 20 December 2018
Correspondence: S. Hahn and S. W. Rossi, Laboratory for Prenatal Medicine, Department of Biomedicine, University of Basel, Basel, Switzerland.
E-mail: Sinuhe.Hahn@usb.ch or simona.rossi@unibas.ch

Summary

Neutrophils are often exclusively considered as a first-line innate immune defence, able to rapidly kill or trap pathogens and causing in case of over-activation tissue damage. In the female reproductive tract, however, the presence and activity of neutrophils seems to be tightly regulated. Major players in orchestrating this regulation are cyclical steroid sex hormones present during the menstrual cycle and pregnancy. This review describes the role of sex hormones in regulating directly or indirectly the functionality of neutrophils, the role of neutrophils during fertilization and pregnancy and in controlling viral, fungal and bacterial infection. This review also discusses the consequence of overt neutrophil activation in pregnancy pathologies.

Keywords: cytokines, inflammation, neutrophils, reproductive immunology

Introduction

Polymorphonuclear neutrophils (PMN) are a vital component of the innate immune system's rapid response team [1–4]. They are characterized by a multi-lobed nucleus, granular cytoplasm and short lifespan. In humans they are particularly numerous, accounting for approximately 70% of circulating leucocytes, while in mice their number is much lower, being of the order of 3% [1–4].

PMN are highly specialized immune effector cells, possessing a diverse number of weapons in their armoury with which they can neutralize or eliminate pathogens [2,5]. These include phagocytosis, the production of reactive oxygen species (ROS), release of potent bactericidal enzymes by degranulation and the formation of neutrophil extracellular traps (NETs) [2,5]. The latter are generated by the extrusion of DNA strands into the extracellular milieu, where they can ensnare invasive pathogens [5,6]. The presence of lytic enzymes such as neutrophil elastase (NE) or myeloperoxidase (MPO) on NETs enhances their anti-microbial activity [5,6]. NETosis may involve neutrophil demise (lytic NETosis) or entail their survival (vital NETosis) [7–9]. The signalling cascade invoked during NETosis includes production of ROS by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, the nuclear translocation of NE and MPO and histone deamination by peptidyl arginine deiminase 4 (PAD4) [10–12]. A novel form of NETosis termed ApoNETosis was discovered recently [13]. This unique form, triggered by UV irradiation, involves concomitant apoptosis and NETosis [13].

PMN potentially contribute to various stages of the reproductive process by assisting with conception and implantation, ensuring fetal wellbeing during pregnancy and finally contributing to parturition and postpartum maternal health (reviewed in [14,15]). Conversely, aberrant PMN activity has been noted in severe pregnancy-related disorders such as pre-eclampsia (PE) [16,17], recurrent fetal loss (RFL) [18] or gestational diabetes mellitus (GDM) [19]. These data suggest that altered or overt PMN activity may play a role in the underlying aetiology of these disorders.

Our intention in this review is to highlight new observations made during discrete stages of the reproductive cycle, focusing particularly on the role of sex hormones and cytokines in modulating neutrophil activity and their potential dysregulation in pathology.

Hormone regulation of the reproductive cycle – introducing the players and setting the stage

The steroid sex hormones play a crucial role in optimizing the milieu of the female reproductive tract (FRT) to ensure conception, implantation, maintenance and growth of the fetus throughout gestation [20–23]. Oestrogen exists in a number of forms, including oestrone (E1), 17-β2-oestradiol (E2), oestriol (E3) and oestetriol (E4) [20–23]. In reproductively active humans the major form is E2, while in pregnancy E3 is the predominant form, being largely produced by the placenta. E4 is unique by being produced by the fetal liver. Like E2, progesterone (P4) is present in both sexes, although it is much higher in females, where it increases post-puberty and diminishes post-menopause. P4 is also produced by the placenta during pregnancy. Akin to E3, the concentrations of P4 increase from the beginning of the second trimester, peaking immediately prior to parturition [20–23].

An interesting interplay occurs in the menstrual cycle between E2 and P4, in that E2 levels peak in the follicular phase prior to ovulation, while P4 concentrations are greatest in the luteal phase, where it would assist with implantation of a fertilized zygote [20–23].

In the oestrous cycle of mammalian therian females, oestrogen produced by the mature follicules initiates the phase of sexual receptivity (oestrus), whereas P4 production either assists with the progression of gestation or a period of inactivity (anoestrus) [24].

A vast array of peptide hormones, many of which are produced by the placenta, contribute to the completion of a successful pregnancy, including human placental lactogen (hPL), calcitonin, activins and inhibins, placenta growth factor (PGF) or leptin. While an essential function of these hormones is to regulate maternal metabolism in order to promote fetal growth or alter the ability of fetal trophoblast cells to invade maternal tissue, several of these factors have been shown to possess additional immune modulatory capacity [25,26]. Of particular interest in this discourse is human chorionic gonadotrophin (hCG) and vasoactive intestinal peptide (VIP) [27]. hCG is produced by the syncytiotrophoblast, the single layer of contiguous fetal tissue directly in contact with the maternal circulation, while VIP is produced by the underlying cytotrophoblast tissue [27]. As discussed below, both exhibit the ability to modify PMN behaviour during pregnancy.

Hormones regulate neutrophil infiltration of the female reproductive tract

The process whereby circulatory PMN migrate to sites of infection in the female reproductive tract is currently unclear, but it would appear that cyclical steroid sex hormones levels play an important role in this process [20,28–31]. Several years ago Tibbetts *et al*. showed that P4 given concurrently with oestrogen to ovariectomized mice for 4 days antagonizes the ability of oestrogen to recruit macrophages and neutrophils into the mouse uterus, and this effect was dependent on progesterone receptors [32].

That the hormonal regulation of PMN influx into the female reproductive tract mucosal tissues can go awry is evident from the predisposition of women to fungal vaginal infections with opportunistic pathogens such as *Candida albicans* [30,31]. Vulvovaginal candidiasis is frequently observed during pregnancy or in the luteal phase of the menstrual cycle, as well as upon prolonged treatment with E2 [30,31].

A recent study addressed the underlying mechanism leading to this enhanced susceptibility, using a translational murine model for candida infection [31]. In this model system, Salinas-Munoz and colleagues noted that vaginal application of E2, which predisposes such mice to infection with *C. albicans*, led to a retention of PMN in the vaginal tissue, while application of P4 permitted their transepithelial migration into the vaginal lumen [31]. An examination of the female reproductive tract histology indicated that PMN accumulation was greatest in the lower female reproductive tract (fornix and ectocervix) in E2 than in mock- or P4-treated mice. They furthermore determined that the failure of PMN to traverse the epithelial cell barrier in E2-treated mice was not due to a decrease in either matrix metallopeptidase 9 (MMP9), the enzyme required for degradation of the basement membrane, or the integrins (CD18/CD11b) required for docking to epithelial cells [31]. Rather, it appeared that epithelial factors required to initiate transepithelial migration were down-regulated by E2 treatment interacting with the oestrogen receptor alpha ESR1. Upon this interaction they observed that expression of CD47, an important PMN docking molecule, and CD44v6, a molecule which facilitates PMN release into the lumen, were decreased by E2 treatment. This effect of E2 appears to be restricted to FRT epithelium, as no similar observation was noted concerning the expression of these molecules in gut epithelia upon application of E2 [31].

A noteworthy observation made during this study was that when PMN remained attached to the epithelium following E2 treatment they were incapable of neutralizing pathogens such as *C. albicans*, whereas those unattached in the FRT lumen, following P4 treatment, displayed a robust candidacidal activity. It thus appears that during

the oestrous cycle, E2 will favour sperm survival and fertilization by retaining PMN in an inactive epithelium-associated state. Upon successful zygote formation, P4 alters this status, allowing the transepithelial migration of the PMN into the mucosal lumen, where they are able to combat pathogenic infections, in order to protect the pregnancy (Fig. 1) [31].

