Contributions to genetics, immunology and nutrition in preeclampsia

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ABBREVATIONS

AMPK: Adenosine monophosphate -activated protein kinase
ARDS: Adult respiratory distress syndrome
BM: Basal membrane
CHOP: Transcription factor C/EBP homologous protein
CRP: C-reactive protein
CT: Cytotrophoblasts, cytotrophoblast cells
DAMP: Damage-associated molecular pattern
ER: Endoplasmic reticulum
EV: Extracellular vesicle
EVT: Extra-villous trophoblast
GW: Gestational week
HELLP: Hemolysis, elevated liver enzymes and low platelet count
HPA axis: Hypothalamic pituitary adrenal axis
HMGB1: High mobility group box 1
IL: Interleukin
ILVs: Intra-luminal vesicles
IR: Insulin resistance
IUGR: Intra-uterine growth retardation
LC3-II; Light chain3-II
Mt-DNA: Mitochondrial DNA
MOF: Multiple organ failure
MOMP: Mitochondrial outer membrane permeabilization
MVBs: Multi-vesicular bodies
MVM: Micro-villous membrane
NF-κB: Nuclear Factor-κB
NK cell: Natural killer cell
PAMPs: Pathogen-associated molecular patterns
PCD: Programmed cell death
PlGF: Placental growth factor
PRR: Pattern recognition receptor
RAGE: Receptor for advanced glycation end products
RCD: Regulated cell death
ROS: Reactive oxygen species
SASP: Senescent-associated secretory phenotype
SIRS: systemic inflammatory response syndrome
SNA: Syncytial nuclear aggregates
SNS: Sympathetic nervous system
ST: Syncytiotrophoblast
sEng: Soluble endoglin
sFlt: Soluble fms-like tyrosine kinase-1 (VEGF-receptor-1).

ST: Syncytiotrophoblast (layer)
SLE: Systemic lupus erythematosus
TLR: Toll-like receptor
Th2; type 2 helper T cell
TGF-β: Transforming growth factor-b
TNF-α: Tumor necrosis factor-a
TRAIL: Tumor necrosis factor (TNF)-related apoptosis-inducing ligand
Treg cell: Regulatory Thelper cell
UPR: Unfolded protein response
VEGF: Vascular endothelial growth factor
WAT: White adipose tissue

NORMAL PREGNANCY

A. INTRODUCTION; THE GESTATIONAL TRAJECTORY

During human pregnancy, a new 'organ' (i.e. placental-fetal unit) is introduced within the woman's body for a transient period of time. Strategically positioned between the mother and the fetus, the placenta forms a barrier to protect the fetus, transfers maternal oxygen/nutrients to and disposes waste from the fetus, and exerts profound endocrine functions. The mother is willing to immunologically accept her intruder and allows it to induce significant changes in her metabolism. The induction of tolerating regulatory T cells (Treg cells) targeted against fetal alloantigens is central in this acceptance, recently reviewed by Robertson et al. (Robertson, 2018) and is initiated even prior to conception during unprotected intercourse (Guerin, 2011, Robertson, 2009).

The average length of human pregnancy is 40 weeks (280 days) (ACOG, 2013), which is the time needed for the neonate to be prepared for the start of its further extra-uterine development (the large human brain requires a long intra-uterine period and is in a relative immature state at birth).

Maternal and gestational tissues are subject to a tightly regulated and coordinated program. Fetal tissues that end their organismal life with delivery, like fetal membranes and placental villi, undergo steps of biologically planned aging (Phillipe 2017). Maternal tissues, including decidual tissue, white adipose tissue (Resi, 2012) (*see background; maternal white adipose tissue*), and the cervix (Keelan, 2018) undergo developmental trajectories of their own.

The placenta/fetal membranes develop, mature, and age in a temporally and spatially coordinated manner. The gestational program makes use of pro-inflammatory pathways (e.g. blastocyst implantation, onset of parturition), whereas gestational tissues (e.g. villous trophoblast) experience well-controlled stresses with the concomitant sending of "danger" signals, notably prior to parturition.

Key decidual processes. Events in early pregnancy are representative of the strict trajectory that turns pregnancy into a success. For the upcoming events, the maternal uterine lining develops into a receptive decidua (beginning at the end of the reproductive cycle) (Moffett, 2015, Pijnenborg, 2006), providing nutritional cover (via decidual glands) for the embryo in the early gestational stages (Burton, 2002, 2017b). Here, conditions are created to tolerate the fetal semi-allograft and to allow the extra-villous trophoblast (EVT) to migrate through the maternal decidua to fulfill crucial functions. For instance, EVT invades decidual glands to provide "histiotrophic" nutrition (Moser, 2015, 2010), and, as recently proposed, invades uterine veins to facilitate waste drainage (Moser, 2017). However, the most renowned function of EVT is its role in the remodeling of maternal spiral arteries and to secure efficient utero-placental blood flow for the remainder of the pregnancy. This is a carefully staged process, and constitutes a joined effort of EVT and

maternal decidual immune cells, in particular natural killer (NK) cells and macrophages (Faas, 2017, Hanna, 2006, Lash, 2016, Pijnenborg, 2011, 2006). Remodeled spiral arteries lose their vascular smooth muscle and elastic material, which is replaced by amorphous fibrinoid material (Pijnenborg, 2011, 2006, Burton, 2017b). These remodeled end-arterioles become unresponsive to constrictive stimuli (Burton, 2009a), and their lumen may become dilated up to 10-fold (Labarrere, 2017). Hence, maternal blood flows under non-turbulent, low-pressure, low-velocity conditions, which secures permanent oxygen provision and prevents damage to the villous tree upon passage through the intervillous space (Burton, 2009a, James, 2010). "Poor placentation" is used to refer to aberrant, less optimal placental development related to defects in remodeling. Poor placentation is associated with various pregnancy complications, including intra-uterine growth retardation (IUGR), preterm delivery and preeclampsia (Labarrere, 2017).

