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DOTTORATO DI RICERCA IN SCIENZE BIOMEDICHE XV CICLO

Neutrophils alter placental glucose metabolism in gestational diabetes mellitus via neutrophil elastase mediated IRS1 degradation

Tesi di Dottorato di:

Franco Grimolizzi

Tutor:

Prof.ssa Franca Saccucci

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Symbols and Abbreviations

A1AT	Alpha-1 Antitrypsin
Akt	Protein kinase B
APC	Antigen-Presenting Cell
CEACAM	Carcinoembryonic Antigen Related Cell Adhesion Molecule
CitH3	Citrullinated Histone H3
CXCL	Chemokine (C-X-C motif) Ligand
DCs	Dendritic Cells
dN	Decidual Neutrophils
G-CSF	Granulocyte Colony-Stimulating Factor
G-CSFR	Granulocyte Colony-Stimulating Factor Receptor
GDM	Gestational Diabetes Mellitus
GLUT4	Glucose Transporter Type 4
h	Hours
HDNs	High-Density Neutrophils
HFD	High Fat Diet
HG	High Glucose
HLA-C	Major Histocompatibility Complex, Class I, C
hPGH	Human placental Growth Hormone
hPL	Human Placental Lactogen
IFN	Interferon
IFX	Infliximab
IGF-1	Insulin Like Growth Factor 1
IIIT	Third Trimester
IIT	Second Trimester
IL	Interleukin
IRS1	Insulin Receptor Substrate 1
IT	First Trimester
LDNs	Low-Density Neutrophils
m	Minutes
MHC	Major Histocompatibility Complex
MOs	Macrophages
MPO	Myeloperoxidase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NE	Neutrophil Elastase
NETs	Neutrophil Extracellular Traps
NF	Nuclear factor
NG	Normal Glucose
NK	Natural Killer
NLR	Neutrophil to Lymphocyte Ratio

NO	Nitric Oxide
NOX	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
OGTT	Oral Glucose Tolerance Test
PADI4	Protein Arginine Deiminases
PDGFR	Platelet-Derived Growth Factor Receptor
PDK1	3-phosphoinositide dependent protein kinase-1
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMNs	Polymorphonuclear Leukocytes
PPROM	Preterm Premature Rupture Of Membranes
RFU	Relative fluorescent units
ROM	Rupture of Membranes
ROS	Reactive oxygen species
RT	Room Temperature
s	Seconds
T2DM	Type 2 diabetes mellitus
Th	Helper T cells
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
Tregs	Regulatory T cells
uNK	Uterine Natural Killer Cells

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ABSTRACT

Human pregnancy is associated with a mild pro-inflammatory state characterized by activation of circulatory neutrophils (PMNs). Skewing of PMNs responses toward to neutrophil extracellular traps generation (NETs) is reflected in an increased of circulating nucleosomes and myeloperoxidase with advancing gestational age. Our data indicated that this pro-NETotic profile is enhanced in women with gestational diabetes mellitus (GDM). Maternal hyperglycemia and increased levels of TNF-α are a hallmark of GDM and we show a synergistic effect of both factors on the priming and release of NETs. Moreover, we hypothesized that systemic activation was associated with activated PMN in placenta. Indeed, we observed a massive infiltration of pro-NETotic PMNs and neutrophil elastase (NE) accumulation along chorionic villi of GDM placentas. To further explore whether hyperglycemia predisposes to exaggerated inflammatory response in placenta we incubated trophoblast BeWo cells in high glucose conditions and we next tested the TNF-α production capacity. Interestingly, TNF-α level was incresed and exert a pro-NETotic effect on PMN with consequent NE release. Recent studies in cancer tissues and diabetes models have described that released NE induce profound changes in the surrounding cells, altering the signal transducing cascade and promoting insulin resistance via degradation of insulin receptor substrate 1 (IRS1). Our in-vitro data indicate that addition of NE to trophoblast cell line BeWo causes degradation of IRS1 with consequent glucose uptake impairment. IRS1 is reduced in GDM placentas when compared to control placentas, suggesting that the presence of NE might be the causal factor. Taken together, our data showed that GDM is characterized by excessive NET formation and by a massive influx of pro-NETotic PMN into placentas. These findings underline the competence of NETs as a highly relevant diagnostic biomarker for GDM and NE as a new potential therapeutic target.

La gravidanza è da considerarsi una condizione pro-inflammatoria dove si osserva un'attivazione dei neutrofili circolanti. Con l'avanzare della gravidanza la concentrazione nel sangue di nucleosomi e mieloperossidasi aumenta e riflette la produzione delle trappole extracellulari dei neutrofili (NETs). Abbiamo dimostrato che in corso di diabete mellito gestazionale (GDM) tale produzione è aumentata in confronto ad una gravidanza fisiologica. Elevati livelli di glucosio e TNF-α, segni tipici presenti in corso di GDM, in vitro agiscono in modo sinergico e sono in grado di pre-attivare i neutrofili ed indurre il rilascio delle NETs. Abbiamo ipotizzato che, lo stato di iperattivazione osservato a livello sistemico possa essere associato ad una aumentata attività leucocitaria a livello placentare. A sostegno della nostra ipotesi, si osserva nei villi coriali isolati da placente GDM un'aumentata infiltrazione di PMNs pro-NETotici in associazione ad un accumulo della neutrofili elastasi (NE). Per valutare un possibile effetto pro-infiammatorio del glucosio a livello placentare, abbiamo incubato cellule di trofoblasto (BeWo) in presenza di un'elevata concentrazione di glucosio e successivamente valutato la produzione di TNF-α. È stato interessante rilevare un aumento nel rilascio di TNF-α tale da indurre un effetto pro-NETotico sui PMN con consequente rilascio della NE. Da recenti ricerche è emerso come in corso di diabete e tumore la NE possa essere internalizzata dalle cellule ed alterare la trasduzione del segnale insulinico attraverso la degradazione del substrato 1 del recettore insulinico (IRS1). Esperimenti in vitro hanno dimostrato che in presenza della NE si osserva una riduzione di IRS1 nelle cellule BeWo ed una diminuzione dell'internalizzazione del glucosio. Poichè l'espressione di IRS1 risulta ridotta nelle placente GDM é verosimile ipotizzare che la massiva presenza di NE ne può essere la causa. In conclusione, i nostri dati suggeriscono che in corso di GDM si verifica un'elevata produzione di NETs ed una massiva infiltrazione di pro-NETotici PMN nella placenta. Queste scoperte dimostrano come la NETosi abbia una significativa utilità diagnostica in corso di GDM e la NE un nuovo potenziale bersaglio terapeutico.

INTRODUCTION

Peeking into the novels aspect of neutrophil behavior

Neutrophil are the most abundant members of the leucocyte population, comprising between 40 and 60% of total white blood cells in normal healthy individuals. They play a crucial role in the immune defence against bacterial and fungal pathogens, and they also participate in the development of the inflammatory reaction (Nathan 2006). Neutrophils develop in the bone marrow from haematopoietic stem cells in a process called "granulopoiesis" and mature neutrophils are characterised by their segmented nucleus and granules that are filled with >700 proteins (Rørvig et al. 2013). Since their nucleus is organised into three to five lobules they are often called polymorphonuclear leukocytes (PMNs).

They were typically considered to be short-lived (few hours) but recently it has been shown that non-activated neutrophils have a longer lifespan (>5 days) (Pillay et al. 2010). Furthermore, neutrophils can not only migrate into tissues in response to inflammatory stimuli, but they are present in non-inflamed tissues where they have an important role in maintaining tissue homeostasis and shaping the immune responses (Ng et al. 2011).

Neutrophil biology has been studied extensively in recent years highlighting additional and unexpected functions of these cells. Neutrophils have been proposed to participate in protection against intracellular pathogens such as viruses and mycobacteria, but also as instructors of the immune system in the context of cancer and autoimmune disorders (Mócsai 2013; Amulic et al. 2012). Finally, neutrophils were found to be involved in physiological and pathological processes beyond the immune system, such as wounds healing, diabetes, atherosclerosis, and thrombus formation (Mócsai 2013; Amulic et al. 2012). The role of PMNs in reproduction is still a largely neglected topic, despite their involvement in various stages of the reproductive cycle and pregnancy-related disorders.

Heterogeneity in function and phenotype

PMNs are an essential component of the human innate immune system since they have a well-established role during fungal and extracellular bacterial infections where they promote bacterial clearance through phagocytosis, degranulation, exocytosis and the release of neutrophil extracellular traps (NETs) (Kruger et al. 2015; Kolaczkowska and Kubes 2013a). Neutrophils are one of the first blood cells to respond to infection and are recruited from the circulating blood into the tissue by chemoattractants. There are numerous host derived factors that trigger recruitment of neutrophils, among them one of the most potent is the interleukin (IL)-8 (Nathan 2006).

Neutrophils exist in one of three states: resting, primed or active (Hallett et al. 1995). In healthy condition, neutrophils exist in circulation in resting state, which ensures that their toxic intracellular contents are not accidentally released to damage host tissue. Resting neutrophils can become primed by agents that include bacterial products, cytokines and chemokines, e.g. TNF-α, G-CSF and IL-8 (Cowburn et al. 2008). This process leads to dramatic changes which enhance neutrophil function and lifespan (Wright et al. 2010). The process of priming alters the properties of the endocytic compartment in neutrophils enhancing dynamin-II-dependent and clathrin-mediated endocytosis (Lamb et al. 2012). Furthermore, the intracellular pools of CD11b and CD66b translocate from the intracellular granules to the plasma membrane to enhance cellular adhesion to integrin ligands (Wittmann et al. 2004). Additionally, phosphorylation of Ser-345 on p47phox is a necessary event in priming of neutrophils by TNF-α and G-CSF (Dang et al. 2006). The primed neutrophils are “ready to go” but awaiting further stimulus before activity is triggered (Hallett et al. 1995).

Upon activation, neutrophils produce ROS in a process called the respiratory burst and use their extensive arsenal to ensnare and kill microbes (Cowburn et al. 2008; Amulic et al. 2012). ROS are generated almost exclusively by an NADPH oxidase that belongs to the family of NOX proteins. The leukocyte specific NADPH oxidase is a multisubunit entity with membrane-bound and soluble components that assemble into a heteromeric complex when the cells are stimulated. ROS are clearly important for neutrophil antimicrobial activity: neutrophils from chronic granulomatous disease (CGD) patients kill microbes poorly, making these patients susceptible to many infections (Segal et al. 2000). By definition, CGD results from failure of activity of the NADPH oxidase caused by defects in the components of this system (Segal et al. 2000). In addition to direct antimicrobial action, ROS can modify host molecules. A well-studied example of ROS in signalling is the reversible regulation of various targets (including phosphatases, metalloproteinases, and caspases) by direct oxidation of cysteine residues (Paulsen and Carroll 2010).

Phagocytosis is the major mechanism used by PMNs to remove pathogens and cell debris. It is an active, receptor-mediated process during which a particle is internalized by the cell membrane into a vacuole called the phagosome. Because particles of widely different natures can be taken up by phagocytosis, it is not surprising that numerous receptor types can mediate this process. Foreign particles such as bacteria, fungi, and parasites display many molecules that are never found in higher organisms. These pathogen-associated molecular patterns (PAMPs) can be detected by several receptors, in particular the Toll-like receptors (TLRs) (Flannagan, Jaumouillé, and Grinstein 2012). Foreign bodies can also be recognized by soluble molecules circulating in the blood and in interstitial fluids. For example immunoglobulins recognize extraneous antigens, and components of the complement cascade deposit on foreign surfaces. Following their deposition on particles, these molecules, termed opsonins, are in turn engaged by specific receptors on the membrane of PMNs, including receptors Fc R-I, -II, and -III and fragments of the third component of complement (Flannagan, Jaumouillé, and Grinstein 2012).

Importantly, besides ROS and phagocytosis, neutrophils possess an array of granules that contain a large number of cytotoxic and immune regulatory molecules that can be released by degranulation or exocytosis. As a neutrophil proceeds through activation, granules are mobilized and fuse with either the plasma membrane or the phagosome, releasing their contents into the respective environment (Borregaard and Cowland 1997). The different classes of granules demonstrate varying propensities for mobilization in response to inflammatory signals (Sengeløv, Kjeldsen, and Borregaard 1993). Because of this varying mobilization propensity, each granule subset has been traditionally associated with a particular stage of neutrophil activation (Borregaard and Cowland 1997). The primary granules are the main storage site of the most toxic mediators, including elastase, myeloperoxidase, cathepsins, and defensins. (Borregaard and Cowland 1997).

Neutrophils are no longer seen as leukocytes with a sole function of being the essential first responders in the removal of pathogens at sites of infection but they can positively or negatively regulate the generation of adaptive immune responses (Jaillon et al. 2013; Leliefeld, Koenderman, and Pillay 2015). Neutrophils were shown to promote the maturation of human monocyte-derived DC through interaction between CD18 and CEACAM1 expressed by neutrophil and DC-SIGN receptor on DC (van Gisbergen et al. 2005). The interaction of neutrophils with DC can also result in NK cell activation increasing the release of IL-12p70 (Costantini et al. 2011). Recently a new population of neutrophils was described as residing around the marginal zone of the spleen called “B-cell helper neutrophils” (NBH) (Puga et al. 2012). These neutrophils colonize the marginal zone during foetal life and become more prominent after postnatal mucosal colonization by bacteria. NBH express higher levels of B cell-

stimulating molecules and B cell chemoattractant molecules such as CXCL12 and CXCL13 compared to circulating neutrophils (Puga et al. 2012). Neutrophils can also modulate T-cells by proteases released during degranulation. These proteases are able to cleave and inactivate essential cytokine receptor on T-cells, such as IL-2 and IL-6 receptor (PMID: 11272269). Further, neutrophils can inhibit T-cell by production of hydrogenperoxide (H_2O_2) through cell-cell contact (Narasaraju et al. 2011).

Interestingly, PMNs use a set of membrane and intracellular molecules to sense signals from their local environment and polarize their phenotype towards a pro-inflammatory (N1) or anti-inflammatory (N2) programs (Fridlender et al. 2009; Sagiv et al. 2015). It was also shown that subpopulations of circulating neutrophils in cancer can be distinguished according to their densities (Sagiv et al. 2015), one with "normal," HD characteristics (HDNs) having antitumor properties, previously described as N1 (Fridlender et al. 2009) , whereas the other (LDNs) being of lower density with features associated with protumor activity (N2). The cytokine involved in neutrophils polarization is TGF-β that can mediate a shift between the different neutrophils subtypes. HDNs can switch to become LDNs in the presence of increasing concentrations of TGF-β, both in vivo and ex vivo (Sagiv et al. 2015). In light of the immature state of a subset of LDNs and the mature state of all HDNs, the low- to high-density transition raises the possibility of neutrophil maturation in the circulation (Galli, Borregaard, and Wynn 2011).

Neutrophil plasticity and diversity in humans is a recent discovery that needs further investigation to define their role in different human pathologies.

Dynamics of Neutrophil Extracellular Traps (NETs) formation

Neutrophil extracellular traps (NETs) formation is a unique form of cell death (NETosis) that is characterized by the release of decondensed chromatin and granular contents to the extracellular space (Brinkmann et al. 2004b). The mechanism of NET formation is clearly distinct from apoptosis because the morphological characteristics of these two forms of active cell death are very different, there is no DNA fragmentation and phosphatidylserine is not exposed before cell death (Fuchs et al. 2007). NETs are produced by neutrophils in contact with pathogens and with a variety of host factors such as activated platelets or inflammatory stimuli (Fuchs et al. 2007). NETs are released particularly in response to large microbial structures that cannot be easily phagocytosed such as *Candida albicans* hyphae and *Mycobacterium bovis* aggregates (Branzk et al. 2014). Only recently, it was recognized that NETs are also generated during viral infection (Saitoh et al. 2012) .

The molecular mechanisms behind NETs formation are still poorly understood but it was shown that chromatin decondensation is an essential step (Y. Wang et al. 2009b). The exact sequence of events leading to NEtosis is not known, although it was confirmed that is dependent upon neutrophil elastase (NE), myeloperoxidase (MPO) and the enzymes peptidyl arginine deiminase 4 (PAD4). NE translocates first to the nucleus, where it digests nucleosomal histones and promotes extensive chromatin decondensation and the late binding of MPO to chromatin enhances the NE activity (Papayannopoulos et al. 2010b; Metzler et al. 2014). NE is essential for chromatin decondensation, indeed elastase KO mice are NETosis incapable (Papayannopoulos et al. 2010b). On the other hand, the role of MPO seems to depend on the nature of the stimulus (Parker et al. 2012b). MPO-deficient neutrophils had no effect on NETs induction by *P. aeruginosa*, *S. aureus* or *E. coli*, but fail to respond to PMA (Parker et al. 2012a).

Initially, it was shown that NETs is a process that is dependent on ROS production. In fact, humans with NADPH-oxidase deficiency could not make NETs (Bianchi et al. 2009). On the contrary, there is now growing evidence suggesting that microbial-specific molecular patterns recognized by Pattern Recognition Receptors (PRRs) induce NETs independently of NADPH oxidase 2 (Pilsczek et al. 2010a). Therefore, based on their requirement for NADPH oxidase (NOX2), in the recent literature, NETs are classified in two types: NOX-dependent and NOX-independent (Pilsczek et al. 2010a; Zhao, Fogg, and Kaplan 2015).

NOX-dependent NETosis occurs following stimulation by PMA, a strong protein kinase C (PKC) agonist. Raf-MEK-ERK pathway is involved through activation of NADPH oxidase and upregulation of antiapoptotic proteins (Hakkim et al. 2011a). To release

NETs, activated neutrophils undergo dramatic morphological changes. During the 1 hour of stimulation the nucleus loses its lobules, the chromatin decondenses, and the inner and outer nuclear membranes progressively detach from each other. After 1 h, the nuclear envelope disaggregates into vesicles and the nucleoplasm and cytoplasm form a homogenous mass. After 2 h, the cell membrane ruptures and the interior of the cell is ejected into the extracellular space, forming NETs (Brinkmann and Zychlinsky 2012).
Besides PMA, a synthetic activator of the PKC family of enzymes, there are several natural inducers lead to PKC activation, such as TNF-α, IL-1ß, IL-8, IL-1 or G-CSF (Newton and Dixit 2012).

NOX-independent NETosis has been reported following both direct microbial exposure and lipopolysaccharide (LPS). For gram-negative bacteria, NETs are induced via Toll-like receptor (TLR) 4 activation of platelets followed by direct neutrophil-platelet interaction via CD11a, whereas both complement receptor 3 and TLR2 are required for vital NETosis following gram-positive infection (Pilsczek et al. 2010b). The regulators of NOX-independent NETosis are largely unknown. Douda et al showed that calcium activated NOX-independent NETosis is fast and mediated by a calcium-activated small conductance potassium (SK) channel member SK3 and mitochondrial ROS (David Nobuhiro Douda et al. 2015). Although mitochondrial ROS is needed for NOX-independent NETosis, it is not mandatory for NOX-dependent NETosis (David Nobuhiro Douda et al. 2015).