These data were underscored by a further recent study documenting the importance of E2 in maintaining the integrity of the vaginal epithelium and regulating PMN flux across this cell barrier [29]. In this study, the oestrogen receptor (ESR1) was conditionally ablated in the murine vaginal epithelium. In such animals, the lack of an oestrogen response was determined to render the vaginal tissue highly susceptible to laceration during mating. This was determined to result from diminished keratin expression and lack of a cornified protective layer [29].

Furthermore, excessive PMN infiltration of the vaginal tissue, readily detectable in vaginal smears, was noted in these mice regardless of the stage of the oestrous cycle. These PMN exhibited enhanced expression of MMP9, the enzyme required for transepithelial migration. ESR1-ablated mice also exhibited reduced levels of bacterial infiltration compared to wild-type animals [29].

In summary, these data therefore provide new insight into the mechanism rendering women sensitive to vaginosis during phases of pregnancy, menstruation or following hormone replacement therapy. They also offer an explanation for the variation in PMN populations due to oscillations in E2 and P4 levels during the menstrual cycle. Furthermore, they reiterate the importance of E2 in maintaining the integrity of epithelial mucosal tissue.

An unanswered question is whether the long-term application of progesterone used to prevent preterm delivery [21,33,34] reduces the susceptibility of such pregnant women to vaginosis.

NETs in the vaginal mucosa tackle viruses

PMN are a crucial component of mucosal immunity, where they dispose of invasive pathogens by phagocytosis or degranulation [28]. A novel aspect is the ability of PMN located in the FRT lumen to employ NETs in order to ensnare and kill invasive pathogens; a process that even appears to apply to viruses such as HIV [35].

Several previous reports have indicated that viral infections can trigger NETosis by PMN attracted to the site of infection, a process which can have severe clinical

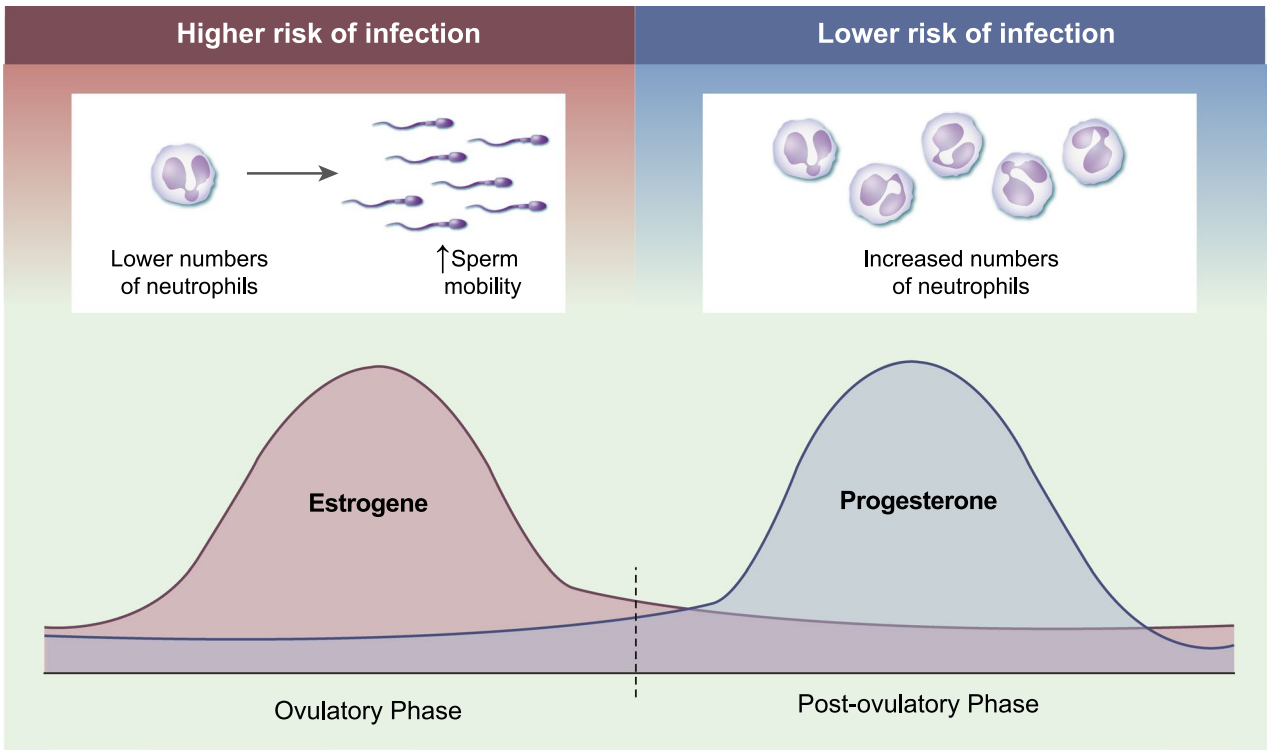

Fig. 1. Cyclic steroid sex hormones regulate the permeability of the female reproductive tract (FRT) allowing neutrophils to access the FRT, but exclusively during the post-ovulatory phase and in pregnancy where progesterone is high. In contrast, to permit the survival of sperm and their mobility, oestrogen decreases the membrane permeability decreasing the migration of neutrophils to the FRT. This can result in increased risk of infection.

consequences, for instance, in pulmonary infections [36–40].

Direct evidence that NETs could trap HIV virions was obtained by Saitoh and colleagues [41], using a technique termed super-resolution structured illumination microscopy (SR-SIM), which permits a greater degree of resolution than conventional fluorescence microscopy (200 nm).

In an initial exploratory investigation, circulatory PMN obtained from healthy donors were stimulated by phorbol ester to trigger the NETotic cascade, following which they were co-cultured with HIV-1 virions. An examination of the NETs in these cultures by SR-SIM ultra-high definition microscopy, as well as scanning electron microscopy (SEM), clearly indicated that individual HIV-1 virions were trapped by the sticky DNA lattice structures. They further determined that HIV-1 virions trapped by NETs were incapacitated by the presence of PMN proteins (MPO and α-defensin), known to coat NETs. Subsequent analyses indicated that HIV-1 virions were capable of activating isolated PMN directly and triggering NETs formation in a process involving Toll-like receptors (TLR)-7 and -8 [41]. As HIV-1 induced NETs lead to reduced infection of co-cultured $CD4^+$ T cells, it is plausible that NETosis may play an important initial role in combating viral infection [39].

Recent data suggest that the interaction between PMN and HIV-1 virions is not restricted to the circulation, but may involve PMN in the FRT mucosa [35]. In their examination, Barr and colleagues observed that when challenged with HIV-1 viral-like particles, PMN isolated from FRT smears rapidly formed NETs that entrapped and incapacitated the virions [35]. Of interest is that viral particle-induced NETosis occurred by ROS-independent means [35].

While these data are encouraging, there appear to be several caveats concerning the interaction of HIV-1 and PMN, the most striking of which may be the report made by Bowers and colleagues, which indicated that HIV-1 may exploit its interaction with circulatory PMN to down-regulate the host's immune response [42]. In this study it was determined that HIV-1 induced expression of programmed cell death ligand 1 (PD-L1) on circulatory PMN [42]. PMN isolated from HIV-1 infected individuals were shown to suppress T cell function by a mechanism involving PD-L1/PD-1 interaction and ROS generation [42].

In the context of the above findings, it would be worthwhile clarifying whether the use of injected contraceptives associated with a moderate increased risk for HIV-1 infection in high-risk populations [43,44] may be due to altered PMN behaviour in the FRT.

Neutrophils in the female reproductive tract – interaction with semen

The process of insemination involves the deposition of millions of sperm into the female reproductive tract, after which an intriguing interplay between the female reproductive tract and semen ensues, whereby the progress of the most motile sperm towards the receptive ovum is promoted, while the passage of any co-deposited pathogens is hindered [15,45,46]. In addition, excess sperm are removed in order to prevent antigenic exposure in the recipient female, failure of which can lead to infertility. Conversely, the presence of PMN has to be regulated in such a manner as to ensure sufficient sperm survival for fertilization [15]. As discussed above, for this reason PMN may be absent from the vaginal lumen during the ovulatory phase: a feature which may render this tissue susceptible to infection.