Un-remodeled spiral arteries remain responsive to vasoconstrictive stimuli, retain a narrow lumen (Pijnenborg, 2006), display high-pulsatile, jet stream flow conditions (low absolute flow), which can induce ischemic-reperfusion injuries to the villous tree (Burton, 2009a, 2009b, James, 2010) and inevitably leads to placental oxidative stress (Burton, 2017a). Remodeling of about 100-110 spiral arteries (Laberrere, 2017, Pijnenborg, 2011) is completed in the second trimester (temporal program), affecting maternal spiral arteries located in the decidua up to the first third of the myometrium, expanding from central to lateral parts (spatial program) (Pijnenborg, 2011, 2006). Such spatial and temporal arrangements enable maternal blood supply to evolve in pace with the higher demands of the developing fetus (Pijnenborg, 2011). Not all maternal spiral arteries are fully remodeled, not even in uncomplicated pregnancies (Moser, 2017).

Placental villi. During the first stage of pregnancy, embryonic/fetal needs are of a qualitative rather than quantitative nature (Hadden, 2009, Herrera, 2006, von Versen-Hoenyck, 2007). Much effort is put into the differentiation, growth, and maturation of the placental villous tree, and building up maternal fat reserves (Hadden, 2009). Profound vascularization and branching lead to the villous-like structure that expands the exchange area considerably (Mayhew, 2002). The functional layer of the placenta, the syncytiotrophoblast (ST), is a unique syncytium of terminally differentiated (non-dividable) continuous epithelium that covers the villous tree (Longtine, 2012a). Fresh organelles are supplied by the underlying cytotrophoblast (CT) layer, which acts as a progenitor pool (Fogarty, 2015).

Changes in endocrine functions start immediately after fertilization, a task primarily executed by the *corpus luteum* and villous tissue thereafter. In early pregnancy, these modifications induce the more subtle changes in maternal metabolism to meet with qualitative fetal needs. In later pregnancy, placental hormones, like the placental-variant of growth hormone (V-GH), trigger the mother to re-allocate her resources (Hill, 2018). An efficient way is the creation of an insulin resistant (IR) state, which redistributes maternal nutritional fluxes in favor of the placental-fetal unit. For instance, the fat reserves build up in maternal white adipose tissue (WAT) in the first part of pregnancy are mobilized in the

later stages to accommodate the increasing needs of the exponentially growing fetus. This "biphasic" WAT response is regulated in a temporal manner (Catalano, 2015) and relies on changes in maternal insulin sensitivity (Barbour, 2007, Lappas, 2014, Resi, 2012). Everything is coordinated in a timely fashion in order to meet with the fetal requirements for that particular stage of pregnancy (*see background; maternal white adipose tissue*).

In its own way, the placenta takes care of the mother. The placenta secretes factors like placental growth factor (PlGF, discussed later), that protect her vascular endothelium. Placental hormones, such as estrogen, provide protection by minimizing unwanted side effects during the maternal efforts to adapt her metabolism in favor of the fetus. For example, estrogens have been implicated in the down-regulation of hepatic lipase (Alvarez, 1996, Kinnunen, 1980), which is essential in minimizing atherogenic risk in the late gestational hyperlipidemia. Finally, the placenta, especially the ST, releases various vesicular entities, some secreted by other cell types and others unique to the placenta. Release of these vesicles has been implicated in many maternal physiological (metabolic) adaptations (Mincheva-Nilsson, 2014) and protection (Chen, 2012, Wei, 2016) (discussed later).

Increasing fetal demands, accumulating over the last trimester, put placental functioning in an 'overdrive'; the placenta has to activate/trigger the mother to intensify her resource re-allocation and to take up, process and transfer oxygen/nutrients to fulfill all fetal needs. This induces a well-controlled, 'physiologically-induced stress' as part of a normal gestational trajectory and results in an aged placenta towards term, i.e. at the end of its lifespan. Onset of human parturition is a synchronized process, integrating various inputs from the fetus to the mother; these include fetal maturation, fetal growth (uterine distension), and fetal stress (activating corticoid production by fetal adrenals), signaling that the fetus is ready for existence outside of the mother's womb (Menon, 2016a, 2016b). If successfully completed, an adequately developed healthy baby is born to a mother, whose resources are extensively but not fully utilized. This allows her to nurture her newborn for a fair period of time.

B. THE PLACENTAL VILLOUS TROPHOBLAST

The functional layer of the placenta, the multi-nucleated ST acts as a true barrier (Huppertz, 2014) and is devoid of inter-cellular gaps. The ST has a maternal-facing micro-villous membrane (MVM, also termed apical membrane) and a fetal-facing basal membrane (BM). The MVM is in direct contact with maternal blood that passes through the intervillous spaces. From early pregnancy to term, the ST area increases by more than 10-fold (Goldman-Wohl, 2014). This is primarily confined to surface area expansion, as the layer actually gets thinner, especially around fetal capillaries, as pregnancy progresses. This facilitates the transfer of nutrients and oxygen across the MVM, BM, and fetal capillary endothelium (Desforges, 2010). Villi in the proximity of maternal spiral arteries will thrive and grow, and those far away will succumb and form the chorionic layer.

The underlying mono-nucleated cytotrophoblast cells (abbreviated CT) act as stem cells and form the progenitor pool for the non-proliferating ST layer (Longtine, 2012a). Throughout pregnancy, CT proliferate continuously, differentiate, and fuse with the ST layer. As such, the ST layer is supplied with fresh nuclei (Huppertz, 2014) and other new organelles, like mitochondria, albeit turnover rates are unknown (Burton, 2017a). Prior to fusion, the number of organelles is increased in differentiated CT and nuclei resemble those in the ST (Burton, 2009c).