Unlike NOX-dependent NETosis, NOX-independent NETosis is accompanied by a substantially lower level of activation of ERK and moderate level of activation of Akt, whereas the activation of p38 is similar in both pathways (David Nobuhiro Douda et al. 2015). ERK activation is essential for the NOX-dependent pathway, whereas its activation is not essential for the NOX-independent pathway. Despite the differential activation, both NOX-dependent and -independent NETosis require Akt activity (David Nobuhiro Douda et al. 2015). Indeed, suppression of Akt switches NETosis to apoptosis (David N Douda et al. 2014). During the NOX-independent NETosis, NETs are extruded during the first few minutes (<30 min) of the neutrophils activation process (Pilsczek et al. 2010a). Moreover, the extrusion of DNA from within the nucleus to the extracellular space involves vesicular trafficking without requiring membrane perforation (Pilsczek et al. 2010a; Zhao, Fogg, and Kaplan 2015). This mechanism spares the cells from self-lysis, thereby allowing the PMN to continue to function, even to the point of becoming anuclear (Pilsczek et al. 2010a). Because these opposing cellular strategies, one causing cell death and the other leaving the cell alive, NOX-dependent netosis is called suicidal and NOX-independent NETosis is called vital (Pilsczek et al. 2010a; Zhao, Fogg, and Kaplan 2015).

In addition, histone citrullination by PAD4 is a prominent posttranslational modification in vital NETosis. Inhibition of PAD4 by Cl-amidine, a PAD inhibitor, disables NET formation (Y. Wang et al. 2009a). Like other PAD isotypes, PAD4 is a Ca^{2+} dependent enzyme, but only PAD4 possesses a classical nuclear localization signal (NLS) (Mastronardi et al. 2006a). The requirement for PAD4 is not universal to all NETotic pathways, as evidenced by the fact that there is a lack of PAD4 activity and histone hypercitrullination in PMA-mediated NETosis (David Nobuhiro Douda et al. 2015)

The research performed by Obermayer et al. has significantly highlighted the structure and possible functions of NETs (Obermayer et al. 2014). NETs have a unique ultrastructure, whose main components are DNA fibres, of diameter 15–17 nm and spherical protein domains of diameter 25–50 nm. Histone proteins such as H1, H2A, H2B, H3, H4 constitute about 70% of all proteins connected with NETs (Obermayer et al. 2014). Because of post-translational modifications that affect histones after the NETosis process, histones which construct neutrophil extracellular traps have a larger cell molecular mass of 2–5 kDa compared with the histones present in the cell nucleus (Papayannopoulos et al. 2010b; Metzler et al. 2014) . Among non-histone proteins present in NETs, neutrophil elastase (NE) and MPO are the most common, with a content of over 5% (Urban et al. 2009b). However, apart from NE and MPO, NETs structure also contains other proteins originating in different cellular organelles of neutrophils (Urban et al. 2009b). Even at low concentrations, they appear to play an important role in host defense (Brinkmann and Zychlinsky 2007), among them we can find (Urban et al. 2009b):

- cathepsin G, defensins, BPI-bactericidal substance-increasing permeability – originating from the primary neutrophil granule;
- alcaic phosphatase, lactoferrins, lysozyme, cathelicidins, collagenase – deriving from the granularity of secondary neutrophils;
- gelatinase, (MMP-9) – matrix metalloproteinase 9 – deriving from the granularity of tertiary neutrophils;
- catalase – deriving from peroxisomes,
- cytokeratin-10, α-actinin 1 and 4, β-actin, γ-actin, myosin-9 – constituting the cytoskeleton of neutrophils;
- some cytoplasmic proteins, among others: proteinase 3, cathelicidin LL-37, tryptase, S100 (S100A8, S100A9, S100A12) group proteins.

Impact and implications of NETs

NETs provides important biological advantage for the host to fight against microbial infections (Branzk and Papayannopoulos 2013a). A variety of gram-negative as well as gram-positive bacteria have been shown to induce NETs formation. *S. flexneri* is trapped and killed by NETs, which contain NE that degrades virulence factors such as IcsA and IpaB (Brinkmann et al. 2004a). Moreover, NETs specifically degrade *S. aureus* virulence factors and thereby help to contain tissue damage (Brinkmann et al. 2004a). Interestingly, the gram-negative bacterium *K. pneumoniae* is not sufficient to induce NETosis in isolated neutrophils ex vivo, but is a good inducer of NETosis in a mouse lung infection model (Papayannopoulos et al. 2010a). These observations suggest that microbial virulence may be an important stimulus in NET induction, as it may induce additional inflammatory cytokines that may act as co-activators.

PMNs play a crucial role in containing fungal infections and NETs appear to be an important part of the neutrophil antifungal arsenal. Since hyphae are too large to be phagocytosed, extracellular killing by release of NETs is an ideal strategy to contain the hyphal form and a number of studies have demonstrated that NETs are sufficient to kill *C. albicans* yeast and hyphae (Urban et al. 2006). Calprotectin is a cytoplasmic protein that is released via NETosis and is found associated with *C. albicans* in NETs (Urban et al. 2009a). The importance of calprotectin in antifungal defense is crucial since calprotectin chelates Mn^{2+} and Zn^{2+} which are required for *C. albicans* growth (Urban et al. 2009a). NETs are also able to trap and neutralize the negatively charged HIV virions, significantly decreasing HIV infectivity. However, in order to suppress NETs formation, HIV engages CD209 on dendritic cells (DCs), leading to production of IL-10 by DCs, which suppresses NETs formation (Saitoh et al. 2012). The role of NETs has also been explored in parasites causing leishmaniasis. Entrapment in NETs leads to decreased viability of the parasites, although authors of different studies conclude that the main function of NETs in *Leishmania* infection is the immobilization of the parasite and containment of the infection (Gabriel et al. 2010) . NETs are also implicated in *Toxoplasma gondii* infection. NETs kill approximately 25 % of the entangled parasites, which indicates that the primary function of NETs may be to physically contain the infection (Abi Abdallah et al. 2012).

These studies suggest that NETs play an important role in the defense against wide range of pathogens (Branzk and Papayannopoulos 2013a). However, too much of a good thing can be a bad thing. NETosis dysregulation has been implicated in severe autoimmune and autoinflammatory disease. For example, systemic lupus erythematosus (SLE) patients exhibit elevated levels of anti-neutrophil cytoplasmic antibodies (ANCAs), antibodies against histones, DNA (ANAs) and ribonucleoproteins (RNP) that target host tissues [81]. NETs are a major source of extracellular chromatin,

neutrophil proteins and microbial co-stimulatory adjuvants, and are therefore suspected as an antigenic source in SLE (Branzk and Papayannopoulos 2013b).

Maintaining the right balance of NETs formation that accumulates in tissues is essential for preventing damage to the hosts. For example it has been observed that in Lewis lung carcinoma and Ewing sarcoma there are large necrotic area of dead neutrophils and NET-like structures (Berger-Achituv et al. 2013). Another example was recently published by Wong et al., they reported that large quantities of NETs were found in excisional skin wounds of diabetic mice and that DNase1, which dismantled NETs, accelerated wound healing (Wong et al. 2015b). The authors attributed the cause of the large NETs quantity to PAD4 and revealed a fourfold upregulation of PAD4 protein expression in the neutrophils from individuals with diabetes compared to healthy controls (Wong et al. 2015b). In a different pathology Gupta et al observed the presence of large numbers of NETs directly in the intervillous space of preeclamptic placentas (A. K. Gupta et al. 2005). Since excessive fibrin deposition and infarction are frequently observed in preeclamptic placentas, NETs are likely to be responsible for exacerbating the occlusion of blood flow through the intervillous space (A. K. Gupta et al. 2005). Neutrophils through the release of NETs may also contribute to the widespread systemic damage to the maternal endothelium observed in preeclampsia since endothelial cells are susceptible to cell death induced by NETs (A. K. Gupta et al. 2010).

Pregnancy and Myth of Fetal Allograft

The myth of “fetal allograft” has created the concept that pregnancy is associated with immune suppression (Ober 1998) and a substantial body of literature exists describing the mechanisms fetuses use to escape the maternal immune surveillance (Warning, McCracken, and Morris 2011). This concept, unfortunately, does not underline that pregnancy represents the most important period for the conservation of the species. It is fundamental for the immune system to strengthen all the means to protect the mother and the offspring from pathogens. Therefore, it is appropriate to refer to pregnancy as a unique immune condition that is “modulated”, but not “suppressed” (Mor et al. 2011).

Maternal mortality is surprisingly high and about 830 women die from pregnancy- or childbirth-related complications around the world every day (Kleppel et al. 2016). Moreover, the world’s number one killer of young children is not an infectious disease but rather is preterm birth. Each day, 3000 children under 5 years of age die from direct complications of being born before 37 completed weeks of pregnancy (Liu et al. 2015). Furthermore, other pregnancy complications can have severe short- and long-term health effects for both the mother and foetus. For example, women who had decreased haemoglobin levels during pregnancy were found to have two times the chance of eventual death by heart disease compared to their counterparts whose haemoglobin levels remained stable (M. Lee et al. 2011; Cirillo and Cohn 2015). And those who had preeclampsia were found to be almost 6 times more at risk of cardiovascular diseases and related death (M. Lee et al. 2011; Cirillo and Cohn 2015). In addition, gestational diabetes mellitus (GDM), besides all of the adverse effects that go along with pregnancy, affects health later in life for both mother and child increasing the risk of developing obesity, diabetes type 2 and metabolic diseases (Bellamy et al. 2009; Lauenborg et al. 2004).

Immunological phases during pregnancy

Although pregnancy is characterized by an enhanced inflammatory state, it is known that inflammation has to be fine modulated at both placenta and systemic level to promote fetal development and avoid pregnancy complications. A common mistake in the field of reproductive medicine is considering from the immunological point of view pregnancy as a single event. In reality, women go through at least three distinct immunological phases during gestational advancement (Mor et al. 2011). Noteworthy, immunological phases at the implantation site are not fully revealed at systemic level in term of circulating serum cytokines (Christian and Porter 2014).

The first trimester (week 1-week 12) of pregnancy is considered as pro-inflammatory at the implantation site. In fact, during implantation the blastocyst has to break through the epithelial lining of the uterus in order to implant. An inflammatory environment is required in order to secure the adequate repair of the uterine epithelium and the removal of cellular debris. A high level of uterine-specific natural killer (NK) cells (65-70%), antigen presenting cells (APC) such as macrophages (MOs) and dendritic cells (DCs) (10-20%), T helper (Th)-1 cells and pro-inflammatory cytokines like TNF-α, IFN-γ, IL-1, IL-1β, IL-6, IL-12 secreted by the endometrial cells characterize the site of implantation (Dekel et al. 2010).

The second trimester (week 13-week 28) is a period of rapid fetal growth and development and is considered an anti-inflammatory state. At the implantation site immune cells that produce IL-4 and IL-10 largely mediate the resolution of inflammation. IL-4 aids in the polarization of antigen-stimulated naïve Th cells into Th2 effector cells and the presence of TGF-b facilitate the polarization of inducible Tregs from naïve CD4+ T cells (Chatterjee et al. 2014). IL-10 primarily exerts its anti-inflammatory effect by inhibiting pro-inflammatory cytokines and blocking antigen presentation by reducing MHC class II expression (Chatterjee et al. 2014).

During the 3rd trimester (week 29-week 40), the fetus complete its development and the mother needs to deliver the baby. Parturition can only be achieved through renewed inflammation to promotes the contraction of the uterus and the expulsion of the baby (Romero et al. 2006).

Besides the immunological phases at the implantation site, maternal immune system undergoes systemically changes characterized by significant increase in serum pro-inflammatory mediators (Christian and Porter 2014). Few studies have examined longitudinal changes in circulating serum cytokines as pregnancy progresses. Although, women showed elevations in TNF-α from early to late pregnancy, both IL-8 and IL-1β showed a U-shaped curve in the serum, with decreases in mid or late

pregnancy compared to early pregnancy. This pattern of response suggest in early pregnancy an initial inflammatory response that is then down-regulated over time (Christian and Porter 2014). On the other hand, trophoblast and macrophage-like cells constitutively produce IL-8 during pregnancy, showing maximal production of IL-8 by second trimester and term placentas (Shimoya et al. 1992).

Future studies are needed to identify the functional effects of specific cytokines during each stage of gestation, both at the peripheral and local level, as these functions should provide insight into mechanisms underlying adverse perinatal health outcomes.

Contributions of PMNs to pregnancy

Immune cells residing in the reproductive tract are T cells, macrophages/dendritic cells, natural killer (NK) cells, mast cells and neutrophils (S. K. Lee et al. 2015). The leukocytes in the reproductive tract play different roles depending from the site they reside: they maintain immunity against vaginal pathogens in the lower tract and establish immune tolerance for sperm and an embryo/fetus in the upper tract. The role of neutrophils in reproduction is still a largely neglected topic, despite the evidence of their importance at various stages of the female reproductive cycle such as menstrual cycle, implantation and pregnancy (Hahn et al. 2012).

Neutrophils generally are barely detectable in normal human endometrium but the number rises up to 6–15% of the total cell number during the menstrual period (Salamonsen and Woolley 1999). In murine systems, the migration of PMNs into the vaginal vault appears to play a crucial role in the continued progression of the oestrous cycle, as their depletion leads to a blocking of the cycle in diestrus (Salamonsen and Woolley 1999). This neutropenic abrogation of the oestrous cycle was accompanied by reduction in the levels of the sex hormones oestradiol and progesterone, implying some form of feedback mediated by endometrial presence of infiltrating PMNs (Sasaki, Nagata, and Kobayashi 2009). During pharmacological miscarriage, Milne et al. (Milne et al. 2005) showed that neutrophil infiltrate into human decidua after administration of a progesterone antagonist (RU-486) and were responsible for the vascular breakdown and decidual shedding. In a recent study, Amsalem and colleagues revealed that PMNs were present in the human decidua during 2nd trimester and that PMNs clusters were frequently located close to spiral arteries (Amsalem et al. 2014). Co-culture experiments determined that decidual neutrophils (dN) were able to promote angiogenic sprouting by uterine microvascular endothelial cells (Amsalem et al. 2014).

In order to maintain an environment favorable to implantation, neutrophils are also recruited into the female reproductive tract following insemination where they play a major role in the removal of excess sperm (Strzemienski 1989; Alghamdi et al. 2009). Indeed, sperm can be antigenic to the recipient female and immunization against them can result in infertility (Alghamdi et al. 2009).
In pregnant women the number, activation state and migratory capacity of PMNs are increased compared to non-pregnant women (Hahn et al. 2012; G. P. Sacks et al. 1998). Interestingly, in non-pregnant women neutrophil apoptosis was significantly delayed and was further delayed in pregnancy with intrauterine growth restriction and preeclampsia (von Dadelszen et al. 1999). The activation of circulating neutrophils in preeclampsia was similar to the activation state of neutrophils found in sepsis (G. P. Sacks et al. 1998). Since preeclampsia is associated with endothelial cell activation and dysfunction (Noris, Perico, and Remuzzi 2005), the increased neutrophils activation may cause the damage of vascular endothelium. Endothelial damage in vitro by complement activated PMNs was originally described by Sacks et al. (T. Sacks et al. 1978). There is now abundant evidence that PMNs mediate endothelial cell/tissue damage in vivo by a wide variety of mechanisms, such as release of damaging oxidants and proteolytic enzymes (Gao, Neff, and Ward 2006).

Despite several studies have aimed to examine the role of circulating PMNs in pregnancy, little is known regarding their effects in human placenta. However, the presence of neutrophils in reproductive tissues at term and their ability to migrate to this region during labor have been well documented in both humans and rodents (Sharp et al. 2016; Giaglis, Stoikou, Grimolizzi, et al. 2016). Neutrophils migration into tissues includes the following steps: tethering, rolling, adhesion, crawling and transmigration. Tethering and rolling of neutrophils, which greatly facilitate subsequent arrest and recruitment, are mediated by selectins (Yago et al. 2010). Neutrophils adhesion and crawling is facilitated by increased expression of integrin at the cell surface such as CD11b and CD66b (Luo et al. 2015). Many molecules are implicated in transmigration (Bayat et al. 2010) and in order to transmigrate across the membranes, neutrophils release specific proteases such as MMPs and serine proteases. These enzymes are able to affect neutrophil migration by the degradation of elastin and collagen, thereby increasing the vascular permeability (Monaco et al. 2006). Interestingly, these proteins are under hormonal regulation during pregnancy (Nekrasova and Shirshev 2013) and participate in the process of labor (Helmig et al. 2002). In human decidual tissues, the number of neutrophils was higher in women with preterm labor associated with chorioamnionitis than in women with term gestations (with and without labor) and in women with spontaneous preterm labor without chorioamnionitis (Hamilton et al. 2012). These observations suggest that neutrophils contribute to the physiological rupture of the fetal membranes (ROM) and

pathological preterm premature rupture of membranes (PPROM) during term and preterm labor (Hamilton et al. 2012).
The precise mechanism leading to the neutrophils migration into the placenta is currently unclear. It may involve the migration of circulatory neutrophils into this placental tissue via chemokines attraction (e.g. IL8) (Christian and Porter 2014). Trophoblast and macrophage-like cells constitutively produce a certain amount of IL-8 during pregnancy. IL-8 increased with development aging of the placenta, showing maximal production of IL-8 by second trimester and term placentas (Shimoya et al. 1992).

The placenta: how to study a highly complex organ?

In 2015 the National Institutes of Health (NIH) has dedicated $41.5 million in research awards for an initiative to understand the development, structure and functions of the human placenta during pregnancy with the goal of improving the health of mothers and children. Many of the objectives of the *Human Placenta Project,* launched by the NIH will necessitate pre-clinical studies and testing in appropriately designed animal models that can be readily translated to the clinical setting.

The placenta is a complex and fascinating organ because during the course of a pregnancy, it acts as the lungs, gut, kidneys and liver of the fetus. There are two placenta components, a fetal and a maternal one that must interact successfully for a healthy pregnancy. The maternal component of the human placenta is the endometrium, which undergoes transition to form the decidua in early pregnancy. The two principal fetal sources are the trophectoderm that forms the wall of the blastocyst, and the underlying extraembryonic mesoderm. The trophectoderm differentiates into trophoblast, which in turn forms the epithelial covering of the placenta and the subpopulation of invasive extravillous trophoblast cells (Graham J Burton and Fowden 2015). Extravillous cells migrate from the tips of the placental anchoring villi into the wall of the uterus as far as the inner third of the myometrium. Uterine natural killer (uNK) cells play a key role in this process that depends on the combination of HLA-C ligands on the trophoblast cells and killer immunoglobulin-like receptors expressed by the uNK cells (Moffett, Hiby, and Sharkey 2015). Far from killing trophoblast cells, the uNK cells encourage migration of the extravillous trophoblast cells into the endometrium through the release of cytokines and chemokines (Moffett, Hiby, and Sharkey 2015). This is one remarkable example of the importance of trophoblast-immune interactions for a proper placentation.

The extraembryonic mesoderm forms the stromal core of the placenta, from which originate the fibroblasts vascular network and resident macrophage population

(Hofbauer cells) (Graham J Burton and Fowden 2015). The placenta has two surfaces, the chorionic plate that faces the fetus where the umbilical cord is attached, and the basal plate that comes into the maternal endometrium. Between these plates is the intervillous space that, combined with the villi, is the functional unit of the human placenta: here maternal fetal metabolic exchange occurs. The epithelial covering of the villous tree is the syncytiotrophoblast, a multinucleated syncytium that is involved in many of the functions of the placenta, such as the synthesis and secretion of large quantities of steroid and peptide hormones (G J Burton and Tham 1992).