Previous studies have indicated that species-specific differences exist between bovine and equine PMN concerning their interaction with spermatozoa during fertilization [45,46]. In this regard, equine PMN strongly interacted with spermatozoa, rapidly forming NETs upon exposure to equine semen [45,46]. This action is countered by the presence of a factor with DNAse I-like activity in equine seminal plasma. This factor was shown to disrupt semen-induced NETs, but not those triggered by *Escherichia coli*. Conversely, while bovine spermatozoa were recognized and phagocytosed by PMN, this interaction was determined to depend upon the presence of seminal plasma, as sperm cleared of seminal plasma were determined to be relatively impervious to PMN action. This difference between the two species may reflect upon the modes of semen deposition. In the equine system the sperm are deposited directly in the uterus, and as such there is a tight limit wherein the maternal immune system can interact with the ejaculum.

In the bovine system, the ejaculate is deposited in the vagina and the sperm need to traverse a lengthy distance in order to reach the ovum. It appears that additional mechanisms to that of seminal plasma are operative to ensure safe passage of the sperm as they traverse the female reproductive tract [45–49]. In this context, *in-vitro* experiments have indicated that the ability of PMN to phagocytose sperm or generate NETs is down-modulated by bovine oviduct fluid, of which alpha 1-acid glycoprotein appears to be a regulatory factor [47,48].

Although these and other reports would suggest that inappropriate interactions between sperm and PMN could contribute to infertility, only one study has examined this aspect in humans [50].

In their report, Zambrano and colleagues co-incubated isolated human PMN with fresh human sperm [50]. This interaction was determined to trigger the generation of NETs in a time- and concentration-dependent manner. As sperm motility was impeded by NETs formation, they concluded that should such an interaction occur on a large scale in the human female reproductive tract, this would reduce the chance of successful fertilization (Fig. 1) [50].

Unfortunately, no clear data currently exist suggesting that human infertility could be attributed to unwarranted interaction in the female reproductive tract between PMN and semen.

The influence of pregnancy on the pro-NETotic behaviour of circulatory neutrophils: interplay between progesterone, oestrogen and granulocyte colony stimulating factor (G-CSF)

Because pregnancy is associated with a subliminal activation of circulatory PMN, evident by increased ROS production and expression of activation markers (CD11b) [51], we recently examined NETotic response during the course of normal healthy pregnancies [52]. Our study confirmed that during pregnancy circulatory PMN displayed an increased activation status, and an increased propensity for degranulation and phagocytosis. We also observed that such PMN exhibited an enhanced tendency to form NETs, which increased throughout gestation, peaking at term [52]. The regulation of this pro-NETotic state was determined to be of a multi-modal nature involving an interaction between G-CSF and steroid sex hormones [52].

It was noted that G-CSF, which increased progressively during gestation, leading to a boost in circulatory PMN numbers, led to a generalized pro-NETotic state. In the first trimester of pregnancy, NETosis was additionally enhanced by the activity of hCG, a peptide hormone produced by the placenta after implantation.

During the remaining period of gestation until term, a complex interaction between G-CSF, E2/E3 and P4 was noted in that the oestrogen molecules exhibited a pronounced pro-NETotic influence. This could be due, in part, to the increased expression of protein arginine deiminases (PAD4), as this gene bears an oestrogen response element, because these cells displayed extensive histone citrullination, a key part of the NETotic cascade [12].

The action of E2/E3 was strongly antagonized by the action of progesterone, in that the PMN were not able to progress down the NETotic pathway. This restraint appeared to be due to an inability of neutrophil elastase (NE) to migrate into the nucleus [52], where it would cleave histone molecules, another vital step required for fulminant NETs extrusion (Fig. 2) [53].

It would thus appear that this sophisticated and complex regulation serves to retain circulatory PMN in a high state of alert, ready to attack invasive pathogens. NETs formation would, however, only be triggered if appropriately challenged. As aberrant NETosis can lead to damage of surrounding tissues, this complex hormonally regulated safeguard could assist in ensuring he optimal safety of mother and unborn child.

Overt PMN activation is associated with excessive NETosis in PE

PE is a severe life-threatening disorder of late pregnancy, characterized by sudden elevation in blood pressure and oedema in previously normotensive pregnant women [54–57]. PE is a leading cause of fetal and maternal mortality, and has a pronounced influence on post-partum health of both parties. PE is a complex disorder with a multi-faceted aetiology, having at least two separate forms based on gestational age at onset of symptoms: early-onset PE (eoPE: < 34 weeks) and late-onset PE (loPE: > 34 weeks) [55,56]. In general, eoPE poses the greater clinical threat in that the maternal symptoms are more severe and the fetus is frequently growth-restricted [56]. Although most cases with PE are with the late-onset form (≈90%), these can still be associated with poor outcome, especially if occurring in tertiary health-care centres [58].

Placental malfunction is proposed to play a key role in the underlying aetiology of eoPE [57]. This is characterized by a defect in trophoblast differentiation and subsequent inadequate modification of the maternal spiral arteries, resulting in an inadequate supply of maternal blood to the fetal compartment of the placenta [57]. Starved of nutrients and oxygen, the syncytiotrophoblast reacts by the excessive shedding of highly inflammatory microparticles into the maternal circulation [59]. These particles have been proposed to contribute to key features of PE, such as endothelial cell damage or inappropriate activation of maternal immune cells, including induction of NETs [17,60]. An overt maternal inflammatory response appears to be crucial for the development of loPE, with risk factors including diabetes prior to conception, gestational diabetes mellitus (GDM), super-obesity or even air pollution [61].

In an extension of their analysis of PMN activity in normal pregnancy, the Oxford research group of Redman and Sargent observed that circulatory PMN were excessively activated in cases with manifest PE [51]. A striking finding aspect of this analysis was that PMN in cases PE were determined to be more highly activated than in cases with sepsis, used a positive control for a highly inflammatory disorder [51]. Furthermore, it appeared that this activation was triggered by the excessive release of highly proinflammatory micro-debris by the placenta, a feature known to occur in PE [62].

A major research focus in our laboratory at the time of these reports was the examination of cell-free nucleic acids in maternal blood as a means for non-invasive prenatal diagnosis [63]. During the course of these investigations, we had observed that manifest PE was associated with a significant elevation in the concentration of both placentally and maternally derived cell-free DNA (cfDNA) [64]. As such, we were very intrigued by the then novel