Supplying fresh nuclei to the ST is of vital importance, since ST nuclei lose their transcriptional activity over time (Ellery, 2009). ST nuclei are dispersed through the syncytium, but also form clusters, i.e. syncytial nuclear aggregates (SNAs), such as syncytial sprouts and syncytial knots. Syncytial sprouts**,** abundantly present in early gestation, are associated with villous growth and proliferation, and harbor recently incorporated, transcriptionally active nuclei (Burton, 2009c, Fogarty, 2013). They can easily detach and end up as "teardrop-like" macro-vesicles in the intervillous space (occurs ~100,000 times a day) (Burton, 2009c). The high frequency of this unique phenomenon may serve functional roles within the maternal body (Chamley, 2011) and reduce numbers of ST nuclei (Burton, 2009c). Syncytial knots are rare before GW 32, are prominent in the last trimester, and increase in number towards term (Burton, 2009c, Fox, 1965). Syncytial knots contain nuclei that are no longer transcriptionally active and show signs of oxidative damage after residing within the ST for a long time (Burton, 2009c, Fogarty, 2013, Jones, 1977). These knots are present in normal placental tissue, but numbers profoundly increase in post-term villi, with a more moderate increase in for instance preeclampsia (Burton, 2009c). Therefore, the "knotting index" may be a marker of an aging placenta, but could also serve as an index for preeclampsia severity (Fogarty, 2013).

1. Syncytiotrophoblast

Due to its syncytial nature, resistance to apoptosis in the ST is fundamental. Spreading of apoptosis through the syncytia would affect the entire functional layer, and thereby render it incompatible with pregnancy continuation (Longtine, 2012a, Burton, 2017a). The ST can be considered as one single multinucleated cell that covers the placental villi (Tong, 2016). But unlike other cells that undergo regulated cell death (executed by molecular machineries) (*see background; cellular death*) as part of their normal turnover or after unresolved stresses (then cells are no longer of use, even confer a "danger"), the life of the ST ends at placental delivery at the final stage of human parturition.

Indeed, caspase-mediated apoptosis is not seen in intact syncytium in normal term villous explants (Longtine, 2012a) or in preeclamptic and IUGR villous explants (Longtine, 2012b). At best, damaged areas are sequestered, which is a prerequisite to undergo caspase-mediated apoptosis (Longtine, 2012a). Apoptotic fragments disperse in the circulation, a process increased during preeclampsia and the areas can evolve in fibrin-type fibrinoid-lesions (Longtine, 2012a, 2012b).

As stated above, the onset of human parturition is induced by various inputs from fetal maturation, growth and stress (Menon, 2016a, 2016b). Inputs involve inter-dependent developmental trajectories, also referred to as "functional clock mechanisms" (Menon, 2016b), with increasing weight approaching term. All inputs are integrated and converge/ synchronize into a functional progesterone withdrawal (*see background; functional progesterone withdrawal*), which unlock a laboring phenotype in the myometrium (rhythmic contractions) and the cervix (shortening, dilating) (Menon, 2016a, 2016b).
Hence, even a dys-homeostatic (distressed) ST will likely 'survive' longer compared to a 'regular' cell in distress, as its death is not be a decision of its own. When occurring early in pregnancy, at a time when other (parturition) inputs have not much weight yet, a distressed ST may 'survive' quite long. As an in-between organ, this can impact both the fetus (e.g. compromised fetal transport) and the mother (e.g. release of adverse stress signals).

Resistance to apoptosis has been confirmed experimentally; intact syncytial ST from villous term explants stimulated with an inducer of apoptosis did not display apoptotic cell death (Longtine, 2012a). In culture, resistance to hypoxia-induced apoptosis develops upon CT differentiation (Levy, 2000).

It has been proposed that ST senescence is induced following CT fusion, based on the expression of senescent markers, such as p16, p21, and p53 observed in term ST (Chuprin, 2013). Senescent cells are resistant to apoptosis (*see background; cellular senescence*) and the induction of a senescent state may support ST "viability" throughout pregnancy (Chuprin, 2013). In addition, the ST apical membrane does not express the death receptors Fas and TRAIL-R (tumor necrosis factor (TNF)-related apoptosis-inducing ligand-receptor); thus the ST is protected against extrinsic apoptosis mediated by the ligands of these death receptors (Stenqvist, 2013). Phenotypical changes in mitochondria during CT differentiation may also contribute to the resistance to apoptosis. Mitochondria play a central role in regulated cell death and once depolarized, are initiators of the intrinsic apoptotic signaling cascade (*see background; mitochondrial-centered death pathways*). Starting in one region it can rapidly spread throughout a cell or syncytium; resistance to apoptosis is pivotal in preventing such a devastating scenario (Longtine, 2012b). Compared to those in the CT, mitochondria in the ST become smaller (Poidatz, 2015) and are more dedicated to steroidogenesis (with the production of considerable amounts of free radicals) rather than energy production (Bustamante, 2014, Martinez, 2015). ST mitochondria appear not well equipped to execute the apoptotic program, exemplified by low expression levels of pro-apoptotic proteins (Bustamante, 2014).

Despite the resistance to apoptosis, the ST does express caspases, which are proteolytic enzymes that mediate the apoptotic cell death program. This is in line with the more appreciated death-unrelated roles of these enzymes (Julien, 2017). For instance, caspase-8 may be involved in CT fusion processes (Black, 2004, Rote, 2010).