In the light of recent debate at the 11th Congress of the European Society for Reproductive Immunology (Budapest, Hungary) it was agreed that no other mammal has a placenta identical to that of the human (Chaouat and Clark 2015). Although, there is an ongoing discussion regarding what represents the best animal model for placenta and pregnancy research, often the discussion is based on availability, housing conditions or financial considerations and not necessarily scientific merit (Chaouat and Clark 2015; Schmidt et al. 2015). Therefore, more representative models are required, such as primary trophoblast, villous explants and cell lines (Orendi et al. 2011).

Primary trophoblasts are routinely isolated in many laboratories and additionally stored to use cells from a single placenta for different projects over time. A major disadvantage of isolated primary trophoblasts is that they do no longer proliferate in culture. Hence, only short term cultures can be performed. At the same time these cells spontaneously syncytialize in culture and thus may be of use to study the release of syncytial fragments and factors in vitro (Tannetta et al. 2008). Villous explants are mostly used to dissect cellular processes such as proliferation, differentiation, apoptosis and syncytial fusion (Ahmed et al. 2016). One of the obvious drawbacks in studies using villous explants is the deficient availability of fresh material for explant cultures. Different to isolated primary trophoblasts, villous explants are not regularly stored. Hence, placental tissues can only be used directly after delivery, and explant cultures using tissues from a single placenta cannot be repeated at a later date. Several cell lines are available for in vitro analysis of placental cell function. The choriocarcinoma cell line BeWo is the most extensively used model since it reveals most of the characteristics of villous trophoblast, including syncytial fusion and secretion of hormones (Omata et al. 2013; Wolfe 2006). The cells were isolated at autopsy from a cerebral metastasis of a choriocarcinoma (Hertz 1959b). Then, the cells were transplanted to the cheek pouch of a hamster and maintained in hamsters through 304 serial transfers over a period of 8 years (Hertz 1959b). Only then Pattillo & Gey (Pattillo and Gey 1968b) removed cells from tumors in the cheek pouch of hamsters, cocultured them with decidual tissues and finally established the BeWo cell line.

In conclusion, many aspects of human placenta can only be understood on the basis of experiments on human cell lines and original placental tissues in combination with human subject studies (Chaouat and Clark 2015; Schmidt et al. 2015).

Immunomodulation of PMNs by placenta

Many factors are released from the placenta that can influence the PMNs behavior. The human placenta constitutively produces IL-8 during pregnancy and enhances its production in chorioamnionitis (Shimoya et al. 1999). Since IL-8 is chemotactic and activating factor for PMNs, placental IL-8 may act as a mediator to recruit and accumulate neutrophils into the feto–maternal area.

G-CSF, known to enhances key functions of PMNs such as superoxide production, phagocytosis and bacteriocidal killing (Cella et al. 2006), is also produced by the placenta (Umesaki et al. 1995). Interestingly, this cytokine has recently been implicated in a murine tumour model where it predispose neutrophil to the generation of NETs (Demers et al. 2012).

Immunomodulatory signals are also released as membrane-associated factors in the form of extracellular vesicles (EVs) (Sarker et al. 2014). Placental EVs are highly stable and contain a wide array of proteins, microribonucleic acids and retroviral proteins with stimulatory or inhibitory activities on immune cells (Tong and Chamley 2015). They are released in large quantities from the syncytiotrophoblast layer and include microparticles (0.2–1 μm) and exosomes (40–150 nm) (Tong and Chamley 2015). In general, microparticles may activate immune effector mechanisms, while exosomes lead towards an anti-inflammatory state. It is speculated that the physiological range of the microparticles/exosomes ratio is disturbed in various pregnancy complications (Mincheva-Nilsson and Baranov 2014). Gupta et al. showed that placentally-derived microparticle efficiently activated neutrophils and triggered NETs formation (A. K. Gupta et al. 2005).

Gestational Diabetes Mellitus: The Dark Side of Glucose

The term Gestational Diabetes Mellitus (GDM) was first used by O'Sullivan in 1961 to describe any degree of glucose intolerance with onset or first recognition during pregnancy (O'Sullivan 1961). The background rate in pregnancy varies between 2 and 14% with incidences reported to be as high as 40% in obese populations (Chu et al. 2007; Kampmann et al. 2015a). GDM is the lacks specific symptoms, therefore, the pregnant woman is usually unaware of her condition until it is diagnosed at routine prenatal screening. Despite being symptom free, serious pregnancy complications are associated with hyperglycemia in pregnancy (Kampmann et al. 2015a). The seriousness of GDM and the dramatically increasing incidence of this condition make it one of the most urgent health challenges of this century.

Screening, diagnosis and treatment: where do we stand?

Screening for GDM is usually done between 24 and 28 weeks of gestation since insulin resistance increases during the second trimester and glucose levels rise in women who do not have the ability to produce enough insulin. Interestingly, the timing of the diagnosis of GDM is significantly associated with preterm delivery. Women who received a diagnosis of GDM before 24 weeks' gestation were 10 times more likely to deliver before 37 weeks' gestation than women who received a GDM diagnosis after 24 weeks' gestation (Catalano and Sacks 2011). Precise level of glucose intolerance characterizing GDM has been controversial over three decades. However during the non-fasting Oral Glucose Tolerance Test (OGTT) the following ranges are recommended: fasting plasma glucose ≥ 5.1 mmol/L (≥ 92 mg/dL), 1 h ≥ 10.0 mmol/L (180 mg/dL), or 2 h ≥ 8.5 mmol/L (153 mg/dL) (International Association of Diabetes and Pregnancy Study Groups Consensus Panel et al. 2010). It has been estimated that with these new diagnostic criteria the prevalence of GDM will increase to nearly 18% (Webber, Charlton, and Johns 2015). From an economic perspective, GDM is associated with an excess of €1549.56 per patient in costs of care during pregnancy and €411.31 per patient in annual healthcare costs post pregnancy (Danyliv et al. 2015).

The main goal of GDM treatment is to prevent macrosomia and pregnancy complications. As a primary treatment of GDM an optimal diet combined to physical activity is generally recommended, aiming to maintain sufficient energy intake and ensure glycaemic control of the mother. This approach has been found to be sufficient in 70–85% of the GDM cases (American Diabetes Association 2016). If these measures are insufficient in terms of achieving optimal glycemic control subcutaneous insulin therapy is the therapy of choice (Vääräsmäki 2016). It is effective for the mother and

safe for the fetus, as it does not cross the placenta. However, insulin is relatively expensive and difficult to administer. It requires education to ensure a safe administration and it is associated with an increased risk of hypoglycemia and weight gain (Kelley, Carroll, and Meyer 2015). Therefore new oral glucose-lowering agents have been studied in the treatment of GDM, such as metformin and glibenclamide (Balsells et al. 2015). The benefits of these treatments during pregnancy include no risk of hypoglycemia or excessive maternal weight gain, are low cost and easy to use. However, both glibenclamide and metformin cross the placenta and their long-term effects on the child have yet to be considered.

Short and long-term consequences of GDM

During GDM, the placenta undergoes a variety of structural and functional changes (Gernot Desoye and Hauguel-de Mouzon 2007). One of the characteristic features of a placenta from GDM is its increased weight, which is accompanied by edematous stroma, increase in the number of syncytial knots and perivillous fibrin deposition (Meng et al. 2015). Further, in GDM, the overexpression of placenta TNF-α is associated with increased fetal adiposity (Radaelli et al. 2003). Noteworthy, in diet-treated GDM patients, the amount of trophoblast insulin receptors is lower than in nondiabetic pregnancies, whereas in insulin-treated GDM, the placenta contains more insulin receptors (Al-Attas 1995).

GDM implies several risks on both mother and baby; for example increases the risk of cesarean and operative vaginal delivery, macrosomia, neonatal hypoglycemia and hyperbilirubinemia (Kampmann et al. 2015b). Moreover, women with GDM are at higher risk of hypertensive disorders including gestational hypertension, preeclampsia, and eclampsia (Kampmann et al. 2015b). GDM is also associated to long term complications for mothers: for example up to 10-fold increased risk for development of T2DM, even though most women return to a euglycaemic state shortly after delivery (Bellamy et al. 2009; Lauenborg et al. 2004). The specific biological link between GDM and T2DM remains unclear. In addition, studies provide evidence that several of the known T2DM risk associated genes are more frequent in women with previous GDM, and many of the risk factors are the same, such as a raised body-mass index, high age, family history of diabetes and Asian and black ethnicity (Chu et al. 2007; Kampmann et al. 2015a). It thus appears plausible that the pathogenesis is overlapping, and GDM may serve to identify women at high risk of future T2DM. Offspring of women with a history of GDM are also at increased long-term risk of developing metabolic diseases such as obesity, metabolic syndrome and up to 6-fold increased risk of T2DM (Bellamy et al. 2009; Lauenborg et al. 2004).

Interestingly, already 36 years ago, Freinkel (Freinkel 1980) proposed the concept of “fuel-mediated teratogenesis”, postulating that increased nutrients have both

immediate and long-lasting adverse consequences for the offspring. One plausible mechanism underlying fetal programing by GDM is that nutrient and hormonal levels in utero affect epigenetic modifications (El Hajj et al. 2014). Epigenetic, i.e. in form of DNA methylation and histone modifications, can cause changes in gene expression, which are transmitted to daughter cells during somatic cell division. It is well known that the epigenome is highly plastic during early development and susceptible to internal and external environmental factors (El Hajj et al. 2014). Epigenetic changes in GDM placentas were reported in several genes involved in metabolic pathways, such as leptin, adiponectin and ATP-binding cassette transporter A1 (Bouchard et al. 2010; Bouchard et al. 2012; Houde et al. 2013).

Noteworthy, although breast feeding is usually recommended, early neonatal ingestion of milk from diabetic mothers appears to increase the risk for obesity and impaired glucose tolerance in the offspring (Plagemann et al. 2002), whereas breast feeding after the first week of life does not affect childhood risk of obesity and diabetes (Plagemann et al. 2002). These observations suggested that the early neonatal period is a particularly critical window for therapeutical interventions to break the “unhealthy” epigenetic programming in utero.

Post-receptor defect in Insulin Receptor Substrates

Insulin is a peptide hormone, secreted by the β cells of the pancreatic islets of Langerhans, responsible to maintain normal blood glucose levels by facilitating cellular glucose uptake, regulating carbohydrate, lipid and protein metabolism and promoting cell division and growth through its mitogenic effects. Interestingly, the increased maternal estrogen and progesterone in early pregnancy promote pancreatic β cells hyperplasia causing an increased insulin release (Rieck and Kaestner 2010).
The first step by which insulin increases energy storage or utilization involves the regulated transport of glucose into the cell that depends on GLUT4 translocation to the plasma membrane (Leto and Saltiel 2012). The stimulation of glucose uptake by insulin is mediated by activation of insulin receptor (IR), which results in receptor auto-phosphorylation on cytoplasmic tyrosine residues and the tyrosine phosphorylation of IR substrates (IRS). This allows association of IRS with the regulatory subunit of phosphoinositide 3-kinase (PI3K), activating protein kinase 1 (PDK1) which in turn phosphorylates Akt, resulting in translocation of GLUT4 by promoting exocytosis of storage vesicles (Leto and Saltiel 2012). There are two main IRS proteins in humans (IRS1 and IRS2) and both are widely expressed. IRS1 is phosphorylated by both the insulin receptor and insulin-like growth factor 1 (IGF-1) receptor. The latter is proposed to be the major IRS phosphorylation in skeletal muscle. IRS2, proposed to be the main IRS in liver, is phosphorylated by the insulin receptor and mediates peripheral actions of insulin and growth of pancreatic β cells (Kido, Nakae, and Accili 2001).
Physiologically, insulin sensitivity is influenced by the interplay of several hormones. A decrease in insulin sensitivity or increased insulin resistance is normally seen during the second and third trimester of pregnancy to spare the glucose for the fetus. This is attributed to the effects of placental hormones, particularly human placental lactogen (hPL) and human placental growth hormone (hPGH) that increases dramatically during gestation (Barbour et al. 2007; Bandyopadhyay et al. 2005). The molecular mechanisms that decrease insulin sensitive in response to elevated hPL or hPGH are not clear but it was suggested that these hormones inhibit PI3-kinase activity while increasing the expression of the p85 subunit of PI3-K resulting in a dominant-negative effect to form a PI3-K heterodimer with the p110 subunit (Bandyopadhyay et al. 2005).

Pregnancy with GDM present β-cell dysfunction (Kautzky-Willer et al. 1997) and extra post-receptor defects in insulin signaling due to decreased IRS1 expression (Friedman et al. 1999; Catalano et al. 2002). In skeletal muscle and adipose tissue of GDM affected women a reduction in IRS1 protein expression was measured (Friedman et al. 1999; Catalano et al. 2002). The decreased insulin sensitivity result in the decreased ability of insulin to mobilize GLUT4 from the interior of the cell to the cell surface

(Catalano 2010). Furthermore, it has recently been shown that a reduction in IRS-1 protein expression is also present in the placenta of women with pregnancies complicated by GDM (Colomiere et al. 2009).

Neutrophil elastase: a new player in the immuno-metabolism field

Immuno-metabolism is an emerging field of investigation at the interface between the historically distinct disciplines of immunology and metabolism. It is now becoming clear that immune cells have direct roles in regulating the cellular metabolism and metabolites can impact directly on immune cell function (Loftus and Finlay 2016).

There is increasing evidence that neutrophil elastase (NE) is a key modulator of chronic metabolic inflammation, insulin sensitivity and glucose metabolism in obesity (Talukdar et al. 2012; Houghton et al. 2010). NE is one of the major protease stored in primary granules of neutrophils, the total amount in a single cell has been estimated at up to 3 pg (Liou and Campbell 1995). High-level transcription of its gene (Takahashi et al. 1988) is limited to the promyelocytic stage of granulocyte development when NE is produced and stored in cytoplasmic azurophilic granules. NE is a powerful enzyme playing a crucial role in killing and degrading the engulfed microorganisms present in the phagolysosome. It has also been implicated in the release of pro-inflammatory cytokines such as IL-6 and IL-8 (Bédard et al. 1993) and in the activation of the pro-form of tumour-necrosis factor (pro-TNF) and interleukin-1beta (pro-IL-1beta) (Wiedow and Meyer-Hoffert 2005). However, in addition to its antimicrobial activity, NE has also been implicated in sterile inflammation (Pham 2006).

Similar to microbial induced inflammation, sterile inflammation is marked by the recruitment of neutrophils and the release of NE. There are several examples of sterile inflammatory diseases (G. Y. Chen and Nuñez 2010) such as obesity-related insulin resistance and type 2 diabetes (Olefsky and Glass 2010; Gregor and Hotamisligil 2011). A number of experimental and clinical data have clearly established that adipose tissue, liver, muscle and pancreas are sites of chronic low grade tissue inflammation in presence of obesity and T2DM (Olefsky and Glass 2010; Gregor and Hotamisligil 2011). Neutrophils are the first immune cells to respond to inflammation, and can promote a chronic inflammatory state by helping to recruit macrophages and other immune cells (Kolaczkowska and Kubes 2013b). Talukdar et al showed that feeding a high fat diet to mice causes an increase in neutrophil recruitment into adipose tissue and liver characterized by an accumulation of NE (Talukdar et al. 2012). Treatment of hepatocytes and 3T3–L1 adipocytes with NE causes cellular insulin resistance and deletion of NE in obese mice leads to decreased tissue inflammation associated with reduced adipose tissue neutrophil and macrophage accumulation (Talukdar et al.

2012). Talukdar et al. showed that NE can gain access to the intracellular space and directly causes insulin resistance through degradation of IRS1 (Talukdar et al. 2012; Houghton et al. 2010) that results in non association of the PI3K complex leading to free PI3K in cytoplasm. Interestingly, in vitro and in vivo tumour models showed that the free form of PI3K can associate with platelet-derived growth factor receptor (PDGFR) skewing the PI3K axis toward cell proliferation (Houghton et al. 2010).

Under physiological conditions, NE activity is tightly regulated by endogenous protease inhibitors, such as A1AT (Mansuy-Aubert et al. 2013) able to block the catalytic function of NE through covalent binding. Notably, NE activity was significantly increased in HFD-induced obese mice, consistent with the reciprocal reductions in the level of the NE inhibitor A1AT (Mansuy-Aubert et al. 2013).

Link between hyperglycemia and TNF-mediated inflammatory response

Hyperglycemia, as one of the key abnormalities in GDM, plays an important role in the development of inflammation in diabetes complication. In both clinical and experimental conditions, this metabolic state is associated with the generation of pro-inflammatory cytokines, such as IL-1ß, IL-6 and TNF-α (Shanmugam et al. 2003; J. Wang et al. 2012; Gonzalez et al. 2012). Although, high glucose has been shown to trigger p38 mitogen-activated protein kinase, protein kinase C (PKC) and nuclear factor-κB (NF-κB) activity (Yang et al. 2008; Ceolotto et al. 1999), the central mechanism underlying the inflammatory effect of glucose is through generation of ROS (Yu, Jhun, and Yoon 2011). Mitochondria are the major source of ROS during hyperglycemia, as high concentration of glucose increases metabolic input into mitochondria, overwhelming the electron transport chain, resulting in mitochondrial hyperpolarization and ROS overproduction (Yu, Jhun, and Yoon 2011).

A number of studies have demonstrated that pro-inflammatory cytokines deleteriously influence insulin sensitivity and β-cell function (Ruotsalainen et al. 2006). One of the most important cytokines during inflammation is TNF-α. It is produced as a membrane-bound pro-form, which needs to be cleaved proteolytically to be released in its major biological active form (Kim, Kwon, and Jue 1993). TNF-α exhibits a pro-apoptotic action on pancreatic β cells (Parkash, Chaudhry, and Rhoten 2005) and it blocks insulin action by inducing serine phosphorylation of IRS1 (Hotamisligil 2003). TNF-α can also affect insulin signaling independent of IRS1. Indeed, treatment of cultured 3T3-L1 adipocytes with TNF-α leads to reduced expression of the insulin receptor, IRS1 and Glut4 mRNA, as well as a decrease in insulin stimulated glucose uptake (Stephens, Lee, and Pilch 1997). The association between GDM and TNF-α concentrations was provided by a meta-analysis which revealed markedly elevated TNF-α in serum of GDM vs. normal pregnancies (Xu et al. 2014). From early developmental stages onward, the secretory activity of placenta cells clearly contributes to increased local and systemic levels of cytokines (Hauguel-de Mouzon and Guerre-Millo 2006; Ma et al. 2006). The Hofbauer cells, syncytiotrophoblast and the cytotrophoblast cells of the placenta produce nearly all known cytokines, including TNF-α (H. L. Chen et al. 1991). Most of the TNF-α produced by the placenta is delivered to maternal circulation and by comparison only a small amount is produced by the fetal compartment (Kirwan et al. 2002; H. L. Chen et al. 1991). There is an overproduction of placental TNF-α during GDM associated with increased fetal adiposity (Radaelli et al. 2003). Furthermore, in vitro experiment placentas from women with GDM released more TNF-α in response to a glucose stimulus than placentas from women with normal glucose tolerance (Coughlan et al. 2001a). Cowburn et al showed that in human neutrophils, TNF-α causes a robust activation of NF-κB promoting neutrophils survival and IL-8 release (Cowburn et al. 2004).