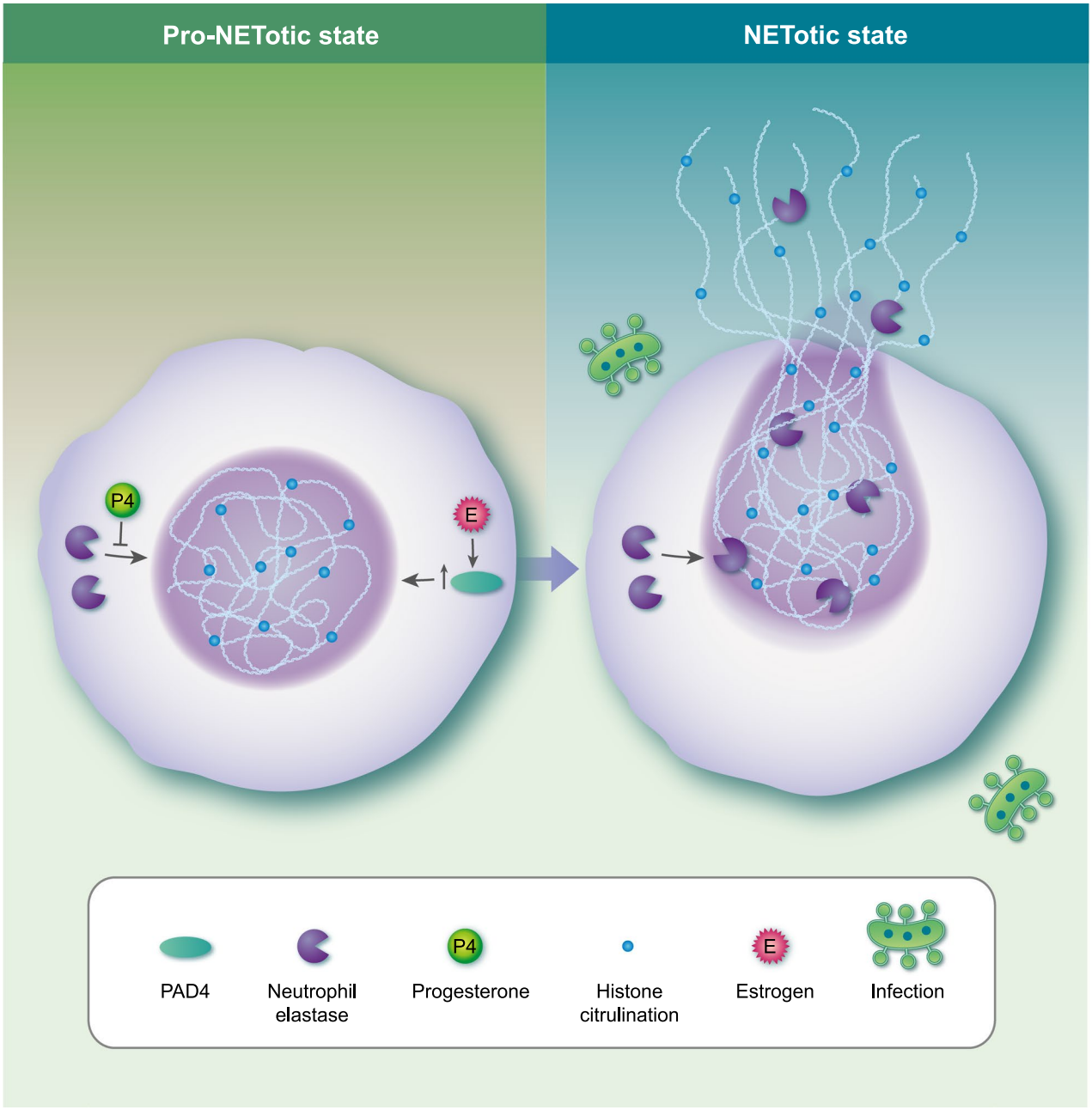

Fig. 2. Progesterone controls the NETotic process in neutrophils blocking the translocation of neutrophils elastase to the nucleus where it is required for histone cleavage.

report that PMN can extrude their genomic DNA into the extracellular environment in the form of NETs [6], and posed the query of whether these could be the source of increased maternal cfDNA in PE [65].

To our surprise, we observed that PMN isolated from normal healthy individuals readily formed NETs *in vitro* upon treatment with placental microparticles of the type used by the Oxford group in their investigations [17]. As placental microparticles are released in increased amounts by the syncytiotrophoblast into the maternal circulation in PE, we examined pertinent placental tissue sections by fluorescent immunohistochemistry, where a vast presence of PMN NETs structures was detected directly in the intervillous space of affected placentae [17].

To address whether the overt presence of NETs in PE could contribute to placental dysfunction, we made use of a translational murine model in collaboration with the

research groups of Wagner and Karumanchi in Boston [66]. This murine model relies upon an imbalance of angiogenic factors, notably soluble fms-like tyrosine kinase 1 (sFlt-1), which has been noted in PE [67]. By antagonizing the action of vascular endothelial growth factor (VEGF), the excessive production of sFlt-1 by the placenta in PE leads to widespread endothelial damage [67].

In this murine model, over-expression of sFlt-1 was shown to lead to a massive influx of PMN and occurrence of NETs in the placentae; a feature associated with reduced litter size and fetal demise [66]. In mice incapable of undergoing NETosis due to genetic ablation of the PAD4 enzyme, an amelioration of the effect of excessive sFlt-1 expression was noted [66]. Although not completely analogous to the situation observed in human pregnancies affected by PE, these data indicate that aberrant NETs formation can contribute to placental dysfunction and potentially ensuing fetal loss. It is currently unknown, and a matter of research, if the PMN response could be causative of the pathological pregnancies or just a consequence of the altered placental environment.

Infiltrating PMN alter placental function in GDM via the action of exogenous NE

During our examination of PMN activity in normal pregnancy we noted an extremely high level of NETosis in one sample [19]. Consultation of the case history indicated that this pregnant woman had been diagnosed with GDM at the time of blood sampling [19]. GDM is a unique form of glucose intolerance, being of a transient nature and only occurring during pregnancy in women with no prior history of diabetes [68–70]. GDM shares a number of features in common with T2DM, such as a systemic inflammation associated with the metabolic syndrome. Like T2DM, GDM occurs with greater frequency in obese individuals [71]. Accordingly, GDM may be viewed as a momentary unmasking of a T2DM-like condition facilitated by pregnancy. GDM does not contain an autoimmune component such as T1DM. Pregnancies affected by GDM are at an increased risk of developing PE, while post-partum both mother and offspring are at an increased susceptibility of developing T2DM [72].

Our analysis of nine cases with GDM verified that NETosis was indeed exceptionally high in such individuals, and that PMN isolated from such cases displayed pronounced activation and spontaneous generation of NETs by isolated neutrophils in *invitro* culture [19]. The latter was determined to be due to the presence of increased concentrations of tumour necrosis factor (TNF)-α in the circulation of pregnant women with GDM, resulting from the placenta as it struggles to cope with altered glucose levels, particularly hyperglycaemia [19,73].

TNF-α is known to have a pronounced influence on PMN priming, rending them highly susceptible to secondary stimuli [74]. In addition, hyperglycaemic conditions were determined to also promote PMN activation [75]. It is therefore possible that these two factors may act in consort to drive excessive PMN activation in GDM [19,76].

Akin to what we had observed in PE, extensive PMN infiltrates could be detected in GDM placentae. These infiltrates could result from TNF-α production by the trophoblast, as this cytokine has pronounced chemokine activity. The degree of NETosis was, however, not as pronounced as in PE. Instead, highly prodigious liberation of NE into the extracellular environment surrounding the infiltrates was noted [19]. Analogous to what had been observed in PMN infiltrates into tumours [77], exogenous NE was taken up by surrounding cells, where key signal transducing components were incapacitated by the action of this potent enzyme [19]. In our case, we noted the enzymatic degradation of IRS1 and GLUT4, resulting in altered response to insulin and glucose by the affected trophoblast tissue [19].

It is currently unclear if these PMN-induced changes in trophoblast signalling and metabolism result in the enhanced deportation of syncytiotrophoblast-derived microparticles. Such an event could play a key role promoting the inflammatory cascade underlying the development of PE. Although it is clear that myriad factors contribute to the increased occurrence of PE in GDM pregnancies, it is plausible that altered PMN activity could provide a causal link between these two pathologies (for an extensive discussion, refer to [76]).

Could a hormone imbalance contribute to the development of pregnancy related pathologies such as PE?

As steroid sex hormones modulate PMN activity in pregnancy, the question arises of whether an imbalance in these factors could contribute to PMN dysregulation in PE or other pregnancy-related complications. Although there is no direct proof that such a mechanism may be operative, there are a number of lines pointing in this direction.

The first is that oestrogens promote placental angiogenesis by enhancing VEGF gene expression and uterine artery vasodilation by increasing nitric oxide (NO) production, key events ensuring a healthy pregnancy outcome [23]. A further primary line of evidence was provided by the detection of reduced catechol-*O*-methyltransferase (COMT) enzyme activity in PE placentae, and that mice lacking this enzyme exhibit PE-like symptoms [23].

Because COMT plays an important role in oestrogen metabolism, Jobe and colleagues performed a detailed

analysis using mass spectrometry of oestrogen subtypes and metabolic derivatives in cases with PE, which were stratified into severe PE (sPE) and moderate PE (mPE) [78]. They determined that all three common forms of oestrogen (E1, E2 and E3) were reduced in the plasma of sPE cases when compared to mPE cases or matching normal healthy pregnancies. In contrast, cases with sPE exhibited significantly higher levels of 16 keto-17β2-oestradiol (16E2) than mPE cases or matching healthy controls. This keto variant appears to be an intermediary form between E2 and E3, being derived from E2 by the action of cytochrome P450. The function of this variant molecule is not clear. As we observed that both E2 and E3 enhanced PMN activation, promoting a pro-NETotic phenotype, it is possible that highly elevated levels of this intermediary form could significantly alter PMN activity.