2. Cytotrophoblasts

In contrast, cytotrophoblasts (CT) can undergo apoptosis. In CT, the apoptotic rate increases from mid-pregnancy until term (and even more so past term) (Leung, 2001a, Sharp, 2010), as part of a normal gestational trajectory. At term, proliferating CT are relatively sparse (Sharp, 2014). However, an intrinsic waning of proliferative capacity has been disputed (Burton, 2009c). Small apoptotic (CT) bodies can disperse within the ST layer and can misinterpreted as indicators of ST apoptosis (Longtine, 2012a). Increased CT apoptosis attenuates the supply of new cellular material to the ST (Sharp, 2014) and may accelerate ST aging.

CT are progenitor cells with the ability to divide and are able to go into cellular senescence (*see background; cellular senescence*). This may be induced by various stress signals (Burton, 2009c) and potentially be part of the normal gestational trajectory. Losing the ability to proliferate, CT senescence reduces the regenerative capacity of the ST, contributing to (normal) aging. In addition, CT senescence may add to the inflammatory burden of the placenta by exhibiting the senescent-associated secretory phenotype (SASP).

Of note, CT produce the majority of lactate, used by the fetus as oxidative substrate, traditionally believed to originate from the ST (Kolahi, 2017). Hence, increased CT apoptosis may also impact substrate delivery to the fetus.

C. THE SYNCYTIOTROPHOBLAST IS SUBJECT TO AGING AND STRESS AND RELEASES UNTIL THE END OF PREGNANCY

Cells are very capable of "keeping themselves clean" (Terman, 2010), as they harbor effective repair machineries to eliminate cellular "waste" (produced due to normal functioning), such as (parts of) defective organelles and modified proteins. For instance, calpains and proteases degrade short-lived proteins. Autophagy targets "worn-out" organelles, long-lived proteins (also targeted by the unfolded protein response, UPR), as well as extensively damaged/modified proteins *(see background; autophagic-lysosomal flux and background; the unfolded protein response, UPR)*. Autophagy works together with the lysosomal compartment to complete full degradation. In addition, dividing cells can redistribute cellular waste over two daughter cells (Terman, 2010).

Terminally differentiated ST is unable to dilute cellular waste by division; and due to the syncytial nature inherent to its barrier function cannot be fully replaced under conditions of stress (see previous section). While the ST acquires fresh new organelles from the underlying CT and can therefore overcome a fair degree of organelle loss, worn out organelles and degenerated cell constituents have to be cleared. For this, the ST relies heavily on its catabolic degradative machinery (Chifenti, 2013), including proteasome activity, autophagic-lysosomal flux and the UPR. In addition, the ST has the capacity to dispose of trophoblastic material into the maternal circulation (discussed in detail later).

Repair and cleaning functions are not impeccable and every cell accumulates waste as they age. Forms of stress, especially oxidative stress, accelerate the aging process (*see background; aging*). The very dynamic ST will accumulate cellular waste over its nine-months existence, which is an integral part of the placental trajectory of aging.

1. Physiological functional stress in an aging syncytiotrophoblast

Remarkably, the ST encounters its most intense functional period towards the end of its lifespan. This coincides with the time that adaptive stress responses and degradative capacity, including autophagy and the UPR, may lose efficacy, as reported in many chronological aging processes (Brown, 2012, Cuanalo-Contreras, 2013, Cuervo, 2005). In addition, the proliferative capacity of the CT progenitor pool attenuates (although disputed by some, Burton, 2009c). Toward the end of pregnancy, fetal oxygen demands increasingly outpace maternal supply, which likely results in oxidative stress in the trophoblast (Burton, 2017a). The ST exhibits low antioxidant capacity (Holland, 2017), which facilitates aging induced by oxidative stress. As mentioned before, ST mitochondria are primarily engaged in steroid production (Martinez, 2015). Steroid synthesis remains active up until placental delivery, since maternal progesterone levels do not drop (Menon, 2016b) *(see background; functional progesterone withdrawal*). This may coincide with high reactive oxygen species (ROS) generation, speeding up the aging process. Physiological oxidative ST stress, if tightly controlled, serves functional roles, and is 'communicated' to the mother (see next section).

The rare presence of lipofuscin (pigment granules composed of lipid containing residues of lysosomes digestion) in term placentas (Cindrova-Davies, 2018, Parmley, 1981, Schröder, 2007) is indicative of increased oxidative stress associated with challenged lysosomal compartments (*see background; aging*). Lipofuscin accumulation has not been observed in placentas obtained from pregnancies around GW 32 (Parmley, 1981).

At a certain stage, substrate supply cannot longer meet with increased fetal oxygen/metabolic needs (Menon, 2016b). Even in normal, uncomplicated pregnancies, the fetus will start to experience fetal stress (Menon, 2016b), indicating that gestation has reached its final stage. A novel concept is that fetal membrane deterioration/senescence perfectly communicates this fetal stress. Together with distension, these signals are believed to be prime instigators of the onset of human term parturition (Menon, 2016a, 2016b, 2017).

2. The syncytiotrophoblast releases until the end of pregnancy

The ST releases various vesicular entities (extra-cellular vesicles, EVs). EVs were thought to represent a mechanism by which trophoblastic debris, like aged/effete ST nuclei, is disposed into the maternal circulation. However, the release of EVs have now been implicated in many maternal physiological adaptations (Mincheva-Nilsson, 2014) and protection (Chen, 2012, Wei, 2016). To date, the release of EVs is recognized as a novel way of inter-cellular communication (Adam, 2017, Mincheva-Nilsson, 2014, van Niel, 2018, Salomon, 2017).