Besides the generation of pro-inflammatory cytokines, hyperglycaemia can directly affect the inflammatory response. Interestingly, prevention of neutrophil death by high glucose concentration was also reported (Healy, Watson, and Newsholme 2002). The protective effect of glucose may be due in part to maintenance of the intracellular ATP concentration as a result of stabilization of mitochondrial function (Healy, Watson, and Newsholme 2002). Prolonged survival of viable active neutrophils can cause considerable damage to host tissues increasing inflammation. Moreover, calcium flux is necessary for efficient NET formation as it promotes production of ROS and PAD4-mediated chromatin citrullination (Y. Wang et al. 2009b) and Alexiewicz et al. described a direct correlation between intracellular calcium levels and fasting serum glucose levels (Alexiewicz et al. 1995).

METHODS

Material

Reagents

Name	Manufacter	Product
[3H] 2-Deoxy-D-glucose	Perkin Elmer	NET328A250UC
16% Formaldehyde Solution (w/v)	Thermo Fisher Scientific	28908
2-Mercaptoethanol	Bio-Rad	1610710
Bovine Serum Albumin	Sigma–Aldrich	A1933
Camptothecin	Sigma–Aldrich	C9911
Cytoseal XYL	Thermo Fisher Scientific	8312-16E
DAPI	Sigma–Aldrich	D9542
Dextran solution from Leuconostoc mesenteroides	Sigma–Aldrich	D8802
DMSO (Dimethyl sulfoxide)	Sigma–Aldrich	472301
EDTA (Ethylenediaminetetraacetic Acid)	Sigma–Aldrich	E9884
Eosin Y	Sigma–Aldrich	230251
Fetal Bovine Serum	Thermo Fisher Scientific	10082147
Ficoll-Paque PLUS	GE Healthcare	17-1440-02
Glucose	Sigma–Aldrich	G8270
Ham's F-12K (Kaighn's) Medium	Thermo Fisher Scientific	21127022
HBSS (Hank's Balanced Salt Solution)	Thermo Fisher Scientific	14025092
Hematoxylin Solution, Mayer’s	Sigma–Aldrich	MHS16
Hydrogen peroxide solution	Sigma–Aldrich	H1009
L-Glutamine	Thermo Fisher Scientific	25030081
Laemmli Sample Buffer	Bio-Rad	1610747
Liquid scintillation cocktail	Perkin Elmer	6013141
Mini-PROTEAN® TGX™ Precast Gels	Bio-Rad	4561021
Mowiol® 4-88	Sigma–Aldrich	81381
MTT (Thiazolyl Blue Tetrazolium Bromide)	Sigma–Aldrich	M5655
MeOSuc-AAPV-AMC	Sigma–Aldrich	M4765
Nonfat dry milk	Bio-Rad	1706404
PBS (Dulbecco’s Phosphate Buffered Saline)	Sigma–Aldrich	D8537
Penicillin-Streptomycin	Thermo Fisher Scientific	15140122
PhosSTOP	Sigma–Aldrich	4906845001
PMA (Phorbol 12-myristate 13-acetate)	Sigma–Aldrich	P8139
RBC lysis buffer	BioLegend	420301
RIPA Lysis and Extraction Buffer	Thermo Fisher Scientific	89900
RPMI 1640 Medium	Thermo Fisher Scientific	32404014
SYTOX® Green Nucleic Acid Stain	Thermo Fisher Scientific	S7020
Tissue-Tek O.C.T. Compound	VWR	4583

Trypan Blue Solution, 0.4%	Thermo Fisher Scientific	15250061
Trypsin-EDTA (0.25%), phenol red	Thermo Fisher Scientific	25200056

Recombinant Proteins

Name	Manufacter	Product
G-CSF	eBioscience	BMS322
Insulin	Novo Nordisk	A10AB01
Neutrophil Elastase	Abcam	ab91099
TNF-α	PeproTech	300-01A

Antibodies

Name	Manufacter	Product
Alexa Fluor 488 anti-rabbit	Thermo Fisher Scientific	A-11034
Alexa Fluor 594 anti-Mouse	Thermo Fisher Scientific	A-21201
Alexa Fluor 647 anti-mouse	Thermo Fisher Scientific	A-21237
Citrullinated Histone H3	Abcam	ab5103
Elastase	Hycult Biotech	HM2174
GLUT-4	Millipore	07-1404
Goat anti-mouse HRP antibody	Santa Cruz Biotechnology	sc-2005
Goat anti-rabbit HRP antibody	Santa Cruz Biotechnology	sc-2030
Infliximab	Remicade	DB00065
IRS1	Santa Cruz Biotechnology	sc-559
β-actin	Sigma–Aldrich	A5441

Primers

Name	Manufacter	Product
ELANE	Thermo Fisher Scientific	HS00236952_m1
RPLP0	Thermo Fisher Scientific	HS99999902_m1
TNF-α	Thermo Fisher Scientific	HS01113624_g1

Kits

Name	Manufacter	Product
ELISA Human Cell Death Detection	Roche Diagnostics	11774425001
ELISA MPO	Hycult	HK324-02
ELISA TNF-alpha	R&D Systems	DTA00C
Pierce™ BCA Protein Assay	Thermo Fisher Scientific	23225
RNeasy Mini	QIAGEN	74106
SuperSignal™ West Pico Chemiluminescent Substrate	Thermo Fisher Scientific	34080
TaqMan RNA-to-C_T 1-Step	Thermo Fisher Scientific	4392653
TiterTACS™ Colorimetric Apoptosis Detection	Trevigen	4822-96-K

Consumables

Name	Manufacter
Blood collection tubes (K2EDTA plasma; SST™ serum)	BD Vacutainer
Cell Scrapers	Corning
Cryo tubes (2 ml)	Sarstedt
Eppendorf tubes (2 ml, 1.5 ml)	Sarstedt
Falcon tubes (15 ml, 50 ml)	BD Falcon
Glass Coverslips BioCoat (12mm; Round)	Corning
Nitrocellulose membrane 0.45 µm	GE Healthcare
Pipette tips	Sarstedt
Polystyrene Cell Culture Plates (96–, 24, 12, 6 wells; flat bottom)	Corning
Polystyrene Plates (96 well; Black or White; flat bottom)	Corning
Sterile Pipettes (5, 10, 25 ml)	Corning
SuperFrost Plus microscope slides	Thermo Fisher Scientific

Subject recruitment

Pregnant women were recruited at the time of their routine examination at the end of the first, second trimesters and at the time of elective caesarean section towards the end of the third trimester. Patient groups were composed of women with gestational diabetes mellitus (GDM) diagnosed according to the new recommendations of European Association for the Study of Diabetes and World Health Organization (WHO) for the 2-h 75-g OGTT as at least two values greater than a fasting glucose of 5.1 mmol/l, a 1-h glucose of 10 mmol/l, or a 2-h glucose of 8.5 mmol/l. For the purpose of the study, controls were selected to match GDM cases with respect to maternal age, gestational age, and BMI at the time of the first prenatal visit. Healthy non-pregnant controls matched for age, were recruited at the Blood Bank of the Swiss Red Cross, Basel. Inclusion criteria for non-pregnant controls were fair general condition, female sex, age ≥ 25 and ≤ 45 years and for blood donors fulfilling national criteria for blood donation. Exclusion criteria were current or previous systemic autoimmune disease, asthma, convalescence after major illness, surgery, current medication with corticosteroids, immunosuppressive agents and malignant neoplasia or chemotherapy within 5 years before recruitment for the study. Exclusion criteria for pregnant subjects included any major complication of pregnancy or coincident disease, such as preeclampsia, pre- or post-term labor (<37 weeks or >42 weeks), intra-uterine growth retardation and viral, bacterial or parasitic infections. There was no ethnic difference between the groups. Informed, written consent was obtained from all participants prior inclusion in the study, which was approved by the Ethical Review Board of Basel/Basel-Land, Switzerland. The number and the main characteristics of participants are summarized in Table 1.

Venous whole blood samples were collected into EDTA tubes and 25 µl of blood was analyzed by a Hemavet 950FS (Drew Scientific) for complete blood cell counts. For serum collection, tubes were inverted five times, allowed 30 minutes clotting time, and centrifuged for 10 minutes at 1000-1300 RCF (g) in a swing bucket centrifuge. Serum aliquots (0.5 ml) were immediately stored at -80°C until analyzed. Human placentas were obtained from pregnant women who, delivered healthy, singleton infants at term (< 37 weeks' of gestation) undergoing elective caesarean section. 5 placenta samples for each group were collected and dissected from the middle cross-section of villus tree within 15 min of the delivery. To compensate for intra-placental variability, we collected 3 independent samples per placenta giving totals of 15 GDM and 15 control samples. Tissue was bluntly dissected to remove visible connective tissue, blotted dry on filter paper, snap frozen in liquid nitrogen and stored at -80 °C. Small pieces of tissues are embedded in optimal cutting temperature (OCT) compound and kept at −80 °C until cryosection.

	Control (n = 45)	**Pregnant**			**GDM** (n = 10)
		IT (n = 15)	**IIT** (n = 25)	**IIIT** (n = 35)	
Age (years)	33 ±8	32 ±4	30 ±5	32 ±4	30 ±3
Gestation (weeks)	-	12.2 ±0.4	24.0 ±0.3	38.4 ±0.4	38.4 ±0.2
Fasting plasma glucose (mmol/l)	4.3 ±0.2	-	4.5 ±0.1	-	5.6 ±0.4
1-h plasma glucose (mmol/l)	6.0 ±0.1	-	6.1 ±0.3	-	10.5 ±0.4 $^{\Psi}$
TNF (pg/ml)	10 ± 1.7	-	18 ±3.5 *	-	45 ±12.3 $^{\Psi}$
PMN count (cells/μl)	3200 ±400	4200 ±200 *	5100 ±300 ‡	6100 ±100 $^{\delta}$	8600 ±1600 $^{\Psi}$

Table. 1. Patient characteristics of study group. Value represent the mean ± SD. * $P < 0.05$ first trimester versus control. ‡ $P < 0.05$ second trimester versus first trimester. δ $P < 0.05$ third trimester versus second trimester. Ψ $P < 0.05$ GDM versus pregnant. IT, first trimester; IIT, second trimester; IIIT, third trimester.

Human neutrophil isolation

Neutrophils are short lived and highly active cells. Therefore, their isolation requires careful handling to yield a good amount of cells within a shorter period of time. To characterize the specific functions of neutrophils, a high purity, fast and reliable method of separating them from other blood cells is desirable for *in vitro* studies. Neutrophils were isolated by Dextran-Ficoll density centrifugation. Whole blood was diluted with PBS without calcium and magnesium containing 2 mM EDTA up to 9 ml. Diluted blood was then layered over 3 ml of ficoll-paque solution and centrifuged in RT at 500 rcf with no brakes for 20 minutes to avoid stress and long contact period of cells with ficoll due to its toxic nature. After centrifugation, four distinct layers were observed which consisted of plasma, peripheral blood mononuclear cells (PBMC), ficoll-paque solution and pellet of sedimented red blood cells (RBC) with granulocytes. The first three layers of plasma, PBMC and ficoll-paque were discarded in order to obtain RBC pellet. The RBC pellet was immediately suspended in 10 ml of 1 x HBSS buffer and on the top of it was layered 1.5 ml of Dextran. After sedimentation (30 minutes at RT), neutrophil rich supernatant at upper layer was collected and centrifuged in RT at 500 rcf with no brakes for 10 minutes. The pellet was then resuspended in 10 ml of RBC lysis buffer and incubate for 5 minutes at RT. The lysis process was stopped using 1 x HBSS buffer and centrifuged for 10 min at 500 rcf with no brakes. After centrifugation, supernatant was discarded and the white pellet consisting of granulocytes was obtained and re-suspended immediately in 5 ml RPMI (Gibco, United Kingdom) in 2% of autologous plasma. Cell viability was assessed by trypan blue dye exclusion in a haemocytometer and was routinely 96–98% with a purity of over 95%.

BeWo cells and co-culture

BeWo choriocarcinoma cells (ATCC CCL-98) were grown at 37°C under a humidified 5% CO2/95% air atmosphere in F-12K Medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. In the present study, cells were grown as a monolayer at a density of 10^7 cells per 75 mm^2 flask with medium changed every 48 h. For passages, cells were detached with trypsin/EDTA at 37°C, then washed in complete culture medium and replated. All the experiments were performed in serum-starved conditions using cells at 90% confluence between passages 30 and 40.

In co-culture experiments, BeWo cells were plated in a 12-well plate until 90-95% confluent and then serum starved overnight in 1 ml of F-12K Medium containing 2mM of L-Glutamine. Then, the purified PMN (2×10^6 cells) were added to with BeWo cells and co-cultured for 4 hours in a final concentration of 1×10^6 cells/ml.

In vitro treatments

2.5 x 10^4 freshly isolated neutrophils were seeded in 96-well plates in and allowed to settle for 15 min at 37°C under 5% CO_2. The cells were treated with of TNF-α (2 ng/ml), and/or D-Glucose (25 mM) diluted with RPMI 2% of autologous plasma for 3 hours. To examine the priming effect in neutrophils, the cells were pre-treated for 60 min with a first stimulus and then for 180 min with the second one. In the experiments with the supernatant from BeWo cells, PMN were treated with 50 ul of supernatant diluited 1:2 with RPMI. To neutralize TNF-α, the PMN were pre-treated for 60 min with 5 ug/ml of chimeric anti-TNF IgG1 antibody infliximab.

BeWo cells were plated in a 12-well plate until 90-95% confluent. During hyperglycaemic incubation, cells were incubated overnight in 1 ml of F-12K Medium containing 2mM of L-Glutamine and 25 mM of Glucose. In the experiments with TNF-α inhibitor, the BeWo cells were incubated for 60 min with 5 ug/ml of chimeric anti-TNF IgG1 antibody infliximab.

The supernatants from all experiments were immediately stored at -80°C until used.

SytoxGreen assay

SytoxGreen is a cell-impermeable dye which increases fluorescence upon DNA binding. 2.5 x 10^4 freshly isolated neutrophils were seeded in 96-well plates in the presence of 0.2 µM SytoxGreen in RPMI with 2% of autologous plasma and allowed to settle for 15 minutes at 37°C under 5% CO_2 before treatments. PMA (20 nM) was used as the positive control. Fluorescence was measured with a multiplate reader (Synergy H4 Hybrid Multi-Mode Reader, BioTek, Germany) at 535 nm with excitation at 480 nm. Data are expressed as relative fluorescence units (RFU). The experiments were performed in triplicate, and the mean±SEM of the data were calculated.

Haematoxylin and Eosin (H&E)

H&E staining is used routinely in most histological laboratories and does not interfere with DNA and RNA preparation. The nuclei are stained blue and the cytoplasm pink/red. The staining method involves application of hemalum, which is a complex formed from aluminium ions and oxidized hematoxylin. This colors nuclei of cells blue. The nuclear staining is followed by counterstaining with an aqueous or alcoholic solution of eosin Y, which colors eosinophilic other structures in various shades of red, pink and orange.

Eight-micrometer cryostat sections were obtained, transferred to SuperFrost Plus microscope slides and stored at -80 °C. Before staining, the sections were thawed, air dried for 1h at room temperature (RT) and transferred into filtered 0.1 % Mayers Hematoxylin for 1 min. After rinsing in cool running ddH_2O water for 5 min, the sections are stained in 0.5% Eosin. Then sections were dipped in 50% ethanol ten times and then 70 % alcohol ten times. After that, the sections were equilibrated in 95% ethanol for 30 s and then 100% ethanol for 1 min. Before mounting in Cytoseal XYL, the sections were dipped in Xylene for 5 times and air-dried.

Immunofluorescence

Eight-micrometer cryostat sections were obtained from placenta tissues frozen in OCT, transferred to SuperFrost Plus microscope slides and stored at -80 °C. Before staining, the sections were thawed, air dried for 1h at room temperature (RT) and fixed in 4% paraformaldehyde/PBS for 10 min. The slides were rinsed 2 times in PBS and permeabilized by incubation with 0.2% Triton X-100 in PBS for 5 min. After rinsing with PBS twice were blocked with 3% BSA in PBS for 30 min and then incubated for 1h at RT with rabbit polyclonal anti-IRS1 (1:200) and mouse monoclonal antibody raised against neutrophil elastase (1:200) diluted in PBS with 3 % BSA and 0.05 % v/v Tween 20 (staining buffer). Negative controls were obtained by incubating with isotype-specific mouse/rabbit IgGs instead of the specific primary antibodies. The sections were then rinsed twice in PBS (at RT, 5 min each) and then incubated for 1h at RT in a light-protected humidified chamber with Alexa 488-conjugated anti-rabbit (1:1000) and Alexa 647-conjugated anti-mouse (1:1000) secondary antibodies diluted in staining buffer. After three PBS washes, sections were stained with DAPI (1: 10,000) for 15 min at RT. The sections were then washed in PBS, mounted in Mowiol and visualized using an Olympus BX61 Diana fluorescent microscope.

Neutrophils were plated in a 96-well black plate at a density of 2.4×10^4 cells per well. Following in vitro treatment cells were fixed in 2% paraformaldehyde/PBS for 15 min, washed three times in PBS and permeabilized by incubation in 0.2% Triton X-100 in PBS for 5 min. The cells were then washed, blocked with PBS containing 3% BSA for 30 min at RT, stained with primary mouse monoclonal antibody directed against human Myeloperoxidase (1:750), rabbit polyclonal against human Histone H3 (1:150) and incubated overnight at 4 °C. Negative controls were obtained by incubating with isotype-specific mouse/rabbit IgGs instead of the specific primary antibodies. The following day, after three washes in PBS, the cells were incubated with goat anti-rabbit AlexaFluor 488-labeled secondary antibody (1:1000) and chicken anti-mouse AlexaFluor 594-labeled secondary antibody (1:1000) for 2 h at RT protect from light. After three PBS washes, PMN were stained with DAPI (1: 10,000) for 15 min at RT. The PMN were then washed twice in PBS, resuspended in 100 ul of PBS and visualized using an Olympus BX83 fluorescent microscope.

BeWo cells were plated on 12 mm round coverslips in a 12-well plate until 90-95% confluent. Following in vitro treatment and co-culture, the cells were fixed in 2% paraformaldehyde/PBS for 15 min, washed three times in PBS and blocked with PBS containing 3% BSA for 60 min at RT. The coverslips were then incubated overnight in a humidified chamber at 4 °C for 2h at RT with mouse monoclonal antibody raised against neutrophil elastase (1:200) and rabbit polyclonal against human Histone H3

(1:150) diluited in blocking buffer. Negative controls were obtained by incubating with isotype-specific mouse/rabbit IgGs instead of the specific primary antibodies. The following day, after washes in PBS, the coverslips were incubated with Alexa 647-conjugated anti-mouse (1:1000) and goat anti-mouse AlexaFluor 488-labeled secondary antibody (1:1000) for 1 h at RT in a light-protected humidified chamber. After three PBS washes, PMN were stained with DAPI (1: 10,000 dilution) for 15 min at RT. The cells were then washed in PBS, mounted in Mowiol and visualized using an Olympus BX61 Diana fluorescent microscope.

