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

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Abstract

Neutrophils are often exclusively considered as 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 seem 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 fertilisation and pregnancy and in controlling viral, fungal and bacterial infection. This review also discusses the consequence of overt neutrophil activation in pregnancy pathologies.

1. 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 life span. 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 antimicrobial 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 (reactive oxygen species) by NADPH oxidase, the nuclear translocation of NE and MPO, and histone deamination by PAD4 (peptidyl arginine deiminase 4) [10-12]. Recently a novel form of NETosis termed ApoNETosis was discovered [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 post-partum maternal health (reviewed in [14,15]). On the other hand, aberrant PMN activity has been noted in severe pregnancy related disorders such as preeclampsia (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, focussing particularly the role of sex hormones and cytokines in modulating neutrophil activity and their potential dysregulation in pathology.

2. 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]. Estrogen exists in a number of forms, including estrone (E1), 17- β 2-estradiol (E2), estriol (E3) and estetriol (E4) [20-23]. In reproductively active humans the major form is E2, whilst 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 albeit it 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, whilst P4 concentrations are greatest in the luteal phase where it would assist with implantation of a fertilized zygote [20-23].

In the estrous cycle of mammalian therian females, estrogen produced by the mature follicles initiates the phase of sexual receptivity (estrus), whereas P4 production either assists with the progression of gestation or a period of inactivity (anestrus) [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 gonadotropin (hCG) and vaso-active 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 is discussed below, both exhibit the ability to modify PMN behaviour during pregnancy.

3. 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 estrogen to ovariectomized mice for 4 days antagonises the ability of estrogen 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 *C. 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*, lead to a retention of PMN in the vaginal tissue, while application of P4 permitted their trans-epithelial 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

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treated mice was not due to a decrease in either 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 trans-epithelial migration were down regulated by E2 treatment interacting with the estrogen 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 candida-cidal activity. It thus appears that during the estrous 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 trans-epithelial migration of the PMN into the mucosal lumen, where they are able to combat pathogenic infections, in order to protect the pregnancy (Figure 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 estrogen receptor (ESR1) was conditionally ablated in the murine vaginal epithelium. In such animals, lack of an estrogen 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 estrous cycle. These PMN exhibited enhanced expression of MMP9, the enzyme required for trans-epithelial migration. ESR1 ablated mice also exhibited reduced levels of bacterial infiltration compared to wild type animals [29].

In summary, these data, hence, 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. They furthermore reiterate the importance of E2 in maintaining the integrity of epithelial mucosal tissue.

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

4. 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 (human immunodeficiency virus) [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 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 to be capable of activating isolated PMN directly and triggering NETs formation in a process involving toll-like receptors (TLR) 7 and 8 [41]. Since 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 combatting viral infection [39].

Recent data suggests 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 PD-L1 (programmed cell death protein ligand 1) 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.

5. 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. On the other hand, the presence of PMN has to be regulated in such a manner 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 *E. coli*. On the other hand, while bovine spermatozoa were recognized and phagocytosed by PMN, this interaction was determined to depend on 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 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. Since 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, that this would reduce the chance of successful fertilization (Figure 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.

6. The influence of pregnancy on the pro-NETotic behaviour of circulatory neutrophils: interplay between progesterone, estrogen and G-CSF

Since 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 what the NETotic response was 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 (granulocyte colony stimulating factor) and steroid sex hormones [52].

It was noted that G-CSF, which increased progressively during gestation, leading to a boost in circulatory PMN numbers, lead to a generalized pro-NETotic state. In the 1st 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 till term a complex interaction between G-CSF, E2/E3 and P4 was noted in that the estrogen molecules exhibited a pronounced pro-NETotic influence. This could in part be to the increased expression of PAD4, as this gene bears an estrogen response element, since 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 (Figure 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. Since aberrant NETosis can lead to damage of surrounding tissues, this complex hormonally regulated safeguard could assist in ensuring optimal safety of mother and unborn child.

7. 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 ($\approx 90\%$), these can still be associated with poor outcome, especially if occurring in tertiary healthcare 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]. It furthermore appeared that this activation was triggered by the excessive release of highly pro-inflammatory 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 report that PMN can extrude their genomic DNA into the extracellular environment in the form of NETs [6], and posed the query 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]. Since 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 on an imbalance of angiogenic factors, notably sFlt-1 (soluble fms-like tyrosine kinase 1) which has been noted in

PE [67]. By antagonizing the action of VEGF (vascular endothelial growth factor), 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 do indicate that aberrant NETs formation can contribute to placental dysfunction and potentially ensuing fetal loss. It is currently not known and matter of research if PMNs response could be causative of the pathological pregnancies or just consequence of the altered placental environment.