Release of extra-cellular vesicles by the ST. The ST releases a heterogeneous population of EVs. Nano-sized (30-100 nm) and micro-sized (0.1-1 μm) vesicles release are shared with many other cell types, whereas larger sized packaged material (20-100 μm; 70 μm on average) is unique to the ST (Dragovic, 2013, Wei, 2016). The larger-sized vesicles (further referred to as macro-vesicles) become entrapped in the first encountered capillary bed, i.e. the maternal lungs as evidenced by autopsy material by Schmorl more than hundred years ago (Lapaire, 2007). Nano- and micro-sized EVs can freely enter the maternal circulation (Knight, 1998, Dragovic, 2013, Tong, 2017a) and are carriers of protein, lipids, as well as RNA species, like mRNA and miRNA (Colombo, 2014, Lo Cicero, 2015, van Niel, 2018).

Release of macro-vesicles has been viewed as a route to dispose aged/effete ST nuclei (Huppertz, 2006) and perhaps other trophoblastic debris. However, these macro-vesicles do serve functional roles in pregnancy (Chamley, 2011, Chen, 2012) and are not just debris that must simply be cleared (e.g. through engulfment by professional phagocytes) without any apparent consequences (see macro-vesicles).

Similarly, release of EVs in the nano- and micro-size range, shared by many other cell types, was initially considered as a way to remove surplus proteins and lipids from the releasing cell (van Niel, 2018). Within recipient cells, most EV cargo seemed to end up in the lysosomal compartment for degradation. However, interactions of EVs with recipient cells can induce functional changes, e.g. miRNA species affecting the recipient gene expression (Colombo, 2014). To date, the release of these EVs is recognized as an inventive way of inter-cellular communication, with the transfer of numerous components as opposed to single molecules (Adam, 2017, Mincheva-Nilsson, 2014, van Niel, 2018, Salomon, 2017). To this aim, EV cargo seems to be selectively "sorted" by specific machineries and the mechanisms underpinning vesicle biogenesis and cargo sorting are increasingly understood (van Niel, 2018). The composition of the EV cargo released may depend on cell type and physiological state (van Niel, 2018). As demonstrated in various settings, exogenous and endogenous stress signals in the releasing cell affect protein, lipid and RNA composition (Kucharzewska, 2013). Therefore, EVs provide valuable information on the condition of the releasing cell and are now being extensively explored as reliable, non-invasive candidates for e.g. surveillance in various disease states (van Niel, 2018).

Plasma membrane-derived micro-vesicles and endosomal exosomes. Nano-vesicles and micro-vesicles are most often classified based on site of origin. First, EVs can be generated via outward budding ("blebbing') of the plasma membrane. These EVs are in the higher sized range (50-1000 nm), and referred to as micro-vesicles (van Niel, 2018). Second, smaller EVs (30-100 nm) can be formed within the endosomal compartment and are defined as exosomes (Mincheva-Nilsson, 2014, van Niel, 2018). In brief, intra-luminal vesicles (ILVs) form within so-called multi-vesicular bodies (MVBs) in late endosomes. Usually these MVBs fuse with the lysosomal membrane, resulting in degradation of the ILV contents. However, some MVBs traffic and fuse with the plasma membrane and

release ILV into the extracellular space; these ILVs are now referred to as exosomes (Colombo, 2013, Mincheva-Nilsson, 2014, van Niel, 2018, Raposo, 2013).

Micro-vesicles and exosomes carry different cargo, but there is also considerable overlap (e.g. share some sorting machineries) (van Niel, 2018), even more than initially anticipated (Colombo, 2013). Given the overlap in cargo and size, and in addition to the lack of a definitive marker to discriminate between exosomes and micro-vesicles, isolation from biological fluids or cultured medium does not render pure enriched subsets (Lo Cicero, 2015). Despite considerable progress in this field (Lai, 2018) this must always be kept in mind when exosomes and micro-vesicles are discussed as individual (sub) groups.

ST-derived micro-vesicles and exosomes. Ultrastructural analysis revealed a well-developed endosomal compartment within many MVBs filled with ILVs and frequent fusion events with the ST apical membrane, indicative of vivid constitutive exosome release from the ST (Mincheva-Nilsson, 2014). Hence, exosomes have been recognized to play an important role in fetal-maternal cross-talk (Mincheva-Nilsson, 2014). Circulating micro-vesicles and exosomes originating from the ST are identified in the maternal circulation by the use of the ST-specific marker placental alkaline phosphatase (PLAP).

ST-derived exosomes (Salomon, 2017, 2014) and micro-vesicles (Germain, 2007) are reported to increase in number as pregnancy progresses, and their relative abundance appears to be of importance (Mincheva-Nilsson, 2014, Redman, 2012, Tannetta, 2017). Generally, ST-derived exosomes are considered immunosuppressive (see exosomes), whereas ST-derived micro-vesicles, especially in late gestation, are pro-inflammatory (Mincheva-Nilsson, 2014, Redman, 2012, Tannetta, 2017, 2013) (see micro-vesicles). The release of pro-inflammatory micro-vesicles has been proposed to parallel oxidative stress in ST towards term (Mincheva-Nilsson, 2014) and as such may serve as a reliable indicator of oxidative stress (Tannetta, 2017).

2a. Communicators of "trophoblast stress"; micro-vesicles and damage-associated molecular patterns (DAMPs)

2a1. Micro-vesicles. Micro-vesicles (synonym micro-particles) are released after "blebbing" events at the plasma membrane as part of normal turnover, upon cellular activation (e.g. activated platelets) and/or cellular stress (e.g. oxidative stress) (Mincheva-Nilsson, 2014, Redman, 2012). Micro-vesicles are formerly known as "platelet dust" and activated platelets are the most common source of circulating micro-particles even during pregnancy (Marques, 2012). After micro-vesicle release from the ST, the apical membrane closes again (Mincheva-Nilsson, 2014).