Significant differences in products of hydroxylation, O-methylation or epimerization were also noted, indicating that the metabolism of steroid hormones is dramatically altered in PE, especially in cases with sPE [78]. Although the PE cases in this study were stratified into moderate or severe, they were all loPE forms. It is thus open to speculation as to how much more extensive the changes in steroid metabolism would be in eoPE cases, which characteristically have more severe clinical symptoms.

Recently, a more extensive analysis of steroid sex hormone levels in pregnancies affected by PE (n = 86) and matching healthy controls (n = 97), was performed by Wu and colleagues [22]. This analysis indicated that both E2 and P4 levels were significantly reduced in PE when compared to healthy controls. These reduced levels of E2 and P4 appeared to be due to a lower production by the placenta, as the levels synthesized by placenta explant *in vitro* cultures were lower in tissues obtained from cases with PE than when using healthy control samples. Of interest is that no significant difference could be discerned between cases with eoPE (n = 35) and loPE (n = 51), regardless of severity [22]. A further striking observation was that no significant difference was apparent in either E2 or P4 levels in pregnancies with PE bearing growth-retarded fetuses. Thus, these data reinforce the notion that an imbalance in sex hormone levels can contribute to the development of PE, but do not provide evidence that this involves some form of placental dysfunction. If this were the case, then it would be expected that this hormone imbalance would be more pronounced in cases with eoPE or exhibiting fetal growth restriction, events clearly associated with placental anomalies. As low E2/P4 levels are associated with both eoPE and loPE cases, it would appear that the influence of reduced hormone levels is more distal and systemic than local to the placenta. It could thus be that this hormone imbalance leads to an altered modulation of the maternal immune system, a feature which appears to be common to both early and late forms of PE [61].

It should, however, be noted that other factors such as lifestyle can influence circulating steroid hormone levels [79]. In this regard, it was noted that obesity is associated with reduced P4 levels, which may influence fetal birth weight [79]. Although the study by Diemert and colleagues was too small (n = 200) to provide any details regarding the incidence of PE in their study cohort, it is noteworthy that obesity has emerged as an important risk factor for the development of loPE, but not eoPE [80]. Furthermore, obesity contributes to the development of GDM, itself a pronounced risk factor for PE [71]. As PMN activity is altered in GDM and may play a role in the subsequent development of PE [76], it may be worthwhile examining the relationship between BMI and E2/P4 levels in this context.

VIP – potent new kid on the block

Originally described as a neuropeptide, VIP has emerged as a very interesting regulatory factor at the feto–maternal interface [27,81]. In human pregnancy, VIP is expressed by the syncytiotrophoblast directly at the site of feto–maternal interaction, highly suggestive of a crucial role in regulating the maternal immune response [81]. In the context of this review, it is noteworthy that its expression is regulated by P4.

By the use of *in vitro* co-culture systems between a trophoblast cell line (Swan 71 cells) and peripheral blood mononuclear cells (PBMCs) it was shown that VIP triggered an increase in forkhead box protein 3 (FoxP3) regulatory T cells [82]. VIP also led to an increase in the expression of immunosuppressive cytokines, such as interleukin (IL)-10, associated with a T helper type 2 (Th2) phenotype known to favour pregnancy.

Initial evidence that VIP induced an immunosuppressive state at the feto–maternal interface and that this had a pronounced effect on pregnancy outcome was obtained in translational rodent model systems [83]. In mice, expression of VIP was detected mid-gestation at the sites of implantation (days 9–11). In these studies use was made of non-obese diabetic (NOD) mice prone to autoimmune complications, including the development of insulin-dependent diabetes mellitus (IDDM). These mice have a poor pregnancy outcome, leading to fetal demise and resorption. This may be due to limited vascular remodelling, or from aberrant immune interactions at the feto–maternal interface, or a combination of both. Tissues were removed from both vital and failed implantation sites of pregnant NOD mice; VIP expression was largely detected on trophoblast

giant cells in the former, while its expression was significantly reduced in the latter. *In vitro* treatment of these tissue biopsies with exogenous VIP led to the increased expression of regulatory cytokines such as IL-10 and transforming growth factor (TGF)-β, associated with a Th2 immunosuppressive phenotype. An increase in the expression of FoxP3 was also noted, indicative of an increased presence of regulatory T cells with immunosuppressive function. In contrast, in failed sites of implantation, a decrease in an immunosuppressive milieu was noted with a concomitant increase in IL-17, a potent proinflammatory cytokine. Treatment of pregnant NOD mice at day 6.5 of pregnancy resulted in an improved pregnancy outcome, accompanied by an increase in IL-10, TGF-β and FoxP3. Hence, these data clearly suggest that VIP is able to promote pregnancy outcome by modulating the maternal immune system and inducing an immunosuppressive state favouring implantation and fetal survival [83].

That VIP can influence PMN behaviour and possibly regulate NETosis was the subject of a recent report by Calo and colleagues [81]. In this study, use was made of first-trimester trophoblast cell lines (Swan-71 and HTR8) in combination with PMN isolated from healthy donor blood samples. It was noted that treatment of isolated PMN with either conditioned medium from trophoblast cultures or exogenous VIP reduced their NETotic response to phorbol ester. This was found to correlate with a decrease in ROS production in treated PMN. This effect was deemed specific for VIP, as it could be reversed by the addition of a VIP receptor antagonist, and was not evident in culture medium from trophoblast cells in which expression of the VIP had been silenced. An interesting aspect of this report was that VIP enhanced PMN apoptosis, especially by reducing the anti-apoptotic effect of lipopolysaccharide (LPS), and promoting their clearance by monocytes [81].

Although these data are truly novel and exciting, it remains to see what role, if any, VIP plays in the development of complex disorders such as PE, and whether its application has any therapeutic merit.

Pregnancy, NETs and thrombosis

Pregnant women face an increased risk (four- to fivefold) for venous thrombosis [84], which is further dramatically increased by the presence of PE [85]. Evidence also suggest that the risk of venous thrombotic embolism is greatest immediately post-partum [84].

In normal pregnancy, it has been suggested that increased venous thrombotic embolism is the result of a state of hypercoagulation existing during pregnancy, a mechanism used to protect women from blood loss during childbirth [84]. The underlying cause for increased venous thrombotic embolism in PE is not clear. However, the observation that NETs promote coagulation and thereby contribute to blood clot formation may shed new light into this enigma [86].

The initial observation was made in the laboratory of Denisa Wagner in Boston, where it was noted that when NETs were perfused with blood this led to platelet adhesion, activation and aggregation [86]. The disruption of NETs by the addition of DNAse, or the addition of heparin, prevented clot formation [86]. This report was followed-up by a series of elegant studies in mice prone to coagulopathies, where it was noted that NETs-induced thrombi were reneged by genetic ablation of the PAD4 gene [87,88].

The mechanism whereby NETs promote coagulation is currently unclear, but may involve interaction with platelets [89]. In this context, it has been observed that NETs can trigger platelet activation and that activated platelets can, in turn, promote NETosis [89,90]. If left unchecked, this could provide the basis for autocatalytic loop with potentially disastrous consequences. Furthermore, it seems that intact NETs may not be required, but rather that isolated NETs components such as cell-free DNA, histones or liberated serine proteases may provide the crucial impetus [90,91].

It may, therefore, be that our own observations indicating that PE is associated with increased concentrations of circulatory cfDNA, as well as liberated NE, may provide vital clues concerning the increased incidence of venous thrombotic embolism in cases with PE [64,92,93]. Additional credence for such a supposition is provided by observations made in a collaborative study with Redman and Sargent in Oxford [94]. In this study it was noted that the concentrations of maternal cfDNA were greatest during labour and immediately post-partum, and that these were significantly greater in cases with severe PE. Should these factors indeed contribute to enhanced coagulation, then our data would fit well with the increased incidence of venous thrombotic embolism under such conditions [93].