Morphometric analysis

The image processing and manipulation is based in ImageJ software (National Institutes of Health). Images were saved with more than 300 dpi (in .tiff uncompressed format) to guarantee the quality of the morphometric analysis. Table. 2. contains the formulas and settings for each analysis:

Analysis	Formula
Primed PMN	$Cells\,(\%) = \frac{Count\ CitH3}{Count\ DAPI} \cdot 100$
NETs	$Area(\%) = \frac{Total\ Area\ MPO}{Total\ Area\ DAPI} \cdot 100$
Extracellular NE	$Area(\%) = \frac{Total\ Area\ NE}{Total\ Area\ DAPI} \cdot 100$
Settings (Analyze Particles):	
Size	Circularity
DAPI, CitH3: 0--50 **MPO, NE: 50--∞**	0.5-1.0 (DAPI, CitH3, MPO, NE)

Table. 2. Formulas and settings of ImageJ used for morphometric analysis.

Analysis were performed on at least 10 randomly selected non overlapping fields taken from each preparation. An example with the sequence of steps is presented in Table. 3. for extracellular NE staining after co-culture BeWo cells with PMNs:

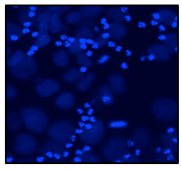
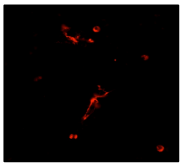
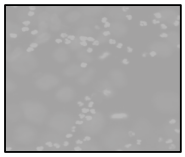
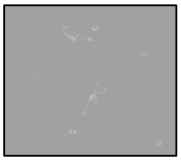
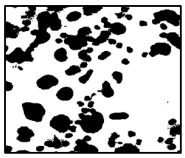
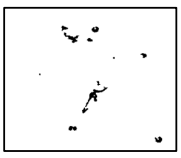
DAPI staining		NE staining
	Threshold the image of staining using manual settings: ***Image → Adjust →Threshold*** and play with sliders to include all stained areas	
	Convert the image to grayscale: ***Image → Type → 8-bit***	
	Analyze Particles: ***Analyze → Analyze Particles***	

Table. 3. Example of extracellular NE morphometric analysis using ImageJ.

Elastase activity

Serum (50 µL) was incubated with 0.25 mM of MeOSuc-AAPV-AMC in 50 µL of PBS for 30 min at 37 °C, 5% CO2 protected from light. Fluorescence was measured with a multiplate reader (Synergy H4 Hybrid Multi-Mode Reader, BioTek, Germany) at 455 nm with excitation at 360 nm. Data are expressed as relative fluorescence units (RFU). The experiments were performed in duplicate, and the mean±SEM of the data were calculated.

Total cellular protein was extracted from placenta using Pierce RIPA Buffer supplemented with PhosSTOP Phosphatase Inhibitor Cocktail Tablets. Frozen tissues were thawed for a few minutes in pre-chilled RIPA buffer and homogenized with a Polytron (Kinematica). Lysates were centrifuged at 14,000×g for 10 min at 4°C and protein levels were tested by Pierce BCA Protein Assay Kit. Lysates (25 ul) were incubated with 0.25 mM of MeOSuc-AAPV-AMC, in 75 µL of PBS for 30 min at 37 °C, 5% CO2 protected from light. Fluorescence was measured with a multiplate reader (Synergy H4 Hybrid Multi-Mode Reader, BioTek, Germany) at 455 nm with excitation at 360 nm. Data are expressed as relative fluorescence units (RFU)/µg protein. The experiments were performed in duplicate, and the mean±SEM of the data were calculated.

Protein isolation and western blotting

Total cellular protein was extracted using Pierce RIPA Buffer supplemented with PhosSTOP Phosphatase Inhibitor Cocktail Tablets. For the placenta, frozen tissues were thawed for a few minutes in pre-chilled RIPA buffer and homogenized with a Polytron (Kinematica). Lysates were centrifuged at 14,000×g for 10 min at 4°C and protein levels were tested by Pierce BCA Protein Assay Kit. Twenty µg total protein mixed 1:4 volume of 4x Laemmeli buffer (Bio-Rad) with at a final concentration of 2.5% β-mercaptoethanol were denatured at 95°C (5 min), separated on MiniProtean TGX precast gels and transferred to a nitrocellulose membrane at 30 V overnight with cooling at 4°C. The membrane was first blocked for 1 h at RT in PBS containing 5% w/v non-fat dry milk and 0.05 % v/v Tween-20, then incubated with the corresponding primary antibodies, diluted in blocking solution, under gentle agitation overnight at 4°C. The antibodies used were rabbit polyclonal anti-IRS1 (1:500) and rabbit polyclonal anti-GLUT4 (1:1000). After incubation with the primary antibody, the membranes were washed three times in PBST (PBS containing 0.05 % v/v Tween-20) and incubated

for 90 min at RT, under gentle agitation, with a goat anti-rabbit HRP antibody (1:5000) or a goat anti-mouse HRP antibody (1:5000) diluted in blocking solution. Following three additional washes with PBST (at RT, 5 min each), bound antibody was detected by chemiluminescence according to manufacturer's instructions, and imaged using a Biorad ChemiDoc MP Imaging System. Band intensities were measured using Image Lab Software (version 5.2.1). Blots were stripped briefly in 0.2 M sodium hydroxide and reprobed for mouse monoclonal β-actin as a loading control.

RNA isolation and quantitative real-time PCR

Total RNA was isolated by using RNeasy Mini Kit. TaqMan real-time quantitative RT-PCR was performed using the StepOne plus real-time PCR thermal cycle system (Applied Biosystems) and TaqMan Gene Expression Assay primer/probe sets. The housekeeping gene RPLP0 (36B4) was used as a reference gene. The relative RNA amount was calculated with the comparative cycle threshold method. The data are presented as the fold change ($2^{-\Delta\Delta CT}$) over control value normalized to RPLP0 where ΔCt = Ct (RPLP0) – Ct (NE) and ΔΔCt = Avg. ΔCt (GDM) – Avg. ΔCt (CTRL). The experiments were performed in triplicate, and the mean±SEM of the data were calculated.

Apoptosis Assay

BeWo cells were grown in a 96-well plate until 90-95% confluent and then incubated overnight in serum starved-condition with different concentrations of NE (20-320 nM). After washing twice with pre-warmed PBS, the assay was performed using the TiterTACS™ Colorimetric Apoptosis Detection Kit according to the manufacturer's instructions. During the process of apoptosis, DNA fragmentation occurs following the activation of endonucleases. The labeling of the 3' ends of DNA fragments provides an easy measure of cells undergoing apoptosis. Modified nucleotides are incorporated at the 3' ends by the activity of terminal deoxynucleotidyl transferase (TdT). These nucleotides are detected using a horseradish-peroxidase detection system and TACS-Sapphire™.

Samples incubated with camptothecin (6 μM) were used as a positive control. The resulting color was measured at 450 nm using a multiplate reader (Synergy H4 Hybrid

Multi-Mode Reader, BioTek, Germany). The absorbance of the control cells was designated as 0%. The experiments were performed in triplicate, and the mean±SEM of the data were calculated.

Assay for reactive oxygen species (ROS)

Generation of reactive oxygen species (ROS) was detected with the cell permeant reagent 2', 7' –dichlorofluorescin diacetate (DCFDA) a fluorogenic dye that measures hydroxyl, peroxyl and other reactive oxygen species (ROS) activity within the cell. After diffusion in to the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2', 7' –dichlorofluorescein (DCF). DCF is a highly fluorescent compound which can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 529 nm respectively.
BeWo cells were grown in a 96-well plate until 90-95% confluent. The cells were then incubated overnight with different concentrations of NE (20-320 nM). The cells were washed twice with pre-warmed PBS, incubated with 10 μM of DCFDA in 100 ul of PBS and incubated for 1 h at 37°C. Samples incubated with of hydrogen peroxide (200 μM) were used as a positive control. Fluorescence was measured with a multiplate reader (Synergy H4 Hybrid Multi-Mode Reader, BioTek, Germany) at 535 nm with excitation at 480 nm. Data are expressed as relative fluorescence units (RFU). The experiments were performed in triplicate, and the mean±SEM of the data were calculated.

MTT Assay

The yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) is reduced by metabolically active cells and converted into a purple colored formazan product with an absorbance maximum near 570 nm. When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells. The exact cellular mechanism of MTT reduction into formazan is not well understood, but likely involves reaction with NADH or similar reducing molecules that transfer electrons to MTT. Speculation in the early literature involving specific mitochondrial enzymes has led to the assumption mentioned in numerous publications that MTT is measuring mitochondrial activity. BeWo cells were grown in a 96-well plate until 90-95% confluent and then incubated

for 16 hours in serum starved-condition with different concentrations (20-320 nM) of NE. After the incubation time, MTT was added at 0.3 mg/ml final concentration. Samples incubated with 6 μM of camptothecin were used as a negative control. Following a 3 h incubation period with MTT, media was removed and the blue formazan crystals trapped in cells dissolved in sterile DMSO by incubating at 37 C° for 30 min. The resulting color was measured at 570 nm using a multiplate reader (Synergy H4 Hybrid Multi-Mode Reader, BioTek, Germany). The experiments were performed in triplicate and the results are expressed as fold change±SEM relative to untreated cells (fold change = 1).

Radiometric Glucose Uptake Assay

BeWo cells were seeded in 12 well and allowed to reach 85–90% confluence and serum-starved for 16–18 h before incubation with 80 nM of human recombinant NE in glucose-free KrebsRinger HEPES buffer (KRH) [25 mM Hepes-NaOH (pH 7.4), 120 mM NaCl, 5 mM KCl, 1.3 mM CaCl2, 1.3 mM KH2PO4] for 3 h. Then cells were rinsed twice with KRH and stimulated in the same buffer containing 100 nM of insulin for 1 h. Following stimulation, the cells were washed and, in order to measure the glucose uptake incubated in KRH buffer supplemented with 1 μCi/ml of 2-[1,2-3H(N)]-deoxy-d-glucose for 10 min. The uptake of glucose was terminated by rapidly aspirating the radioactive incubation medium followed by three rapid washes with PBS. Cells were lysed by the addition of 200 uL/well of RIPA Buffer, scraped with a cell scraper and centrifuged at 14,000×g for 10 min at 4°C. Approximately 150 uL cell lysate was treated with 4 mL of scintillation fluid and vortexed. Radioactivity was measured in a liquid scintillation analyzer (Packard 1900 TR). Protein values used for expressing the data were determined from the remaining supernatants using the BCA Protein Assay Kit according to manufacturer's specifications. Glucose uptake was expressed as counts per minute (cpm)/mg protein and samples were measured in duplicate. Data was corrected for background levels using scintillation fluid plus RIPA Buffer as a blank. The experiments were performed in triplicate, and the mean±SEM of the data were calculated.

Statistical analysis

All data are presented as mean ± SEM. Descriptive statistics for continuous parameters consisted of median and range, and categorical variables were expressed as percentages. Comparisons between patients and healthy controls were carried out by the Mann-Whitney U test with a Welch post- test correction. Statistical significance in multiple comparisons was by one-way analysis of variance (ANOVA) followed by Bonferroni's post-test correction. P values under < 0.05 were considered statistically significant: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Data were processed in GraphPad Prism version 6.0 for MacOSX.

RESULTS

Skewing of PMNs toward a pro-NETotic profile during pregnancy

In pregnant women the number, priming and migratory capacity of PMNs are increased compared to non-pregnant women (G. P. Sacks et al. 1998). However, nothing was reported regarding their NETosis activity.

For this purpose, freshly isolated neutrophils were incubated at 37 °C, 5% CO_2 for 3 hours to enable the formation and the spontaneous release of extracellular DNA filaments. In order to monitor the PMNs activity through the various stages of pregnancy, neutrophils were isolated from first-, second- and third trimester of gestational age. The DNA was stained using SytoxGreen, a cell impermeable DNA dye. As shown in **Fig. 1A**, PMNs isolated from non-pregnant women are unable to release NETs after the incubation suggesting that the cells are in the resting state. On the other hand, PMNs isolated from pregnant women exhibited a pro-NETotic profiles that increase compared to healthy controls during the advancement of pregnancy.

To confirm that these filamentous structures stained by SytoxGreen, were indeed NETs, we used immunofluorescent microscopy to stain myeloperoxidase (MPO). Since MPO is required for chromatin and nuclear envelope breakdown the co-localization of DNA with MPO is generally seen as evidence for NETosis (Papayannopoulos et al. 2010b; Metzler et al. 2014). MPO staining confirmed the increased tendency for neutrophils to undergo NETosis with advancing gestational age (**Fig. 1B**).

NETosis is mediated by a variety of molecular mechanism that involve an increase in MPO expression and the citrullination of histone H3, driven probably by the enhanced PADI4 expression. Western blot analysis revealed significantly increased of PADI4, citH3 and MPO in neutrophil isolated from pregnant women compare to those isolated from non-pregnant women (**Fig. 1C**).

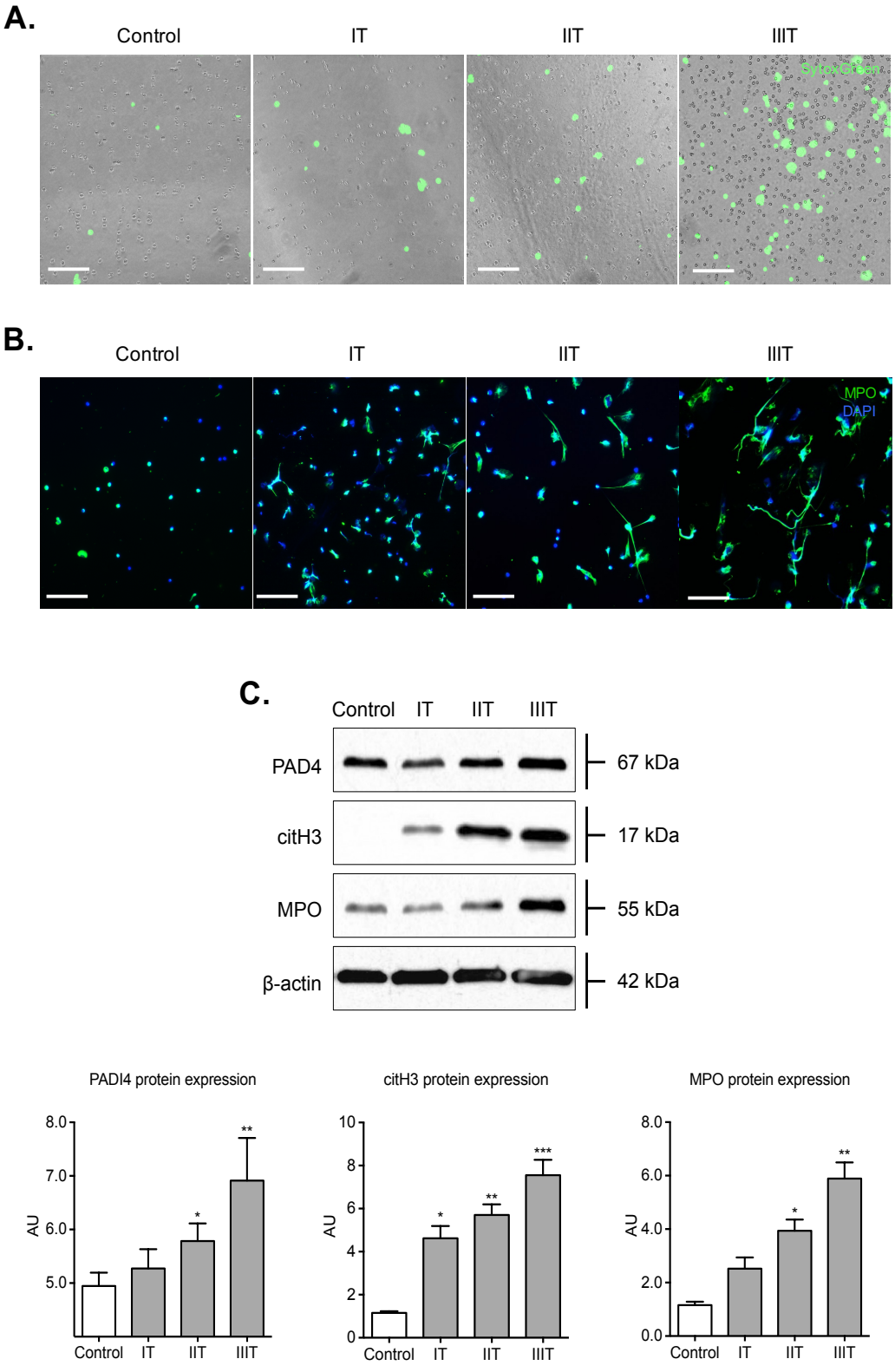


Fig. 1. Skewing of PMNs responses toward a pro-NETotic profile during pregnancy. (**A**) Assessment of spontaneous NETs by fluorescent microscopy using SytoxGreen dye in neutrophils from non-pregnant control group and pregnant women across trimesters with 180 minutes incubation time. Scale bars: 100 µm. (**B**) Assessment of spontaneous NETs by immunofluorescence staining for MPO (green) and DNA counterstain with DAPI (blue) in neutrophils as in (A). Scale bars: 50 µm. (**C**) Western blot and densitometric analysis of PAD4, citH3, MPO and ß-actin protein expression levels in neutrophils from non-pregnant control group and pregnant women across trimesters immediately after isolation. IT, first trimester; IIT, second trimester; IIIT, third trimester; AU, arbitrary unit.

Increased levels of NETosis markers during GDM

There is growing consensus in the literature that inflammation plays a central role in the pathophysiology of GDM (Shanmugam et al. 2003; J. Wang et al. 2012; Gonzalez et al. 2012). The first indicator of acute inflammation is evaluated as an increase in the white blood cell count in the peripheral blood. Indeed, compared to healthy pregnancy, women with GDM exhibited significantly increased neutrophil counts (**Table 1**). Besides high PMNs count, we wanted to investigate the degree of their NETotic activity into the bloodstream of GDM women.

Circulating nucleosomes together with circulating markers for neutrophil activation, such as MPO and NE, have been reported to be robust markers to measure NETs formation (Borissoff et al. 2013). The results showed that GDM is associated with elevations in cell-free nucleosome (**Fig. 2A**) and MPO (**Fig. 2B**) levels in the serum compared to those from women with healthy pregnancies. Furthermore, sera isolated from GDM women exhibit higher NE activity as determined using a fluorogenic peptide substrate (**Fig 2C**).

Interestingly, these findings support our ex vivo NETs formation data that showed an increased tendency of PMNs from pregnant women to form NETs in comparison with PMNs from non-pregnant women (**Fig. 1**). Indeed, levels of MPO and NE are significant higher during pregnancy in comparison with non-pregnant control group. However, NE activity, was slight but non-significant elevated during healthy pregnancies (**Fig 2C**).