Significant levels of ST-derived micro-particles can be detected in the maternal circulation during the second trimester and increase over the third trimester (Germain, 2007). Micro-vesicles may be instructed to home to specific tissues (Tong, 2017a, 2016). *In vitro*, micro-vesicles obtained from term placentas have been shown to induce pro-inflammatory cytokine production (e.g. IL-6 and IL-8) in peripheral mononuclear blood

cells, like monocytes (Germain, 2007, Messerli, 2010, Southcombe, 2011), and are able to activate neutrophils (Gupta, 2005). These micro-vesicles carry anti-angiogenic (later discussed) factors (Tannetta, 2013, Guller, 2011) and active tissue factor (Gardiner, 2011, Guller, 2011).

Thus, late gestational micro-vesicles harbor pro-inflammatory and pro-coagulant factors and indeed can serve as a "read out" (Tannetta, 2017) of the late oxidative ST stress (Mincheva-Nilsson, 2014). Pro-inflammatory/pro-coagulant micro-vesicle release is an entirely physiological event (Mincheva-Nilsson, 2014), contributing to the well-controlled systemic inflammation in late pregnancy (Messerli, 2010, Southcombe, 2011) (see the low-grade maternal (systemic) inflammatory response). Likewise, it contributes to the physiological pro-coagulant state (Gardiner, 2011), which prepares pregnant women for the upcoming parturition as it can minimize blood loss.

2a2. Release of damage-associated molecular patterns (DAMPs). Like every other stressed cell, the ST can start to release damage-associated molecules patterns (DAMPs) (Nadeau-Vallée, 2016), also known as danger signals, danger molecules, or alarmins. This may be part of the normal gestational trajectory, inflicted by the physiologically-induced ST stress and persists for the remainder of the ST's lifespan. DAMPs can be released as isolated molecules, or within extra-cellular vesicles, like micro-vesicles (Redman, 2008). DAMPs are normally stored away in a cell, within the confinement of organelles. For example, ATP and mitochondrial DNA reside within the mitochondrial matrix, high mobility group box 1 protein (HMGB1) and genomic DNA are confined to the nucleus, and calreticulin is found inside the endoplasmic reticulum (ER). Upon organelle stress/damage, these danger molecules are liberated into the cytosol and subsequently induce adaptive responses (like autophagy) in an attempt to restore cellular homeostasis (Kroemer, 2010, Ma, 2013, Sica, 2015). If homeostasis is not restored, stressed cells can secrete these danger molecules (dys-homeostatic DAMPs) into the extracellular environment and to become accesible for immune cells. When the cell actually dies, DAMP release is intensified (Kepp, 2014).

DAMPs and pathogen-associated molecules patterns (PAMPs) are recognized by the same receptors, including pattern recognition receptors (PRRs, like Toll-like receptors). However, downstream pro-inflammatory responses are less pronounced after DAMP stimulation as compared to those in response to PAMP. PAMPs and DAMPs alert and attract (innate) immune cells to a site of pathogenic invasion and endogenous danger, respectively, with the intention to restore tissue homeostasis (Giaglis, 2016, Pittmann, 2013, Tan, 2017) *(see background; damage-associated molecular patterns, DAMPs)*.

DAMP release during pregnancy. The best studied DAMP during pregnancy is cell-free fetal DNA. Maternal levels of other DAMPs, like HMGB1 (Pradervand, 2014) and calreticulin (Gu, 2008) were higher in pregnant as compared to non-pregnant women, but did not change over the course of pregnancy. In contrast, cell-free fetal DNA appears in the maternal circulation (Lo, 1997) as early as GW 6 (Rijnders, 2003), its levels modestly

increase up to GW 20 and in considerable increments thereafter, and peak just prior to parturition (Phillipe, 2014). Clearance of cell-free fetal DNA is rather rapid (1-2 days after delivery) (Yu, 2013), and a continuous release of cell-free fetal DNA is present in pregnancy (Bischoff, 2005). The low levels of circulating cell-free fetal DNA in earlier stages of pregnancy may simply be a reflection of the turnover of nuclear material shed by the ST (*see background; circulating cell-free DNA*). Stress within the ST may eventually lead to the marked increases in circulating cell-free fetal DNA. Term villous explants *in vitro* actively secrete fragmented cell-free fetal DNA (Gupta, 2004). This secretion is increased under hypoxic-reoxygenation conditions that mimic oxidative stress (Tjoa, 2006). Release of genomic DNA fragments due to loss of nuclear integrity is uneventful in the multinucleated ST. Fetal membranes are important additional sources contributing to peak levels just prior to parturition onset (Phillipe, 2014).

DAMPs are used inventively. DAMP release by the ST will attract maternal innate immune cells towards the site of danger, in this case the intervillous space. Unlike classic DAMP signaling, these attracted maternal innate immune cells will not massively infiltrate into the DAMP release site, i.e. the ST, in order to 'resolve the danger'. Rather, DAMP release by a stressed ST will result in more activated maternal immune cells in the maternal circulation adding to the heightened maternal inflammatory state during late pregnancy (see the low-grade maternal (systemic) inflammatory response). In addition, attracted by the right (temporally regulated) chemotactic gradients, these activated maternal immune cells may populate and play functional roles in tissues like the decidua, myometrium and cervix (Gomez-Lopez, 2014).

Importantly, the decidua and myometrium are DAMP-sensing tissues (Nadeau-Vallée, 2016), and can take up DAMPs released by the ST directly. This all contributes to the buildup of a maternal pro-inflammatory load (Gomez-Lopez, 2014, Keelan, 2018, Menon, 2016b) in preparation for the upcoming parturition.