The use of surrogate markers to assess NETotic activity – a word of caution

The implication of aberrant NETosis in a wide variety of disorders has led to a veritable boom in the number of analyses addressing this issue. Unfortunately, the quantitation of NETs is not simple, relying on the isolation of PMN from blood samples, their *in-vitro* stimulation and detection [95,96]. Furthermore, the presence of NETs needs to be verified in order to ensure that the correct structures are being examined and not some type of artefact [97].

As these procedures are fraught with difficulty, prone to operator error and not amenable to routine clinical practice, the need has been voiced for alternatives. Because we and others have previously observed that pathologies such as PE or rheumatoid arthritis are associated with increased levels of cfDNA, and that these elevations may correlate with enhanced neutrophil NETosis, it is enticing to use cfDNA as a surrogate marker for NETs formation [17,65,98,99].

Accordingly, many researchers have turned to the analysis of cfDNA or histone/DNA complexes as surrogate markers for NETs, as the quantity of these can be readily determined using quantitative reverse transcription–polymerase chain reaction (qRT-PCR), fluorescent DNA binding dyes or appropriate enzyme-linked immunosorbent assays (ELISAs) [98,99].

While this practice is widely used, it has to be used with a degree of caution, as the source of cfDNA in the sample being examined is not clear. In order to confirm that cfDNA is indeed of NETotic origin, it is necessary to use immunoassays which detect complexes of cfDNA associated with NET components, such as MPO [98,99]. A further alternative is by the detection of citrullinated histone molecules in the cfDNA fraction, as histone citrullination is a prerequisite for the NETotic process [100]. In this manner, the presence of these cfDNA complexes serves to indicate that this material is derived from NETosis and not from apotosis or other forms of cell death. Unfortunately, the accuracy of these alternate assays has not been explored in large-scale studies.

Another concern is the indiscriminate use of plasma or serum for sample collection and analysis, a practice that can lead to vastly different results, as a small perusal of the extensive literature concerning the use for cfDNA for non-invasive prenatal diagnosis or cancer monitoring will reveal [101]. In brief, these collective data indicate that cfDNA molecules in rapidly processed plasma samples are indicative of the steady state evident in the circulation at the time of blood sampling. By the use of transplant recipients, both solid organ and haemopoeitic stem cells, it was shown that the vast proportion of this material was of haemopoeitic origin, probably resulting from the enucleation of erythroblasts as they mature to anuclear erythrocytes [101].

Among this material, traces could be accounted for by organs such as kidneys, increases of which were suggestive of transplant failure or rejection [102]. It was also shown that cfDNA in the circulation has an extremely short half-life, being of the order of 15 min, indicating that the pool of cfDNA is constantly being replenished.

In serum, the quantity of cfDNA is much higher due to the demise of blood cells during the clotting procedure. In this instance it appears that the levels of cfDNA may be higher under conditions where PMN exhibit an increased pro-NETotic tendency, such as rheumatoid arthritis, pregnancy or PE [98,99]. This was evident in the increased proportion of nuclear material complexed to cytoplasmic granular components such as MPO or NE. This facet was, however, best observed in serum samples permitted to coagulate normally at room temperature without the presence of accelerators.

Summary and conclusions

In this review we have attempted to provide an overview of the diverse actions carried out by PMN in ensuring successful reproduction and species propagation. The initiation of this process starts in the FRT, where PMN serve to remove excess spermatozoa and engage with pathogens including viruses, thereby rendering an optimal environment for implantation of the fertilized zygote. The sex hormones, E2 and P4, play a key role in this process by regulating the tightness of epithelial junctions, thereby controlling transepithelial migration.

During pregnancy, PMN activity and NET-forming ability is tightly controlled in an almost ying–yang-like manner, whereby P4 counteracts the pro-NETotic influence of E2 and G-CSF. The skewing of this process in GDM by TNF-α and hyperglycaemia may contribute to the development of PE. The presence of a pro-NETotic state in pregnancy and its deregulation in PE may provide the basis for the increased incidence of VTE in affected pregnant women.

The observation that VIP may hinder or reduce NETosis opens up the avenue for new biological therapeutics, a long-awaited and much-needed development in obstetrics [103].

Disclosure

None.

References

1 Mocsai A. Diverse novel functions of neutrophils in immunity, inflammation, and beyond. J Exp Med 2013; **210**:1283–99.
2 Borregaard N. Neutrophils, from marrow to microbes. Immunity 2010; **33**:657–70.
3 Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. Nat Rev Immunol 2011; **11**:519–31.
4 Mayadas TN, Cullere X, Lowell CA. The multifaceted functions of neutrophils. Annu Rev Pathol 2014; **9**:181–218.
5 Brinkmann V, Zychlinsky A. Beneficial suicide: why neutrophils die to make NETs. Nat Rev Microbiol 2007; **5**:577–82.
6 Brinkmann V, Reichard U, Goosmann C *et al*. Neutrophil extracellular traps kill bacteria. Science 2004; **303**:1532–5.

7 McDonald B, Urrutia R, Yipp BG, Jenne CN, Kubes P. Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. Cell Host Microbe 2012; **12**:324–33.

8 Pilsczek FH, Salina D, Poon KK *et al*. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. J Immunol 2010; **185**:7413–25.

9 Yipp BG, Petri B, Salina D *et al*. Infection-induced NETosis is a dynamic process involving neutrophil multitasking *in vivo*. Nat Med 2012; **18**:1386–93.

10 Fuchs TA, Abed U, Goosmann C *et al*. Novel cell death program leads to neutrophil extracellular traps. J Cell Biol 2007; **176**:231–41.

11 Gupta A, Giaglis S, Hasler P, Hahn S. Efficient neutrophil extracellular trap induction requires mobilization of both intracellular and extracellular calcium pools and is modulated by cyclosporine A. PLOS ONE 2014; **9**:e97088.

12 Wang Y, Li M, Stadler S *et al*. Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. J Cell Biol 2009; **184**:205–13.

13 Azzouz D, Palaniyar N. ApoNETosis: discovery of a novel form of neutrophil death with concomitant apoptosis and NETosis. Cell Death Dis 2018; **9**:839.

14 Giaglis S, Stoikou M, Grimolizzi F *et al*. Neutrophil migration into the placenta: good, bad or deadly? Cell Adh Migr 2016; **10**:208–25.

15 Hahn S, Giaglis S, Hoesli I, Hasler P. Neutrophil NETs in reproduction: from infertility to preeclampsia and the possibility of fetal loss. Front Immunol 2012; **3**:362.

16 Redman CW, Sacks GP, Sargent IL. Preeclampsia: an excessive maternal inflammatory response to pregnancy. Am J Obstet Gynecol 1999; **180**:499–506.

17 Gupta AK, Hasler P, Holzgreve W, Gebhardt S, Hahn S. Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. Hum Immunol 2005; **66**:1146–54.

18 Girardi G, Bulla R, Salmon JE, Tedesco F. The complement system in the pathophysiology of pregnancy. Mol Immunol 2006; **43**:68–77.

19 Stoikou M, Grimolizzi F, Giaglis S *et al*. Gestational diabetes mellitus is associated with altered neutrophil activity. Front Immunol 2017; **8**:702.

20 Wira CR, Rodriguez-Garcia M, Patel MV. The role of sex hormones in immune protection of the female reproductive tract. Nat Rev Immunol 2015; **15**:217–30.

21 Makieva S, Saunders PT, Norman JE. Androgens in pregnancy: roles in parturition. Hum Reprod Update 2014; **20**:542–59.

22 Wan J, Hu Z, Zeng K *et al*. The reduction in circulating levels of estrogen and progesterone in women with preeclampsia. Pregnancy Hypertens 2018; **11**:18–25.

23 Berkane N, Liere P, Oudinet JP *et al*. From pregnancy to preeclampsia: a key role for estrogens. Endocr Rev 2017; **38**:123–44.

24 Kobayashi Y. The novel roles of neutrophils via opioid peptides: regulation of the estrous cycle and pain. Arch Immunol Ther Exp (Warsz) 2013; **61**:187–91.

25 PrabhuDas M, Bonney E, Caron K *et al*. Immune mechanisms at the maternal-fetal interface: perspectives and challenges. Nat Immunol 2015; **16**:328–34.