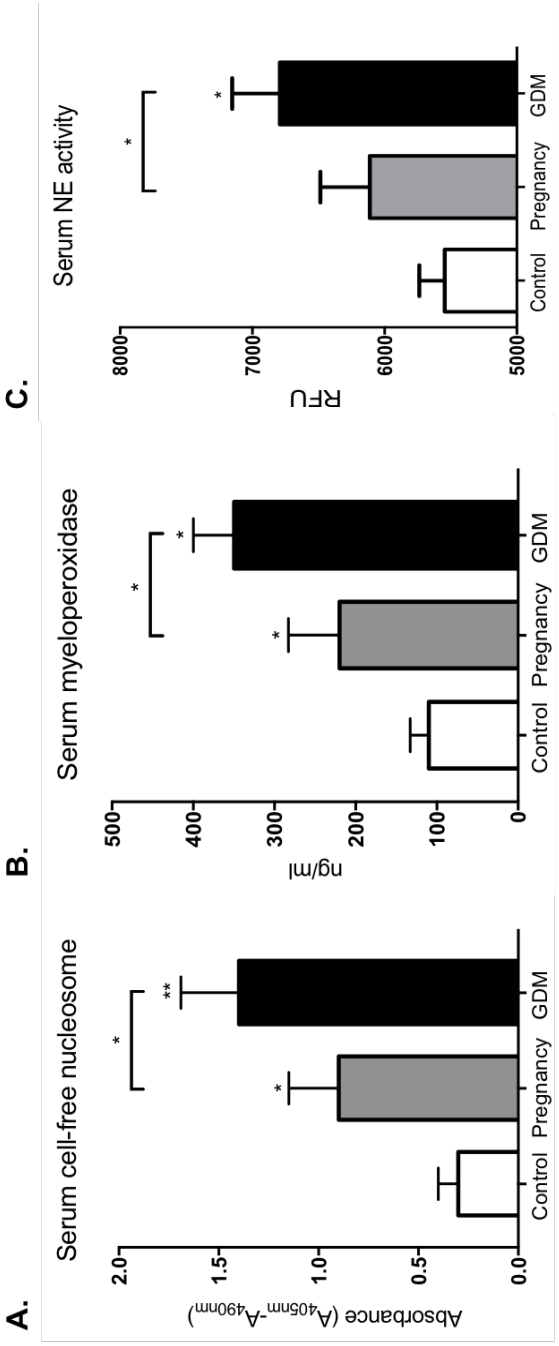


Fig. 2. Increased levels of NETosis markers during GDM. (**A**) Detection of serum cell-free nucleosome levels by ELISA in neutrophils from non-pregnant control group, healthy pregnancies and GDM group. (B) Detection of serum myeloperoxidase levels by ELISA in neutrophils as in (A). (C) Detection of NE activity with MeOSuc-AAPV-AMC in neutrophils as in (A). Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one-way ANOVA followed by Bonferroni's multiple comparison post-test). GDM, gestational diabetes mellitus; RFU, relative fluorescent unit.

Neutrophils from GDM women exhibit excessive NETs formation

Our results showed that markers of NETs are independently associated with GDM (**Fig. 2.**), suggesting an abnormal PMNs behaviour in the peripheral circulation. To provide a better understanding of the potential origin of extracellular DNA traps, we compared the ability to spontaneously release extracellular DNA filaments of ex vivo PMNs isolated from women with GDM with PMNs from healthy pregnant women.

Freshly isolated PMNs were incubated at 37 °C, 5% CO_2 for 3 hours to enable the formation and the spontaneous release of extracellular DNA filaments. NETs formation was quantified with SytoxGreen and immunofluorescence staining for MPO. As shown in **Fig. 3A**, extracellular DNA is present in higher number in neutrophils isolated from women with GDM and is associated with MPO staining (**Fig. 3C**), confirming NETs origin. Statistical analysis of SytoxGreen staining was performed using the fluorescence intensity of the samples quantified using a microplate reader (**Fig. 3B**). Morphometric analysis of MPO staining (**Fig. 3D**) was used as alternative measurement method to compare the fluorescence intensity of SytoxGreen staining. Statistical analysis of two different methods confirmed the exacerbated ex vivo NETosis activity of PMNs isolated from GDM women.

Priming of neutrophils toward NETosis was studied by immunofluorescence staining for histone H3 citrullination (**Fig. 3C**) and subsequent morphometric analysis (**Fig. 3D**). The number of primed cells ($citH3^+$) was higher in PMNs isolated from GDM women.

We have further stained PMNs for neutrophil elastase. This serine proteinase is essential for chromatin decondensation during NETs formation (Papayannopoulos et al. 2010b) and there is increasing evidence that is a key modulator of chronic metabolic inflammation (Talukdar et al. 2012; Houghton et al. 2010). As shown in **Fig. 3F**, NETs appear as irregular cloudy structures in which dense clusters of brightly stained NE are present in higher number in neutrophils isolated from women with GDM.

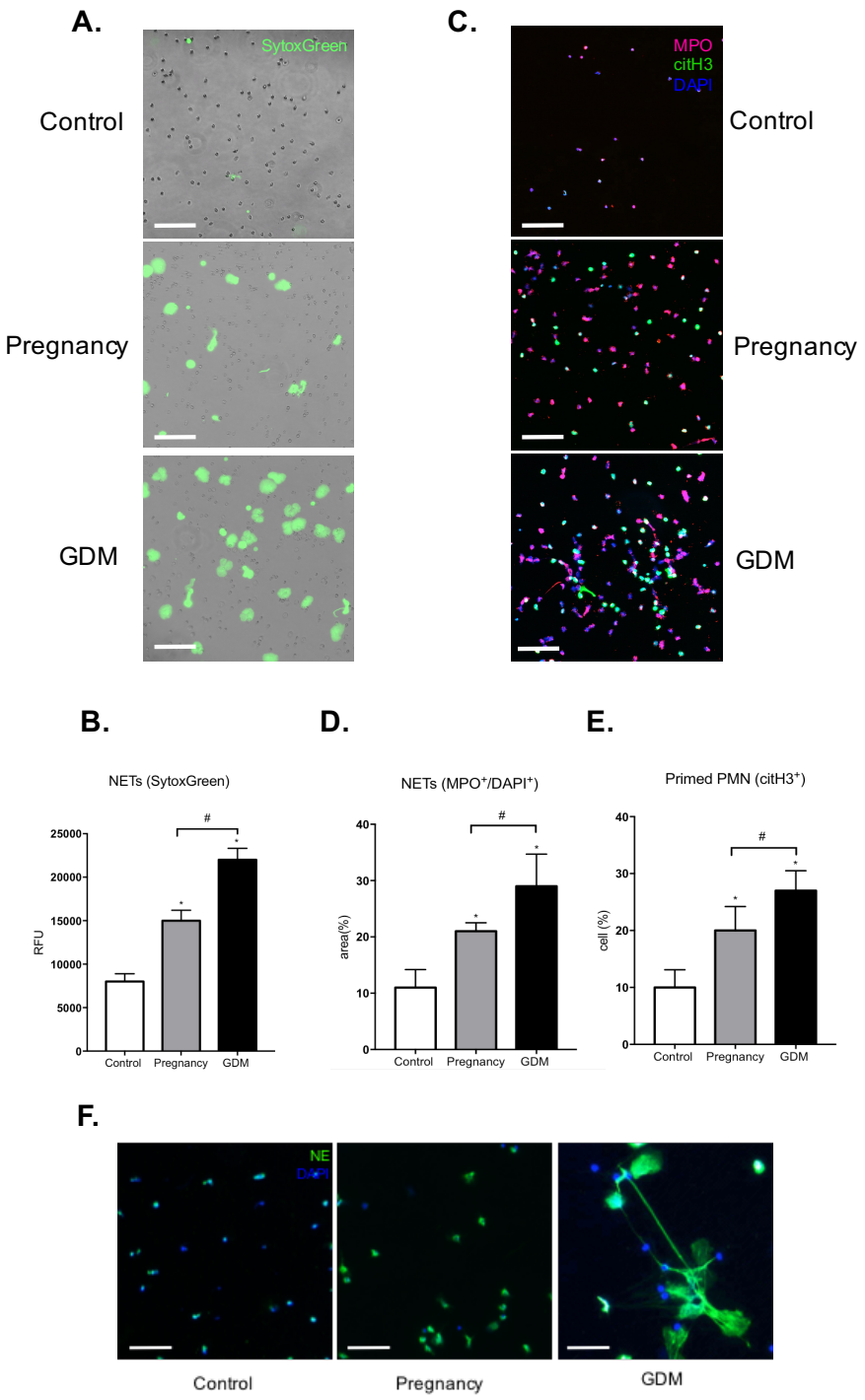


Fig. 3. Neutrophils from GDM women exhibit excessive NET formation (**A**) Assessment of spontaneous NETs by fluorescent microscopy using SytoxGreen dye in neutrophils from non-pregnant control group, healthy pregnancies and GDM group with 180 minutes incubation time. Scale bars: 100 µm. (**B**) Assessment of spontaneous NETs by fluorescence microplate reader using SytoxGreen dye in neutrophils as in (A). (**C**) Assessment of spontaneous NETs by immunofluorescence staining for MPO (red), citH3 (green) and DNA counterstain with DAPI (blue) in neutrophils as in (A). Scale bars: 50 µm. (**D**) Morphometric analysis of NETs (MPO^+/DPI^+) in neutrophils as in (C) using ImageJ software. (**E**) Morphometric analysis of pro-NETotic neutrophils ($citH3^+$) in (C) using ImageJ software. (**F**) Assessment of spontaneous NE release by immunofluorescence staining for NE (green) and DNA counterstain with DAPI (blue) in neutrophils as in (A). GDM, gestational diabetes mellitus; RFU, relative fluorescent unit.

Glucose and TNF-alpha act additively to drive NETs release

GDM is characterized by hyperglycaemia that results from tissue resistance to the glucose-lowering effects of insulin and inadequate pancreatic B-cell compensation (Rieck and Kaestner 2010). Furthermore, TNF-α, which primes neutrophils for NETosis (Brinkmann et al. 2004b), has been associated with insulin resistance in GDM (Shanmugam et al. 2003; J. Wang et al. 2012; Gonzalez et al. 2012). Thus, we hypothesized that diabetic microenvironment may facilitate NETosis in women with GDM.

To determine whether, hyperglycaemia and TNF-α have synergistic effects to NETosis, we isolated neutrophils from healthy controls and incubated 3 hours in the presence of either high glucose or TNF-α. We noticed by SytoxGreen assay (**Fig. 4A**, **Fig. 4B**) and immunofluorescence staining for MPO (**Fig. 4C**, **Fig. 4D**) that glucose and TNF-alpha exhibit an additive, and not synergistic effect on NETs production.

This additive effect was not observed in terms of pro-NETotic priming as detected by immunofluorescence staining for histone H3 citrullination (**Fig. 4E**). Indeed, citH3 staining was higher when the cells were incubated with TNF-α alone.

To mimic ex vivo treatments as close as possible to in vivo conditions and to prove the synergistic effects of hyperglycemia and TNF-α to NETosis, we used neutrophils isolated before and during the oral glucose tolerance test (OGTT) from non-pregnant, healthy pregnant and GDM women. One hour after exposure to 75 g of oral glucose the serum glucose concentration reaches the highest value (**Table 1**). Isolated PMNs were incubated at 37 °C, 5% CO_2 for 3 hours to enable the formation and the spontaneous release of extracellular DNA filaments.
In the GDM group, PMNs isolated at 1 hour during the OGTT test exhibited an increase in NETosis rate (**Fig. 4F**, **Fig. 4G**) if compared to PMNs isolated from fasted women. No statistical differences were found in non-pregnant and healthy pregnant women. These findings follow the same trends as the histone H3 citrullination (**Fig. 4H**).

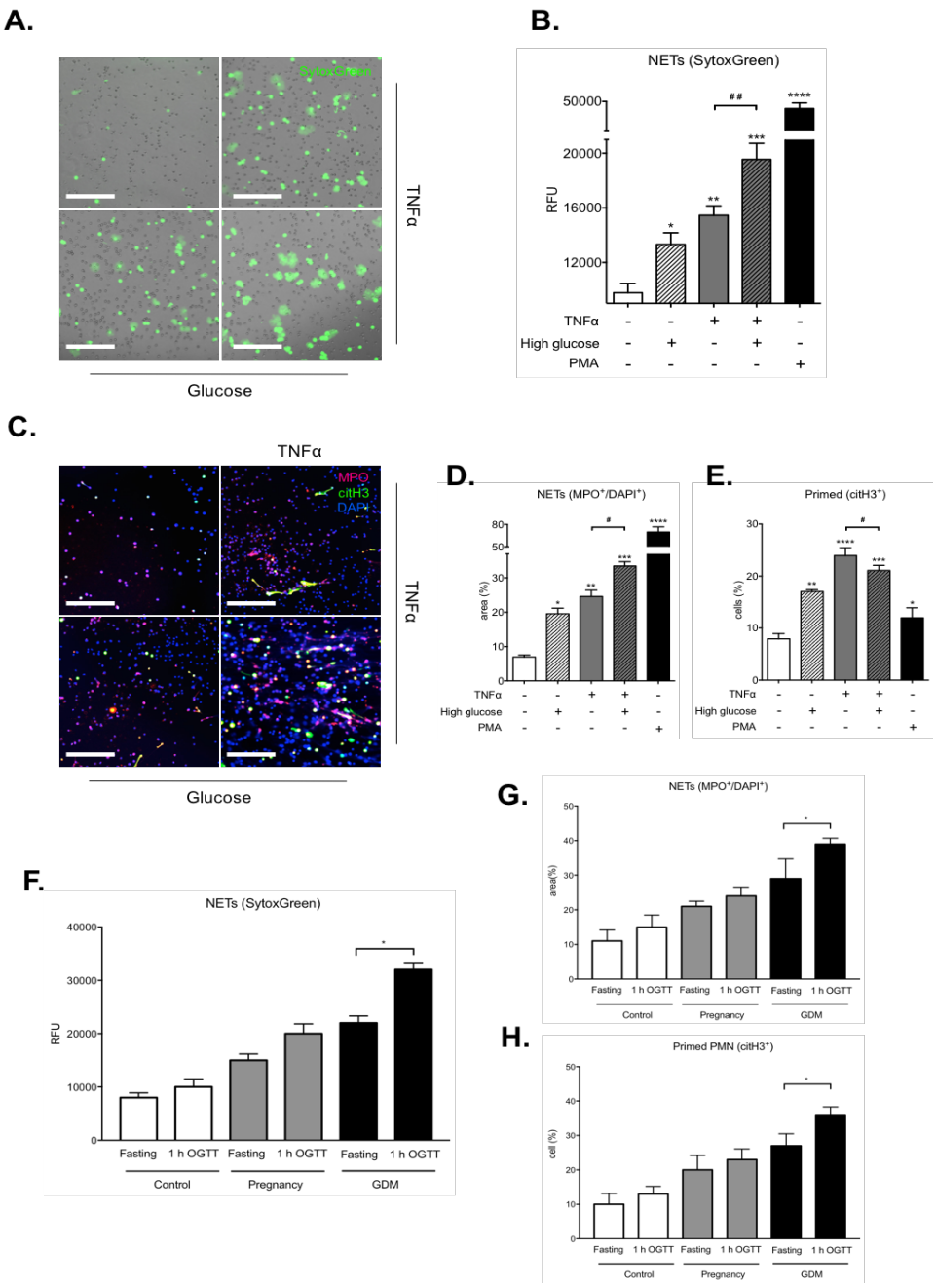


Fig. 4. Glucose and TNF-alpha act synergistically to drive NETs release. (A) Assessment of spontaneous NETs by fluorescent microscopy using SytoxGreen in neutrophils from non-pregnant control group incubated with glucose (25 mM) and/or TNF-α (50 ng/ml) with 180 minutes incubation time. Scale bars: 100 µm. (**B**) Assessment of spontaneous NETs by fluorescence microplate reader using SytoxGreen dye in neutrophils as in (A). (**C**) Assessment of spontaneous NETs by immunofluorescence staining for MPO (red), citH3 (green) and DNA counterstain with DAPI (blue) in neutrophils as in (A). Scale bars: 50 µm. (**D**) Morphometric analysis of NETs (MPO^+/DPI^+) in neutrophils as in (C) using ImageJ software. (**E**) Morphometric analysis of pro-NETotic neutrophils ($citH3^+$) in (C) using ImageJ software. (**F**) Assessment of spontaneous NETs with 180 minutes incubation time, by fluorescence microplate reader using SytoxGreen dye, in neutrophils from non-pregnant control group, healthy pregnancies and GDM group before and 1 hour after drinking the high glucose drink. (**G**) Morphometric analysis, using ImageJ software, of spontaneous NETs (MPO^+/DPI^+) in neutrophils staining for MPO (red), citH3 (green) and DNA counterstain with DAPI (blue) in neutrophils as in (F). (**H**) Morphometric analysis, using ImageJ software, of pro-NETotic neutrophils ($citH3^+$) staining as in (G). GDM, gestational diabetes mellitus; RFU, relative fluorescent unit.

PMNs leads to excessive NE release and IRS1 degradation in placenta

GDM leads to abnormal placental environment, which causes some structural alterations (Fig. 5B) and affect its development and function. Moreover, may have profound effects on the presence and action of immune cells in the placenta.

To determine whether in GDM the high glucose environment affects the infiltration and/or the NETs activity of PMNs we stained MPO and citH3 in the placentas of GDM affected women and placentas from normal pregnancy collected at delivery. The result showed (**Fig. 5A**) a significant increase in the area of MPO stained GDM affected placentas compared to that from normal pregnancy. In addition, positive co-staining for MPO and citH3 of the filamentous structure (**Fig. 5A**) let us suppose an increase of NETs along chorionic villi of GDM placentas.

Although MPO expression correlated with infiltrating neutrophils in the placenta, is also express to a lesser extent in monocytes and some macrophages. Therefore, qPCR (**Fig. 5C**) and enzymatic activity (**Fig. 5D**) for NE was performed to confirm neutrophils infiltration. Once again, levels of NE were higher in the placentas from GDM women.

In mice on HFD, Talukdar et al showed that NE causes cellular insulin resistance through degradation of IRS1 (Talukdar et al. 2012). Based on this, we hypothesize that the increased level of NE may have the same deleterious effect on GDM placentas. Immunofluorescence staining for NE and IRS1 revealed a marked accumulation of NE in GDM that is associated with a reduced IRS1 presence (**Fig. 5F**). The lower expression of IRS1 in GDM placentas was confirmed by Western blot analysis (**Fig. 5E**).