Release of DAMPs, such as fetal-cell free DNA (Phillipe, 2014) and HMGB1 (Menon, 2017, 2016a, 2016b) by fetal membranes is believed to signal fetal stress (Menon, 2016b) and has been implicated as a primary instigator of parturition onset by triggering functional progesterone withdrawal (Menon, 2017, 2016a, 2016b) (*see background; functional progesterone withdrawal*). The loss of anti-inflammatory actions of progesterone further increasing the inflammatory state within the gravid uterus (Menon, 2016b), intensifying the recruitment of maternal leukocyte populations into the decidua, myometrium and cervix, as such, propagating labor. Neutrophils seem to arrive late indicating a key role in tissue repair during labor and post-partum (Gomez-Lopez, 2014).

Taken together, by releasing micro-vesicles and DAMPs, the aging and stressed ST may trigger the mother to mobilize her resources (see a metabolic role for maternal systemic inflammation?) and prepare her for the upcoming parturition.

2b. Release of macro-vesicles and exosomes; waste disposal routes or fetal-maternal communication?

2b1. Macro-vesicles. Macro-sized packaged material is being shed into the maternal circulation from as early as 6 weeks of pregnancy (Covone, 1984). Macro-vesicles have been implicated in fetal-maternal communication, supporting the mother in her adaptive endeavors from the first trimester on (Mincheva-Nilsson, 2014, Tong, 2017a, 2016). The entrapment of macro-vesicles into the maternal lungs may optimize responses of her pulmonary endothelium by rendering the endothelium less responsive to activating stimuli, as evidenced *in vitro* (Chen, 2012). Macro-vesicles have been proposed to contribute to maternal cardiovascular adaptations by for instance, promoting vasodilatory responses of endothelial cells (Wei, 2016). In addition, macro-vesicle release is an opportunity to present (minor) fetal alloantigens to the mother (Lindscheid, 2015) in a safe context and may contribute to maternal tolerance (Abumaree, 2012, Chamley, 2011). Yet, macro-vesicle shedding is not a simple disposal route (Chamley, 2011, Wei, 2016), in which the shed material is just debris that must be cleared from the maternal circulation. In addition, most macro-vesicles have a normal appearance (Burton, 2009c) and represents detached syncytial sprouts, undoubtedly in first half of gestation when syncytial knots are rarely present (Burton, 2009c), and possibly throughout the entire gestational period (Burton, 2011, 2009c). This is not in line with the view (Huppertz, 2006) that aged/effete nuclei are shed into the maternal circulation as part of normal turnover, which has recently been called into question (Burton, 2009c, Calvert, 2016, Coleman, 2013, Fogarty, 2015, 2013).

Aged/effete ST nuclei in knots may be recycled within the ST itself (Burton, 2009c). The observed presence of autophagic vacuoles in close proximity of syncytial knots suggests a role for the autophagic-lysosomal machinery in the degradation of aged/effete nuclei. Many nuclei in syncytial knots display oxidative DNA damage (Fogarty, 2013), which is a known trigger for autophagy (Sica, 2015). We argue that only when the intrinsic degradative capacity to handle aged/effete knots nuclei is impaired, more actual nuclear debris will be exported. This may be the case at the end of normal pregnancy, when the autophagic-lysosomal capacity is attenuated in the aged ST. The number of syncytial knots markedly increases in post-term villi (Burton, 2009c), which could indicate a rather rapid deterioration of degradative capacity past term. This is in line with profound lipofuscin staining found in post-mature placentas (7-20 days past due date) compared to occasional staining in term placentas (Cindrova-Davies, 2018).

2b2. Exosomes. The ST vividly releases exosomes (Mincheva-Nilsson, 2014), which is thought to be related to functional ST area (Salomon, 2014). Exosomes have been implicated in placenta/fetal-maternal cross talk, contributing to many physiological maternal adaptations (Mincheva-Nilsson, 2014). For instance, RNA species within the exosome may reprogram the recipient maternal cells and its metabolic potential (Mincheva-Nilsson, 2014). As early as 6 weeks of gestation, ST-derived exosomes can be detected in the maternal circulation (Sarker, 2014) and numbers increase as pregnancy progresses (Salomon, 2017, 2014, Sarker, 2014). Exosomes may relocate to a specific

tissue and/or a specific cell type (Mincheva-Nilsson, 2014, Rana, 2012).

Exosomes released by epithelial cells are immuno-suppressive, including those released by the ST, respecting its epithelial origin (Mincheva-Nilsson, 2014). Epithelial-derived tumors can produce high amounts of immunosuppressive exosomes to evade host immunity (Andreu, 2014, Kucharzewska, 2013). Similarly, ST-derived exosomes may modulate maternal immunity via several mechanisms. For example, by contributing to the Th2 bias of normal pregnancy (Mincheva-Nilsson, 2014). *In vitro* evidence indicates that ligands on ST-derived exosomes down-regulate the activating NKG2D receptors on NK cells, cytotoxic T cells and γδ-T cells and inhibit cytotoxic activity, contributing to the Th2 bias (Frängsmyr, 2006, Hedlund, 2009). Syncytin-1 is sorted into ST exosomes and recombinant syncytin-1 reduces LPS-stimulated release of the Th1 cytokines interferon-γ (IFN-γ) and TNF-α in human whole blood (Tolosa, 2012). In addition, death receptor ligands (FasL, TRAIL) expressed on exosomal membranes (Sabapatha, 2006, Stenqvist, 2013) allow ST-derived exosomes to induce apoptosis of activated T cells, as evidenced *in vitro* (Stenqvist, 2013). Exosomal B7-H1 (PD-L1) and B7-H3 (Kshirsagar, 2012) can suppress T cell activation (Adam, 2017). Exosome concentration is highest within the intervillous space (Mincheva-Nilsson, 2014). Hence, this represents the prominent site of interaction/communication between bypassing maternal immune cells to meet exosomes (although exosomes and maternal immune cells do interact throughout the mother's body). Tumor-derived exosomes also bind leukocytes and instruct them not to adhere to (TNF-α-) activated endothelial cells (Lee, 2010). Such reduced adherence to endothelium has been reported for third trimester neutrophils (Krause, 1987).