26 Lash GE, Ernerudh J. Decidual cytokines and pregnancy complications: focus on spontaneous miscarriage. J Reprod Immunol 2015; **108**:83–9.

27 Ramhorst R, Calo G, Paparini D *et al*. Control of the inflammatory response during pregnancy: potential role of VIP as a regulatory peptide. Ann NY Acad Sci 2019; **1437**:15–21.

28 Wira CR, Fahey JV, Rodriguez-Garcia M, Shen Z, Patel MV. Regulation of mucosal immunity in the female reproductive tract: the role of sex hormones in immune protection against sexually transmitted pathogens. Am J Reprod Immunol 2014; **72**:236–58.

29 Li S, Herrera GG, Tam KK *et al*. Estrogen action in the epithelial cells of the mouse vagina regulates neutrophil infiltration and vaginal tissue integrity. Sci Rep 2018; **8**:11247.

30 Fidel PL Jr, Cutright J, Steele C. Effects of reproductive hormones on experimental vaginal candidiasis. Infect Immun 2000; **68**:651–7.

31 Salinas-Munoz L, Campos-Fernandez R, Mercader E *et al*. Estrogen receptor-alpha (ESR1) governs the lower female reproductive tract vulnerability to Candida albicans. Front Immunol 2018; **9**:1033.

32 Tibbetts TA, Conneely OM, O'Malley BW. Progesterone via its receptor antagonizes the pro-inflammatory activity of estrogen in the mouse uterus. Biol Reprod 1999; **60**:1158–65.

33 Martinez de Tejada B, Karolinski A, Ocampo MC *et al*. Prevention of preterm delivery with vaginal progesterone in women with preterm labour (4P): randomised double-blind placebo-controlled trial. Br J Obstet Gynaecol 2015; **122**:80–91.

34 Hermans FJ, Karolinski A, Othenin-Girard V *et al*. Population differences and the effect of vaginal progesterone on preterm birth in women with threatened preterm labor (.). J Matern Fetal Neonatal Med 2016; **29**:3223–8.

35 Barr FD, Ochsenbauer C, Wira CR, Rodriguez-Garcia M. Neutrophil extracellular traps prevent HIV infection in the female genital tract. Mucosal Immunol 2018; **11**:1420–28.

36 Raftery MJ, Lalwani P, Krautkrmer E *et al*. Beta2 integrin mediates hantavirus-induced release of neutrophil extracellular traps. J Exp Med 2014; **211**:1485–97.

37 Schonrich G, Raftery MJ. Neutrophil extracellular traps go viral. Front Immunol 2016; **7**:366.

38 Hahn S, Giaglis S, Chowdury CS, Hosli I, Hasler P. Modulation of neutrophil NETosis: interplay between infectious agents and underlying host physiology. Semin Immunopathol 2013; **35**:439–53.

39 Jenne CN, Kubes P. NETs tangle with HIV. Cell Host Microbe 2012; **12**:5–7.

40 Cheng OZ, Palaniyar N. NET balancing: a problem in inflammatory lung diseases. Front Immunol 2013; **4**:1.
41 Saitoh T, Komano J, Saitoh Y *et al*. Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. Cell Host Microbe 2012; **12**:109–16.
42 Bowers NL, Helton ES, Huijbregts RP *et al*. Immune suppression by neutrophils in HIV-1 infection: role of PD-L1/PD-1 pathway. PLOS Pathog 2014; **10**:e1003993.
43 Hofmeyr GJ, Singata M, Sneden J. Hormonal contraception for women exposed to HIV infection. Cochrane Database Syst Rev 2014; **5**:CD009741.
44 Nene Z, Hofmeyr GJ, Patel M *et al*. Changes to the World Health Organization guideline on hormonal contraceptive eligibility for women at high risk of HIV: South African perspective and response. S Afr Med J 2018; **108**:629–31.
45 Alghamdi AS, Foster DN. Seminal DNase frees spermatozoa entangled in neutrophil extracellular traps. Biol Reprod 2005; **73**:1174–81.
46 Alghamdi AS, Lovaas BJ, Bird SL *et al*. Species-specific interaction of seminal plasma on sperm–neutrophil binding. Anim Reprod Sci 2009; **114**:331–44.
47 Liu J, Marey MA, Kowsar R *et al*. An acute-phase protein as a regulator of sperm survival in the bovine oviduct: alpha 1-acid-glycoprotein impairs neutrophil phagocytosis of sperm *in vitro*. J Reprod Dev 2014; **60**:342–8.
48 Marey MA, Liu J, Kowsar R *et al*. Bovine oviduct epithelial cells downregulate phagocytosis of sperm by neutrophils: prostaglandin E2 as a major physiological regulator. Reproduction 2014; **147**:211–9.
49 Marey MA, Yousef MS, Liu J *et al*. Endothelin-1 downregulates sperm phagocytosis by neutrophils *in vitro*: a physiological implication in bovine oviduct immunity. J Reprod Dev 2016; **62**:151–7.
50 Zambrano F, Carrau T, Gartner U *et al*. Leukocytes coincubated with human sperm trigger classic neutrophil extracellular traps formation, reducing sperm motility. Fertil Steril 2016; **106**:1053–60.e1051.
51 Sacks GP, Studena K, Sargent K, Redman CW. Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis. Am J Obstet Gynecol 1998; **179**:80–6.
52 Giaglis S, Stoikou M, Sur Chowdhury C *et al*. Multimodal regulation of NET formation in pregnancy: progesterone antagonizes the pro-NETotic effect of estrogen and G-CSF. Front Immunol 2016; **7**:565.
53 Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol 2010; **191**:677–91.
54 Chaiworapongsa T, Chaemsaithong P, Korzeniewski SJ, Yeo L, Romero R. Pre-eclampsia part 2: prediction, prevention and management. Nat Rev Nephrol 2014; **10**:531–40.
55 Chaiworapongsa T, Chaemsaithong P, Yeo L, Romero R. Pre-eclampsia part 1: current understanding of its pathophysiology. Nat Rev Nephrol 2014; **10**:466–80.
56 Myatt L, Roberts JM. Preeclampsia: syndrome or disease? Curr Hypertens Rep 2015; **17**:83.
57 Brosens I, Pijnenborg R, Vercruysse L, Romero R. The 'Great Obstetrical Syndromes' are associated with disorders of deep placentation. Am J Obstet Gynecol 2011; **204**:193–201.
58 Kenneth L, Hall DR, Gebhardt S, Grove D. Late onset preeclampsia is not an innocuous condition. Hypertens Pregnancy 2010; **29**:262–70.
59 Tannetta D, Masliukaite I, Vatish M, Redman C, Sargent I. Update of syncytiotrophoblast derived extracellular vesicles in normal pregnancy and preeclampsia. J Reprod Immunol 2017; **119**:98–106.
60 Gupta AK, Rusterholz C, Huppertz B *et al*. A comparative study of the effect of three different syncytiotrophoblast micro-particles preparations on endothelial cells. Placenta 2005; **26**:59–66.
61 Hahn S, Lapaire O, Than NG. Biomarker development for presymptomatic molecular diagnosis of preeclampsia: feasible, useful or even unnecessary? Expert Rev Mol Diagn 2015; **15**:617–29.
62 Sacks G, Sargent I, Redman C. An innate view of human pregnancy. Immunol Today 1999; **20**:114–8.
63 Hahn S, Lapaire O, Tercanli S, Kolla V, Hosli I. Determination of fetal chromosome aberrations from fetal DNA in maternal blood: has the challenge finally been met? Expert Rev Mol Med 2011; **13**:e16.
64 Zhong XY, Laivuori H, Livingston JC *et al*. Elevation of both maternal and fetal extracellular circulating deoxyribonucleic acid concentrations in the plasma of pregnant women with preeclampsia. Am J Obstet Gynecol 2001; **184**:414–9.
65 Gupta A, Hasler P, Gebhardt S, Holzgreve W, Hahn S. Occurrence of neutrophil extracellular DNA traps (NETs) in pre-eclampsia: a link with elevated levels of cell-free DNA? Ann NY Acad Sci 2006; **1075**:118–22.
66 Erpenbeck L, Chowdhury CS, Zsengeller ZK *et al*. PAD4 deficiency decreases inflammation and susceptibility to pregnancy loss in a mouse model. Biol Reprod 2016; **95**:132.
67 Levine RJ, Karumanchi SA. Circulating angiogenic factors in preeclampsia. Clin Obstet Gynecol 2005; **48**:372–86.
68 Sacks DA. Gestational diabetes – whom do we treat? N Engl J Med 2009; **361**:1396–8.
69 Buchanan TA, Xiang A, Kjos SL, Watanabe R. What is gestational diabetes? Diabetes Care 2007; **30**(Suppl 2):S105–11.
70 Huhn EA, Massaro N, Streckeisen S *et al*. Fourfold increase in prevalence of gestational diabetes mellitus after adoption of the new International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria. J Perinat Med 2017;**45**:359–66.
71 Janevic T, Zeitlin J, Egorova N, Balbierz A, Howell EA. The role of obesity in the risk of gestational diabetes among immigrant and U.S.-born women in New York City. Ann Epidemiol 2018; **28**:242–8.
72 Bartsch E, Medcalf KE, Park AL, Ray JG, High Risk of Pre-eclampsia Identification Group. Clinical risk factors for pre-eclampsia determined in early pregnancy: systematic review and meta-analysis of large cohort studies. BMJ 2016; **353**:i1753.