8. 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 like 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 9 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 *in vitro* culture [19]. The latter was determined to be due to the presence of increased concentrations of 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 pronounced influence on PMN priming, rendering 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, since 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 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 a 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]).

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

Since steroid sex hormones modulate PMN activity in pregnancy, the question arises 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 being that estrogens promote placental angiogenesis by enhancing VEGF gene expression and uterine artery vasodilation by increasing NO production, key events ensuring a healthy pregnancy outcome [23]. A further primary line of evidence was provided by the detection of reduced COMT (catechol-*O*-methyltransferase) enzyme activity in PE placentae, and that mice lacking this enzyme exhibit PE-like symptoms [23].

Since COMT plays an important role in estrogen metabolism, Jobe and colleagues performed detailed analysis using mass spectrometry of estrogen 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 estrogen (E1, E2 and E3) were reduced in the plasma of sPE cases when compared to sPE cases or matching normal healthy pregnancies. In contrast cases with sPE exhibited significantly higher levels of 16 keto-17 β 2-estradiol (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 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, yet 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. Since 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 life style 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]. Since PMN activity is altered in GDM and may play a role in the subsequent development of PE [76], it may worthwhile examining the relationship between BMI and E2/P4 levels in this context.

10. VIP – potent new kid on the block

Originally described as a neuropeptide, VIP (vasoactive intestinal peptide) has emerged as a very interesting regulatory factor at the fetomaternal interface [27,81]. In human pregnancy, VIP is expressed by the syncytiotrophoblast directly at the site of fetomaternal 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 PBMCs (peripheral blood mononuclear cells) it was shown that VIP triggered an increase in Fox3p regulatory T cells [82]. VIP also lead to an increase in the expression of immunosuppressive cytokines, such as IL-10, associated with a Th2 phenotype known to favour pregnancy.

Initial evidence that VIP induced an immunosuppressive state at the fetomaternal 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 NOD (non-obese diabetic) mice, prone to autoimmune complications including the development of IDDM (insulin dependent diabetes mellitus). These mice have poor pregnancy outcome, leading to fetal demise and resorption. This may be due to limited vascular remodelling, or from aberrant immune interactions at the fetomaternal 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 lead to the increased

expression of regulatory cytokines such as IL-10 and 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 pro-inflammatory cytokine. Treatment of pregnant NOD mice at day 6.5 of pregnancy resulted in improved pregnancy outcome, accompanied by an increase in IL-10, TGF β , and Foxp3. These data, hence, 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 1st 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 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.

11. Pregnancy, NETs and thrombosis

Pregnant women face an increased risk (4 to 5 fold) 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 hyper-coagulation existing during pregnancy, a mechanism used to protect women from blood loss during childbirth [84]. It is not clear what the underlying cause for

increased venous thrombotic embolism in PE is. However, the observation that NETs promote coagulation and thereby contribute to blood clot formation may shed new light onto 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 negated by genetic ablation of the PAD4 gene [87,88].

The mechanism whereby NETs promote coagulation is currently not clear at all, 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 an autocatalytic loop with potentially disastrous consequences. It furthermore 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].

12. 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 artefact [97].

As these procedures are fraught with difficulty, prone to operator error and not amenable to routine clinical practise, the need has been voiced for alternatives. Since 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, since the quantity of these can be readily determined using qRT-PCR, fluorescent DNA binding dyes or appropriate ELISA assays [98,99].

While this practise is widely used, it has to be used with a degree of caution, since it is not clear what the source of cfDNA in the sample being examined is. 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, since 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 apoptosis 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 practise 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 hemopoietic stem cells, it was shown that the vast proportion of this material was of hemopoietic origin, probably resulting from the enucleation of erythroblasts as they mature to anuclear erythrocytes [101].

Amongst 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 furthermore shown that cfDNA in the circulation has an extremely short half-life, being of the order of 15 minutes, 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.

13. 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 trans-epithelial 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 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].

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Figure Legend

Figure 1: Cyclic steroid sex hormones regulate the permeability of the FRT tract 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 decrease the membrane permeability decreasing the migration of neutrophils to the FRT. This can result in increased risk of infection.

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