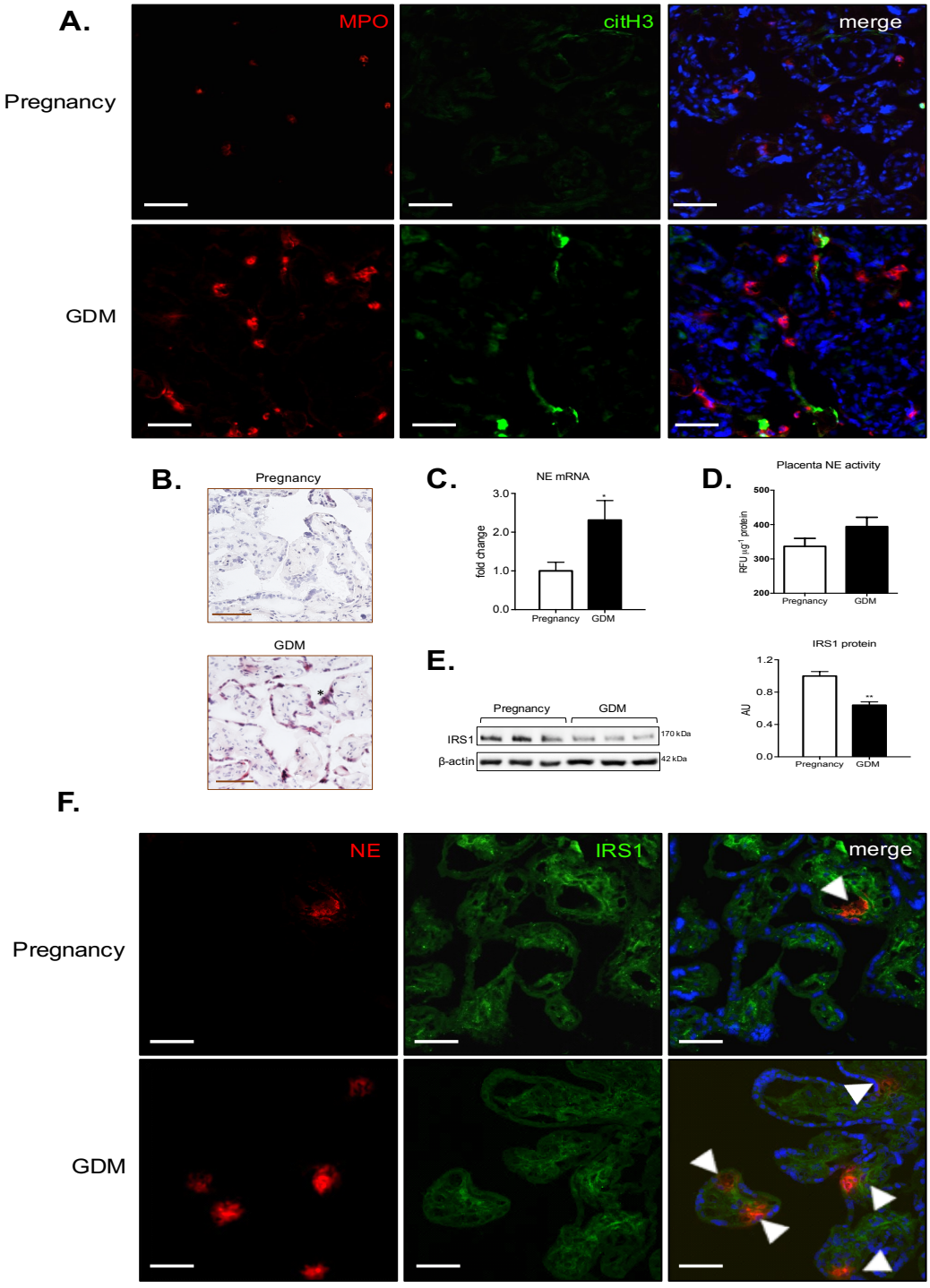


Fig. 5. PMNs leads to excessive NE release and IRS1 degradation in placenta (**A**) Immunofluorescence staining for MPO (red), citH3 (green) and DNA counterstain (blue) of placental tissue of placental tissue collected at the term of pregnancy from normal controls and GDM women. Scale bars: 50 µm. (**B**) H&E staining of placental tissue as in (A). (*): syncytial knotting. Scale bars: 50 µm. (**C**) mRNA expression level of NE determined by qPCR in placental tissue as in (A). (**D**) Detection of NE activity with MeOSuc-AAPV-AMC in placental tissue as in (A). (**E**) Western blot and densitometric analysis of IRS1 and ß-actin protein expression levels in placental tissue as in (A). (**F**) Immunofluorescence staining for NE (red), IRS1 (green) and DNA counterstain (blue) of placental tissue as in (A). Scale bars: 100 µm. GDM, gestational diabetes mellitus; RFU, relative fluorescent unit; AU, arbitrary unit.

NE impairs glucose uptake in BeWo cells

Neutrophil elastase is a highly destructive enzyme, capable of degrading a great variety of different proteins such as fibronectin, collagen, elastin, and cytokines, including G-CSF (El Ouriaghli et al. 2003). In addition to its proteolytic activity, there is increasing evidence that neutrophil elastase (NE) is a key modulator of chronic metabolic inflammation, insulin sensitivity and glucose metabolism in obesity (Talukdar et al. 2012; Houghton et al. 2010). At high concentrations, NE induces apoptosis, as well as other neutrophil serine proteases (Oltmanns et al. 2005). However, at low concentration can be internalized into cells and affects the cell metabolism (Talukdar et al. 2012; Houghton et al. 2010).

To investigate the impact of NE on trophoblast we incubated BeWo cells, placental cell line that has been widely used as an in vitro model for placental villous trophoblast, with recombinant NE. In order to determine the ability of cells to survive and keep their metabolic activity in presence of NE, we incubated for 16 h BeWo cells with different concentrations of recombinant NE (20-320 nM). As expected, high concentrations of NE (> 80 nM) induce apoptosis in BeWo cells (**Fig. 6A**). However, under low NE concentrations (40 and 80 nM) BeWo cells exhibited higher metabolic activity compared to untreated cell, measured by MTT assay (**Fig. 6B**). Because changes in metabolic rate can directly modify ROS production, we evaluated intracellular levels of ROS using DCFDA probe. As shown in **Fig. 6C**, NE has been shown to increase ROS production at concentration of 40 and 80 nM.

Next, we added NE to test its effect on insulin sensitivity. We observed a marked decrease in IRS1 protein content, consistent with enhanced degradation in GLUT4 (**Fig. 6D**). Moreover, the acquisition of glucose by the BeWo appears to be directly regulated by insulin (**Fig. 6B**). Radiometric analysis of glucose uptake showed similar results (**Fig. 6E**) to that observed with Western blotting (**Fig. 6D**).

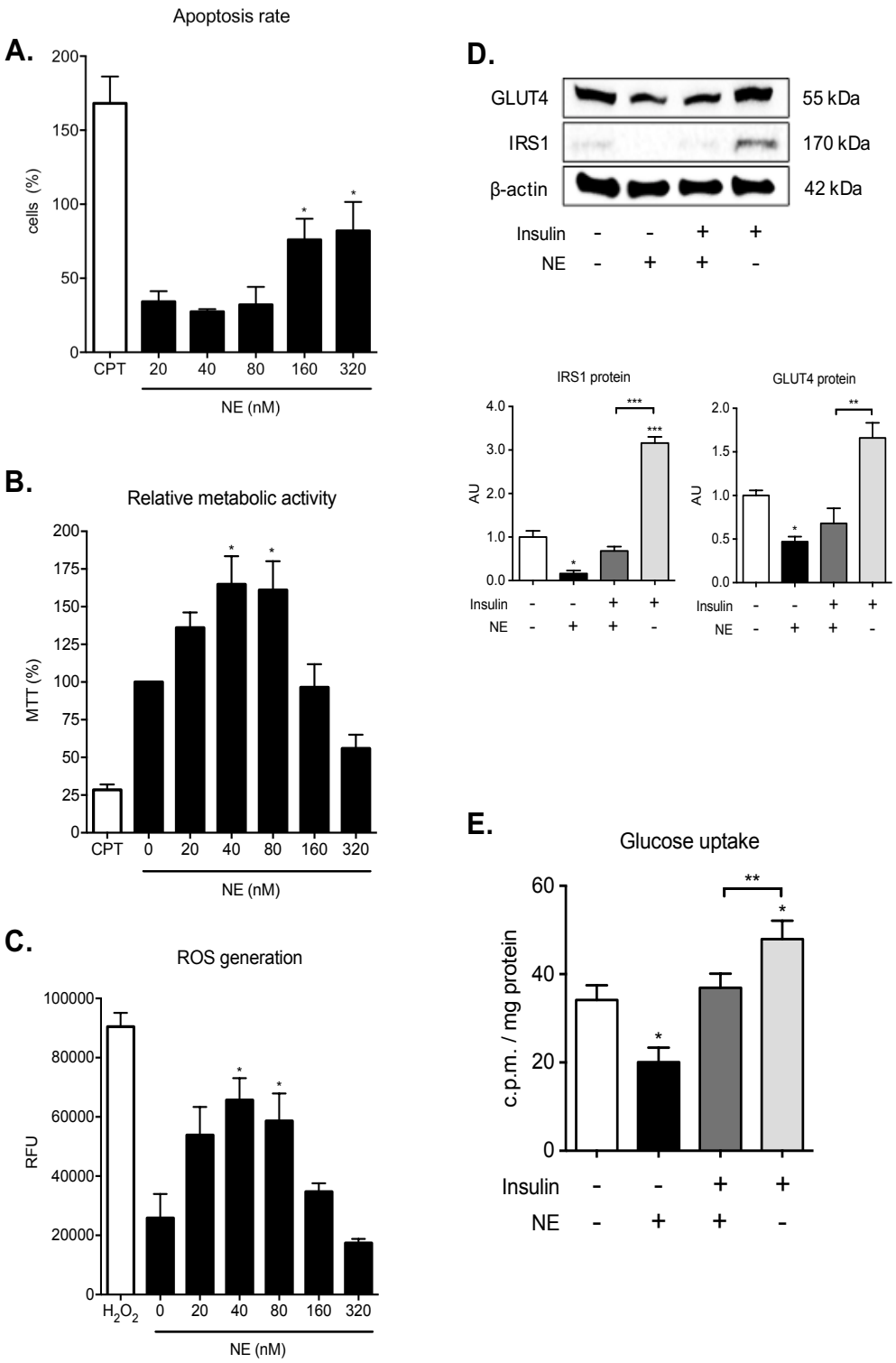


Fig. 6. NE impairs glucose uptake in BeWo cells. (**A**) Apoptotic effect of NE in BeWo cells at concentration 20, 40, 80, 160 and 320 nM after incubation for 16 h (**B**) Viable cell metabolism assessed by MTT assay in BeWo cells incubated as in (A). (**C**) Measurement of cellular ROS by DCFDA assay in BeWo cells incubated as in (A). (**D**) Western blot and densitometric analysis of GLUT4, IRS1 and ß-actin protein expression levels in BeWo cells incubated with NE (80 nM) and/or insulin (100 nM). (**E**) Radiometric Glucose Uptake in BeWo cells incubated as in (D). RFU, relative fluorescent unit; AU, arbitrary unit.

Hyperglycemia triggers TNF-mediated activation of PMNs in BeWo cells

In both clinical and experimental conditions hyperglycemia is associated with the generation of pro-inflammatory cytokines. In order to quantitate the impact of high glucose on the production of TNF-α from trophoblast during GDM, we incubate BeWo cells in the presence of both 5 mM (NG) and 25 mM (HG) of glucose for 4, 8 or 12 h. A considerable production of TNF-α occurred in culture after 12 h of incubation at the mRNA (**Fig. 7A**) and protein levels (**Fig. 7B**). Incubation with mannitol had no effect on TNF-α secretion demonstrating that the high glucose-induced TNF-α secretion was not due to osmotic pressure.

To determine the functional role of TNF-α released from BeWo, we treated neutrophils with BeWo supernatants collected after 12 h incubation in the presence of NG or HG conditions. Neutrophils were incubated at 37 °C, 5% CO_2 for 3 hours and stained using SytoxGreen. From the SYTOX green stained cells recorded at the end of the 3 h culture period it is evident an increase of NETs in presence of HG (**Fig. 7C**, **Fig. 7D**). By using an anti-TNF-α neutralizing antibody it was demonstrated that the biological activity measured was due to the presence of TNF-α alone (**Fig. 7C**, **Fig. 7D**). In fact, in the presence of Infliximab (IFX) SytoxGreen staining was significantly reduced (**Fig. 7C**, **Fig. 7D**).

In order to mimic the interactions between trophoblast and PMNs, we established a co-culture of BeWo cells and neutrophils from healthy women controls. After 12 h of glucose pre-treatment of BeWo with NG, HG or with HG BeWo that had been supplemented with IFX 5 ug/ml PMNs were exposed to the culture. After 4 h of co-culture, immunofluorescent microscopy for NE and H3 citrullination staining reveled that hyperglycaemia environment leads to an increased pro-NETotic activity of neutrophils.
In fact, as shown in **Fig. 7E**, co-culture of PMNs with BeWo in HG conditions results in a higher neutrophil elastase release. Inhibition of TNF-α with neutralizing antibody significantly prevents the staining of extracellular NE (**Fig. 7E**, **Fig. 7F**).

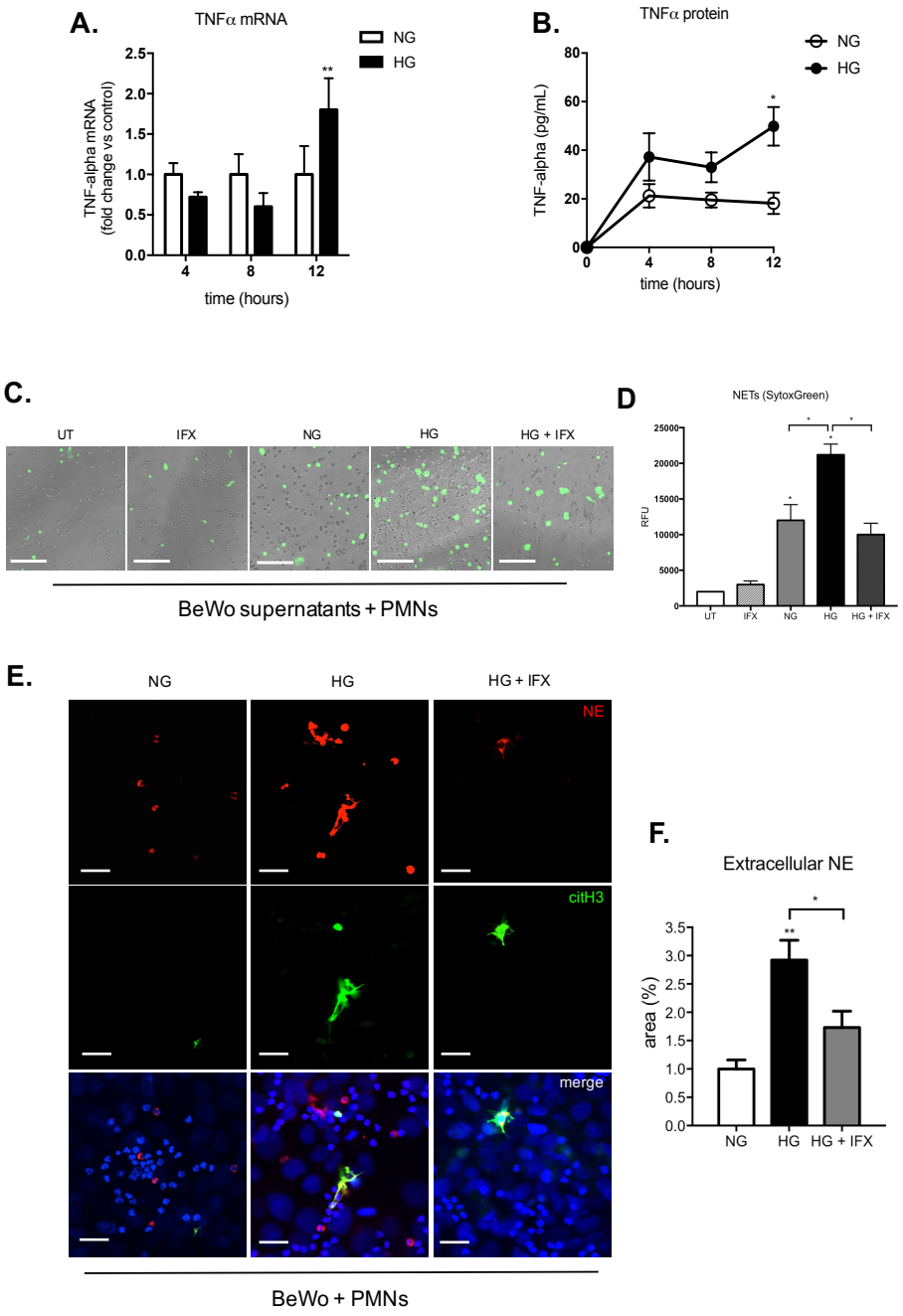


Fig. 7. Hyperglycaemia triggers TNF-mediated activation of PMN in BeWo cells. (**A**) mRNA expression level of TNF-α determined by qPCR in BeWo cells incubated with glucose (25 mM) for 4, 8 and 12 hours. (**B**) Detection of TNF-α by ELISA in BeWo cells incubated as in (A). (**C**) Assessment of spontaneous NETs by fluorescent microscopy using SytoxGreen in neutrophils from non-pregnant control group incubated with BeWo supernatants isolated after 12 h incubation with HG (25 mM). Scale bars: 100 µm. (**D**) Assessment of spontaneous NETs by fluorescence microplate reader using SytoxGreen dye in neutrophils as in (C). (**E**) Immunofluorescence staining for NE (red), citH3 (green) and DNA counterstain (blue) in PMN–BeWo cell co-culture after incubation of PMNs for 3 hours in BeWo cells treated with HG for 12 hours. Scale bars: 50 µm. (**F**) Morphometric analysis of extracellular NE (NE^+/DPI^+) in neutrophils as in (E) using ImageJ software. UT, untreated; IFX, infliximab (5 ug/ml); NG, normal glucose (7 mM); HG, high glucose (25 mM).

DISCUSSION

Over the course of a normal, healthy pregnancy, the body undergoes substantial immunological and metabolic changes (Koren et al. 2012). Pregnancy complications, such as GDM, are associated with an increase of pro-inflammatory mediators that alter the complex balance ensuring maternal immune and metabolic health. Since NETs are recognized as an important neutrophils trapping mechanism and act as first barrier in defense of the organism they also may have a clinical impact shaping both immune and metabolic responses. Understanding the role of NETs during pregnancy will help to clarify the link between metabolism and the immune system during GDM. This study is the first to systematically compare the NETosis activity of PMNs, of 75 pregnant women from the 1st trimester to 3rd trimester of pregnancy. Furthermore, the study was conducted to test the degree of NETosis in women affected by GDM including its effect on placenta glucose metabolism.

We could show that NETosis capacity rises progressively during pregnancy, reaching highest activity in the 3rd trimester. Since maternal immune cells need to protect the mother and the growing foetus from infection during pregnancy, NETs could help neutrophils carry out their duty. As compared with nonpregnant women, pregnant women are more severely affected by infections with some organisms, including influenza virus, hepatitis E virus (HEV), herpes simplex virus (HSV), and malaria parasites (Kourtis, Read, and Jamieson 2014). NETs represent an important anti-microbial host defence based on the immobilization of invading pathogens. Extracellular NET-mediated entrapment has been shown for parasites, viruses, fungi and bacteria (Branzk and Papayannopoulos 2013a) contributing to growth inhibition and/or killing of the pathogen (Urban et al. 2009a). The placenta is an active immunologic site, capable of interacting with and responding to pathogens. The placental tropism of specific pathogens (e.g., listeria or *P. falciparum*) affects the susceptibility and severity of certain infectious diseases during pregnancy, as well as pregnancy outcomes (Mor and Cardenas 2010).