73 Coughlan MT, Oliva K, Georgiou HM, Permezel JM, Rice GE. Glucose-induced release of tumour necrosis factor-alpha from human placental and adipose tissues in gestational diabetes mellitus. Diabet Med 2001; **18**:921–7.

74 Thelen M, Rosen A, Nairn AC, Aderem A. Tumor necrosis factor alpha modifies agonist-dependent responses in human neutrophils by inducing the synthesis and myristoylation of a specific protein kinase C substrate. Proc Natl Acad Sci USA 1990; **87**:5603–7.

75 Fadini GP, Menegazzo L, Scattolini V *et al*. A perspective on NETosis in diabetes and cardiometabolic disorders. Nutr Metab Cardiovasc Dis 2016; **26**:1–8.

76 Vakalova L, van Breda SV, Ye XL *et al*. Excessive neutrophil activity in gestational diabetes mellitus: could it contribute to the development of preeclampsia? Front Endocrinol 2018; **9**:542. https://doi.org/10.3389/fendo.2018.00542.

77 Houghton AM, Rzymkiewicz DM, Ji H *et al*. Neutrophil elastase-mediated degradation of IRS-1 accelerates lung tumor growth. Nat Med 2010; **16**:219–23.

78 Jobe SO, Tyler CT, Magness RR. Aberrant synthesis, metabolism, and plasma accumulation of circulating estrogens and estrogen metabolites in preeclampsia implications for vascular dysfunction. Hypertension 2013; **61**:480–7.

79 Diemert A, Goletzke J, Barkmann C *et al*. Maternal progesterone levels are modulated by maternal BMI and predict birth weight sex-specifically in human pregnancies. J Reprod Immunol 2017; **121**:49–55.

80 Mbah AK, Kornosky JL, Kristensen S *et al*. Super-obesity and risk for early and late pre-eclampsia. Br J Obstet Gynaecol 2010; **117**:997–1004.

81 Calo G, Sabbione F, Vota D *et al*. Trophoblast cells inhibit neutrophil extracellular trap formation and enhance apoptosis through vasoactive intestinal peptide-mediated pathways. Hum Reprod 2017; **32**:55–64.

82 Fraccaroli L, Grasso E, Hauk V *et al*. VIP boosts regulatory T cell induction by trophoblast cells in an *in vitro* model of trophoblast–maternal leukocyte interaction. J Leukoc Biol 2015; **98**:49–58.

83 Hauk V, Azzam S, Calo G *et al*. Vasoactive intestinal peptide induces an immunosuppressant microenvironment in the maternal–fetal interface of non-obese diabetic mice and improves early pregnancy outcome. Am J Reprod Immunol 2014; **71**:120–30.

84 James AH. Pregnancy, contraception and venous thromboembolism (deep vein thrombosis and pulmonary embolism). Vasc Med 2017; **22**:166–9.

85 Egan K, Kevane B, Ni Ainle F. Elevated venous thromboembolism risk in preeclampsia: molecular mechanisms and clinical impact. Biochem Soc Trans 2015; **43**:696–701.

86 Fuchs TA, Brill A, Duerschmied D *et al*. Extracellular DNA traps promote thrombosis. Proc Natl Acad Sci USA 2010; **107**:15880–5.

87 Demers M, Krause DS, Schatzberg D *et al*. Cancers predispose neutrophils to release extracellular DNA traps that contribute to cancer-associated thrombosis. Proc Natl Acad Sci USA 2012; **109**:13076–81.

88 Martinod K, Demers M, Fuchs TA *et al*. Neutrophil histone modification by peptidylarginine deiminase 4 is critical for deep vein thrombosis in mice. Proc Natl Acad Sci USA 2013; **110**:8674–9.

89 Pfeiler S, Stark K, Massberg S, Engelmann B. Propagation of thrombosis by neutrophils and extracellular nucleosome networks. Haematologica 2017; **102**:206–13.

90 Schulz C, Massberg S. Demystifying the prothrombotic role of NETs. Blood 2017; **129**:925–6.

91 Noubouossie DF, Whelihan MF, Yu YB *et al*. *In vitro* activation of coagulation by human neutrophil DNA and histone proteins but not neutrophil extracellular traps. Blood 2017; **129**:1021–9.

92 Gupta AK, Gebhardt S, Hillermann R, Holzgreve W, Hahn S. Analysis of plasma elastase levels in early and late onset preeclampsia. Arch Gynecol Obstet 2006; **273**:239–42.

93 Hahn S, Giaglis S, Buser A *et al*. Cell-free nucleic acids in (maternal) blood: any relevance to (reproductive) immunologists? J Reprod Immunol 2014; **104-105**:26–31.

94 Reddy A, Zhong XY, Rusterholz C *et al*. The effect of labour and placental separation on the shedding of syncytiotrophoblast microparticles, cell-free DNA and mRNA in normal pregnancy and pre-eclampsia. Placenta 2008; **29**:942–9.

95 Brinkmann V, Laube B, Abu Abed U, Goosmann C, Zychlinsky A. Neutrophil extracellular traps: how to generate and visualize them. J Vis Exp 2010; pii: 1724.

96 Brinkmann V, Goosmann C, Kuhn LI, Zychlinsky A. Automatic quantification of *in vitro* NET formation. Front Immunol 2012; **3**:413.

97 Krautgartner WD, Klappacher M, Hannig M *et al*. Fibrin mimics neutrophil extracellular traps in SEM. Ultrastruct Pathol 2010; **34**:226–31.

98 Sur Chowdhury C, Giaglis S, Walker UA *et al*. Enhanced neutrophil extracellular trap generation in rheumatoid arthritis: analysis of underlying signal transduction pathways and potential diagnostic utility. Arthritis Res Ther 2014; **16**:R122.

99 Sur Chowdhury C, Hahn S, Hasler P *et al*. Elevated levels of total cell-free DNA in maternal serum samples arise from the generation of neutrophil extracellular traps. Fetal Diagn Ther 2016; **40**:263–7.

100 Thalin C, Lundstrom S, Seignez C *et al*. Citrullinated histone H3 as a novel prognostic blood marker in patients with advanced cancer. PLOS ONE 2018; **13**:e0191231.

101 Jiang P, Lo YMD. The long and short of circulating cell-free DNA and the ins and outs of molecular diagnostics. Trends Genet 2016; **32**:360–71.

102 Li Y, Hahn D, Zhong XY *et al*. Detection of donor-specific DNA polymorphisms in the urine of renal transplant recipients. Clin Chem 2003; **49**:655–8.

103 Hahn S. Preeeclampsia – will orphan drug status facilitate innovative biological therapies? Front Surg 2015; https://doi.org/10.3389/fsurg.2015.00007.