High citrullination levels of histone H3 were present in the plasma at late stage of pregnancy priming PMNs towards a pro-NETotic state. We hypothesize that pro-NETotic state observed during pregnancy develops in order to react immediately to a pathogenic threat. It is likely that a combination of pro- and anti-NETosis mediators are involved in fine-tuning of PMNs priming and NETs release during healthy pregnancy. As gestational age progresses, hormone levels, as well as cytokines and chemokines, change dramatically shaping neutrophil responses (Giaglis, Stoikou, Sur Chowd, et al. 2016, in revision). In general, circulatory G-CSF promotes NETs formation and its concentration in the blood increases during gestation. Other pregnancy hormons influence NETosis troughout gestation: one is chorionic gonadotropin, that increases NETosis capacity, and its level are very high during the first trimester. The second is the combination of estrogen and estradiol, which increases their concentration in the blood during gestation and stimulate neutrophils towards

NETosis. Our laboratory also identified the important modulatory role played by progesterone during gestation. Primed neutrophils exposed to progesterone are not able to translocate NE to the nucleus and do not decondens the nucleus. In this way neutrophils are primed but keept in the activatory state waiting for a secondary stimulus (i.e. PMA or bacterial products) to undergo NETosis (Giaglis, Stoikou, Sur Chowd, et al. 2016).

In the case of GDM, NETs are significantly increased in the peripheral blood compared to controls and even the ex vivo production with or without secondary stimulus (PMA) is increased compared to normal healthy pregnancy. The diabetic environment and the involvement of an inflammatory switch in GDM may contribute to the NETs imbalance observed.

T2D patients showed increased plasma NE, mono- and oligonucleosomes and dsDNA compared with non-diabetic control individuals (Menegazzo et al. 2015). In vitro data clearly showed how neutrophils release nuclear material (histones and DNA) and elastase upon high glucose stimulation (Menegazzo et al. 2015). Increases in inflammation is associated with both type 1 and type 2 diabetes and GDM. Many inflammatory component were extensively reported, particularly IL-6, TNF-α and IL-1β as characterizing of insulin resistance (King 2008). In the placenta and the subcutaneous adipose tissue in women affected by GDM a significant increases of TNF-α production was detected by Couglan et al. According to the authors, these observations suggest that the tissues of women with GDM increase the release of TNF-α in response to hyperglycaemia (Coughlan et al. 2001b). Because TNF-α is involved in the metabolic regulation of glucose, lipids, and insulin resistance, these data are consistent with the hypothesis that TNF-α is involved in GDM development (M et al. 2006; Coughlan et al. 2001b).

Since TNF-α and hyperglycemia in GDM coexist, we inquired if both factors are involved in the pro-NETotic priming of PMNs. We show that hyperglycemia and TNF-α synergistically increase NETosis and blockade of TNF-α can partially revert this effect. However, our in vitro experiments did not show an additive effect in citrullination of H3 compared to TNF-α treatment alone. Indeed, it was previously shown in another context that exposure of oligodendrocyte cells to TNF-α in culture increased PADI4 translocation into the nucleus leading to histone citrullination (Mastronardi et al. 2006b). It is plausible that TNF-α participate to the formation of the diabetic environment responsible for the phenotypic and functional attributes of neutrophils during GDM.

Furthermore, our data provide a comprehensive evaluation of the effect of hyperglycemia on NET release during in-vivo testing of GDM in healthy and affected

women. We analized NETosis in PMNs isolated from peripheral blood of women undergoing the oral glucose tolerance test routinely performed in the clinic to diagnose GDM. Comparing ex vivo spontaneous NETosis between PMNs isolated from fasting blood and those isolated 1-hr after exposure to high glucose concentrations we observed only in GDM group a significant increase of NETs products and citrullination of H3. Indeed, Wong et al. previously reported that neutrophils from diabetic mouse models revealed an increase in H3Cit+ when compared to normoglycemic mice and produced NETs after incubation in vitro without stimulation (Wong et al. 2015a).

However, many questions remain about the details of the molecular events underlying the release of NETs in response of TNF-α and glucose.

TNF-α can exert opposing, concentration-dependent effects on neutrophils to either accelerate their apoptosis or enhance their survival (Cross, Moots, and Edwards 2008). At a concentration lower than 10 ng/mL TNF-α enhances PMNs survival increasing the rate of turnover of Mcl-1 (Cross, Moots, and Edwards 2008). Notably, Mcl-1 is upregulated soon after PMA. (Hakkim et al. 2011b). Given that Mcl-1 is an antiapoptotic protein that plays a key role in neutrophil survival, further experiments are required to clarify the role of Mcl-1 in inhibition of apoptosis to allow NETosis. Since Mcl-1 interacts with voltage-dependent anion channel (VDAC) facilitating mitochondrial Ca^{2+} uptake and ROS generation in NSCLC cells (Huang et al. 2014), we hypothesised that a similar mechanism is at play for the induction of ROS-mediated NETosis in neutrophils. This signalling cascade needs further studies in the GDM contest.

The mechanisms whereby high glucose promotes NETosis are also unclear and deserve appropriate investigation. The ability of glucose to mimic PMA induced NETosis may be related to its effect on PKC (Ceolotto et al. 1999). Increases in PKC activity are found in a wide variety of tissues and cultured cells isolated from diabetic animals and humans exposed to high glucose levels (Giacco and Brownlee 2010). In addition, glucose-induced NADPH oxidase hyperactivity may be involved, as this enzyme activates granular proteases and is involved in NETs generation (Pilsczek et al. 2010a).

NE is one of the major granular proteases stored in primary granules of PMNs and it is implicated in chromatin decondensation, this is an essential step during the process of NETs formation. NE translocates to the nucleus and partially degrades core histones (Papayannopoulos et al. 2010b; Metzler et al. 2014). NE is also present on the NETs influencing the protease/antiprotease balance of this particular structure (Mansuy-Aubert et al. 2013). Enhanced activity of neutrophil elastase was detected in both serum and placenta tissue lysate from GDM affected women. Furthermore, placenta

villi derived from GDM affected women exhibit typical features of NETosis, such as MPO and citrullinated histone H3. MPO drives NETosis independently from its enzymatic activity (Papayannopoulos et al. 2010b). The neutrophil-specific protease escapes from azurophilic granules into the cytoplasm and translocates to the nucleus where is involved in histones degradation in a mechanism that involves synergy with NE (Papayannopoulos et al. 2010b; Metzler et al. 2014). MPO together with neutrophil elastase represents a content of over 5% among non-histone proteins present in NETs and therefore commonly used as a marker for NETosis (Urban et al. 2009b). Measuring the presence of citrullinated histone H3 in conjunction with the presence of MPO helps clarify the origin of the DNA released. The core histones are the major protein components in NETs and H3 represents 14.50% of total protein content (Urban et al. 2009b). Higher CitH3 levels were detected in diabetic mice subjected to excisional skin wounds compared to the normoglycemic mice (Wong et al. 2015a). DNase 1, which disrupts NETs, accelerated wound healing in diabetic and normoglycemic WT mice. Thus, NETs impair wound healing, particularly in diabetes where neutrophils are more susceptible to NETosis due to elevated glucose (Wong et al. 2015a).

These observations raise the intriguing question of whether a diabetic milieu in the placenta could drive PMNs toward a NETotic phenotype. Consequently, given the fact that high glucose promotes the generation of pro-inflammatory cytokines (Shanmugam et al. 2003; J. Wang et al. 2012; Gonzalez et al. 2012), we evaluated the capacity of trophoblast to respond to glucose stimulation with increased TNF-α production. By using trophoblast BeWo model, we have shown that cells exposed to high glucose (25 mM) increased the levels of TNF-α mRNA and protein secretion contributing to the secretion of NETs and the release of NE. The precise mechanisms by which hyperglycaemia regulates TNF-α expression in trophoblast have not yet been elucidated, although the generation of ROS by high glucose concentrations is believed to contribute to hyperglycaemia-induced inflammatory responses (Yu, Jhun, and Yoon 2011; Coughlan et al. 2001a). Future experiments should clarify the role of ROS in this system. However, we think that the pro-inflammatory effect of glucose on trophoblast may be a fundamental contributor to the development of NETosis observed in GDM villi.

The placenta expresses high amounts of insulin receptors relative to other tissues in the body. Although insulin was able to stimulate glucose transport in the first trimester placental villous explants (Anette Ericsson et al. 2005), previous reports have described that glucose transport in human term placental explants or trophoblast cell lines is not regulated by insulin (Boileau et al. 2001). This may be explained by the absence of the insulin-sensitive glucose transporter GLUT4 in the syncytiotrophoblast of the term placentas (A. Ericsson et al. 2005), whereas the syncytium in early

pregnancy expresses two insulin-sensitive glucose transporters, GLUT4 and 12 (Gude et al. 2003; A. Ericsson et al. 2005). However, placental insulin signaling has been reported to be altered in pregnant women with obesity or diabetes, both conditions associated with fetal overgrowth. The placentas of these women exhibit decreased protein expression of IRS1 and PI3K regulatory subunit p85α (Colomiere et al. 2009). Collectively, these studies suggest that IRS1 plays a crucial role in fetal development but the factors that regulate its expression are currently unknown. Since neutrophil elastase leads to proteolysis of IRS1 targeting the cleavage-site motif Ala-Ala-Pro-Val (Ostergaard, Nielsen, and Flodgaard 1992), we assumed that the enhanced activity of NE detected in GDM placenta may be linked to reduced expression of IRS1 in villi of those women. It was previously shown that treatment of hepatocytes and hepatocytes with NE causes cellular insulin resistance consistent with a marked decrease in IRS1 protein content. Deletion of NE in obese mice leads to decreased tissue inflammation associated with improved adipose insulin sensitivity (Talukdar et al. 2012). Indeed, we observed the loss of IRS1 expression and inhibition of glucose uptake in BeWo cells that were treated with recombinant NE. Interestingly, we also observed under NE treatment a reduction in GLUT4 expression despite not being specifically targeted like IRS1. However, placental GLUT4 mRNA and protein expression were not significantly modified in human pregnancies complicated by impaired glucose homeostasis (Xing et al. 1998). Whether GLUT4 perturbation in BeWo cells is causally related to insulin resistance or whether it reflects a coordinate regulation mediated by NE remains to be established. This discrepancy could be due to the model we use, the BeWo cells. Bewo represent first trimester placentas and are a good model for the study of the development of GDM. However, BeWo may respond differently than trophoblast cells isolated from placentas collected at delivery (Hertz 1959a; Pattillo and Gey 1968a),

Overall, it is tempting to speculate that the possible role of NE in contributing to the etiology of inflammation-induced insulin resistance is caused by mediation of IRS1 degradation. In consequence IRS1 degradation could be linked to the placental metabolic changes observed in GDM placentas. Colomiere et al showed that the decrease in IRS1 protein expression is usually compensate by the increase in IRS2 protein expression (Colomiere et al. 2009). During diabetes, Rodinone et al, described the increase in IRS2 expression in adipocytes as compensatory mechanism for the defective insulin signaling via IRS1 protein (Rondinone et al. 1997). IRS1 and IRS2 are not completely redundant, but rather are selectively linked to specific actions of insulin (Matsumoto et al. 2002). For example, ablation of the IRS1 gene markedly impaired insulin-induced adipocyte differentiation, whereas a deficiency of IRS2 in these cells had little effect on this ability (Fasshauer et al. 2001). The mechanisms by which each IRS protein contributes differentially to various actions of insulin remain to be determined. Noteworthy, degradation of IRS1 results in failure to form the

association complex of IRS-PI3K leading to free PI3K in cytoplasm (Houghton et al. 2010). In several tumour models, the free form of PI3K can associate with platelet-derived growth factor receptor (PDGFR) causing the growth of tumour mass (Houghton et al. 2010). It follows that IRS1 degradation in may provide a mechanistic link between the insulin resistance and the increased weight of GDM placentas. Also this point needs further studies.

Although, to date, few studies have investigated the impact of maternal or placental inflammation on offspring development and later health, it is known that children born to these GDM mothers have an increased risk of the development of obesity and type 2 diabetes. Since, there is a shift in control of insulin-dependent processes from the mother at the beginning of pregnancy to the foetus at the end (G. Desoye et al. 1997; G. Desoye et al. 1994), the NETs insult in the placenta may lead to important consequences for the growth and development of the fetus and health of the offspring in adulthood. Catalano et al. showed that newborn infants of women with GDM have 20% higher body fat than infants of women with normal glucose tolerance, regardless of birth weight (Catalano et al. 2003).

Pre-eclampsia has been frequently reported as a complication of gestational diabetes (Ostlund, Haglund, and Hanson 2004) but the relationship between these two conditions is not well understood. Several studies suggest underlying common pathophysiology, including insulin resistance, chronic inflammation and endothelial dysfunction (Borzychowski, Sargent, and Redman 2006; Ostlund, Haglund, and Hanson 2004). Since NETs and neutrophil elastase were also identified in the intervillous space of preeclampitc placentas (A. Gupta et al. 2006), our new findings suggest a common pathophysiology linking GDM to hypertensive complications. Further studies are needed to determine whetever interventions to reduce NETs in GDM also reduce preeclampsia risk.

Our new findings suggest that reducing NE activity may improve pregnancy outcomes in women with gestational diabetes mellitus and offer therapeutic potential to break the harmful consequences in the placenta reducing the risk of diabetes in late life for both the mother and foetus.

CONCLUSIONS

According to our study, NETosis activity differs significantly between pregnant and non-pregnant women and increases over time from early to late-pregnancy. NETs were significantly higher in the GDM compared to the healthy pregnant women at both systemic and placenta level. Our study indicates a link between increased TNF-α and glucose concentration and NETs formation. Finally, in GDM placentas, we found a negative correlation between NE levels and IRS1 expression that links neutrophils activity to metabolic changes occurring during pregnancy in the diabetic environment of the placenta.

Altogether, our findings provide a new link among hyperglycemia, inflammation and NETosis. However, to understand the functions and dynamics of PMNs during pregnancy and their related complications, such as GDM, more basic research is needed. In particular, is necessary to identify the signal transduction pathways triggered by maternal metabolites that are responsible to drive NETosis and migration of PMNs. Moreover, it is crucial determining the specific origins of the regulatory factors involved in the initiation and progression of hyperglycemia and that participate in the development of an abnormal inflammation response. Prospective studies are also needed to assess whether the increased presence of NETs components can be used as early biomarkers to diagnose GDM in pregnant women. Whether potential therapeutic interventions to inhibit aberrant NETosis or block the activity of NE linked to the pathogenesis of post receptor insulin resistance will lead to improved treatment of this complication without markedly decreasing mother's immune system and/or affects fetus development, also needs further examination.

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CURRICULUM VITAE

FRANCO GRIMOLIZZI
Mobile: +41779073359 Email: franco.grimolizzi@unibas.ch

Contact information

Name: Franco Grimolizzi
Address: Froburgstr. 19, 4052, Basel, Switzerland
Mobile: +41779073359
Email: franco.grimolizzi@unibas.ch
Nationality: Italian
Birthdate: 1987-06-13

Profile

I consider myself as open minded and innovative. I have strong organization skills and able to handle multiple responsibilities. I am determined and enthusiastic. I am confident working independently or as part of a team and I have working experience in a variety of international environments.

Education

- 02.2015-present: **Visiting PhD student,** as part of the European PhD program, at the Department of Biomedicine, Prenatal Medicine Lab (PI Prof. Dr. Sinuhe Hahn), Basel, Switzerland. Project: Neutrophil–trophoblast interactions in gestational diabetes mellitus: key role for neutrophil elastase.
- 09.2013 – 12-2014: PhD student in Biomedical Sciences at the Department of Clinical Sciences, Medical Genetic Lab (PI Prof Franca Saccucci), Ancona, Italy. Project: Immunosuppressive effect of placental exosomes through adenosine production.
- 07.2013: **Master Degree in Applied Biology** at the Marche Polytechnic University, Ancona, Italy. Thesis: Aberrant exosomal expression of miR-126 in patients with non-small-cell lung cancer.
- 03.2011: **Bachelor's Degree in Science Biology** at the Marche Polytechnic University, Ancona, Italy. Thesis: Role of transposable elements in gene regulation and in the evolution of mammalian vertebrates.

Work Experience

- 06.2015-10.2016: **Research assistant** assistant at the Department of Biomedicine, Childhood Leukemia Lab (PI Prof. Dr. med. Jürg Schwaller), Basel, Switzerland. Responsabilities: genotyping, gel electrophoresis, immunoblotting, sequencing, quantitative real-time PCR, mouse dissection.
- 09.2013 – 12-2014: **Undergraduate Tutor** at the Marche Polytechnic University, Ancona, Italy. Responsabilities: help and guide students on all matters related to their academic experience, including advice on progression and careers.
- 06.2012-12.2012: **Laboratory assistant** at the Laboratory Analyses AB, Ancona, Italy. Responsabilities: blood typing, genotyping of human coagulation factors SNPs, fecal occult blood test.
- 01. 2010-06.2010: **Laboratory assistant** at the University Hospital, Ancona-Italy. Responsabilities: quantitative analysis of immunosuppressant drugs in whole blood, urinalysis.

CURRICULUM VITAE
FRANCO GRIMOLIZZI
Mobile: +41779073359 Email: franco.grimolizzi@unibas.ch

Publications

- **Grimolizzi F*,** Stoikou M*, Rossi S, Hoesli I, Lapaire O, Hasler P, Giaglis S and Hahn S (2016). Neutrophils alter placental glucose metabolism in gestational diabetes mellitus via neutrophil elastase mediated IRS1 degradation [in preparation]
- Giaglis S, Stoikou M, Sur Chowdhury C, **Grimolizzi F**, Schaefer G, Rossi S, Hoesli I, Lapaire O, Hasler P, and Hahn S (2016). Multimodal regulation of NET formation in pregnancy: progesterone antagonizes the pro-NETotic effect of estrogen and G-CSF. Frontiers in Immunology [under revision].
- Giaglis S, Stoikou M**, Grimolizzi F**, Subramanian Y B, Hasler P, Hoesli I, Lapaire O, Than G, and Hahn S (2015). Neutrophil migration into the placenta: good, bad or deadly? Cell Adhesion & Migration.

Conference Participation and Training

- May 2016: Annual Meeting of the **International Society for Extracellular Vesicles**, Rotterdam, Netherlands
- September 2015: **Basel Immunology Focus Symposium**, University of Basel, Switzerland
- July 2015: **CTR Placental Biology Course**, University of Cambridge, England
- October 2014: **Ethical and Regulatory Aspects of Clinical Research Course,** Marche Polytechnic University, Ancona, Italy.

Languages

- **Italian**: Mother tongue
- **English**: Proficient
- **Spanish**: Basic

Laboratory and software skills

- Molecular/Cell Biology: DNA and RNA isolation from diverse biological sources, gene cloning from bacteria, PCR, qPCR, DNA sequence analysis, gel electrophoresis, sequencing, cell culturing of adherent and suspension cell lines, primary cell culture, Flow cytometry.
- Biochemystry/Histology: Spectrophotometry, Protein purification, Enzyme assays, Ultracentrifuge, SDS-PAGE, Western Blotting, Enzyme-linked immunosorbent assay (ELISA), Immunoprecipitation, Tissue embedding, Cryostat sectioning, immunohistochemistry (IHC), immunocytochemistry (ICC), Fluorescent Microscopy.
- Background on working with a mouse model, genotyping, dissection.
- Software: MS Office (IC3 certification), GraphPad Prism, ImageJ, FlowJo, Primer3, Image Lab

Hobbies

Swimming, Cooking, Reading, Cycling

References can be provided upon request