The immuno-modulatory ligands FasL, TRAIL and PD-L1 are absent from the apical ST membrane. These ligands may be sorted from the Golgi apparatus into exosome precursors after *de novo* production (Mincheva-Nilsson, 2014). This could contribute to the specific immunosuppressive potency of exosomes.

Exosomes isolated from first trimester plasma increases HUVEC migration in a similar fashion as VEGF, and the authors speculated that exosomes are involved in vascular adaptations (Salomon, 2014).

As discussed previously, exosomes are formed within MVBs in late endosomes (Mincheva-Nilsson, 2014). Usually these bodies fuse with the lysosomal membrane, resulting in the degradation of ILVs (van Niel, 2018). However, some locate to the plasma membrane, fuse and release ILVs as exosomes (van Niel, 2018). The exact underlying regulatory mechanism driving MVB fate is not known (van Niel, 2018). As a result of lysosomal defects, more MBVs may locate to the plasma membrane, exporting cargo originally destined for degradation (such as defective/misfolded proteins) (Borland, 2018, Eitan, 2016, van Niel, 2018). Such a scenario has been proposed in neurodegenerative diseases, where exosomes export misfolded/toxic protein aggregates (such as β-amyloid) out of neuronal cells. This prevents cytotoxicity, and at the same time "spread" these toxic aggregates among other neuronal cells (Borland, 2017, van Niel, 2018). Indeed, brain exosomes (obtained post-mortem from Alzheimer's patients) carry more oligomeric β-amyloid as compared to brain exosomes from control subjects (Sardar Sinha, 2018).

Placental nano- (not specified to exosomes) and micro-vesicles carry aggregated transthyretin, a phenomenon increased in preeclamptic nano-vesicles (Tong, 2017e) (see preeclampsia, failing degradative capacity, a catabolic component in preeclamptic pathophysiology). A similar scenario as proposed for neurodegenerative diseases may then be involved in this pregnancy complication, with exosomes eliminating cellular waste from the ST.

3. The low-grade maternal (systemic) inflammatory response

In the late 1990s it became clear that as pregnancy progresses, pregnant women display a pro-inflammatory, low-grade, systemic response, characterized by a rise in activation markers of monocytes and granulocytes (Redman, 1999). The ST contributes to this systemic maternal response, as it is subject to aging ("inflammaging") and physiologically-induced stresses, with the release of pro-inflammatory cytokines (Hauguel-de Mouzon, 2006), ST micro-particles and DAMPs. Senescent CT cells (SASP), fetal membranes (just prior to delivery), maternal endothelial cells and the maternal white adipose tissue (WAT) compartment (*see background; maternal white adipose tissue*) contribute to the heightened maternal inflammatory state as well. Systemic increases in pro-inflammatory cytokines, including IL-6 and TNF-α, have been demonstrated longitudinally (Christian, 2014, Kirwan, 2002, Stewart, 2007), with an IL-6, IL-8 "surge" (but not TNF-α) approaching term (Ellis, 2001). A well-controlled and perfectly timed pro-inflammatory response may be highly functional in normal pregnancy. It has long been viewed as a physiological compensation for suppressed T and NK cell mediated immunity (Mincheva-Nilsson, Sacks, 1998, Tannetta, 2017). It may also be involved in the preparation for parturition (discussed previously), and we argue that increased levels of maternal inflammatory cytokines contribute to maternal nutritional/energy allocation in favor of the fetal-placental unit.

3a. A metabolic role for maternal systemic inflammation?

Timing of the maternal systemic low-grade inflammatory response coincides with increasing demands of the exponential growing fetus and the induction of insulin resistance (IR) in late pregnancy.

Pro-inflammatory cytokines have been proposed as initiators of the so-called "energy appeal reaction" during an immune response, i.e. to alongside the neuroendocrine system (HPA-axis and SNS, see abbreviations) allocate energy/nutrients to serve the high immunological demands (Straub, 2012, 2010). In addition, pro-inflammatory cytokines (e.g. IL-6, IL-15) released by hard working muscle have been proposed to trigger mobilization of distant white adipose tissue stores (lipolysis) to cover the extra muscular energetic needs (Eckardt, 2014, Pedersen, 2008). Another well-studied mechanism by which inflammatory mediators trigger energy/nutrient allocation is via the induction of IR (*see background; insulin resistance*). In brief, IR causes redistribution of dietary nutrients in favor of an energy/nutrient consuming event (like pregnancy is), mobilizes nutrients from (maternal) body storage and stimulates *de novo* production of e.g. glucose.

IR in late gestation was first recognized in the 1960´s (Burt, 1963) and later conclusively conirmed (Catalano, 1999, Homko, 1999, Kirwan, 2002, Ryan, 1985). IR leads to gross adaptions of maternal metabolism, notably the prioritization of dietary glucose in favor of the fetal-placental unit and the mobilization of maternal white adipose tissue stores, contributing to the late gestational hyperlipidemia. If nutrients prioritized/allocated are consumed, IR is a well-adapted physiological metabolic state allowing the body to cope with the (temporary) extra demands.

The late gestational IR state of pregnancy is multifactorial, with contribution of placental hormones, especially the placental-variant of growth hormone (GH-V) (Hill, 2018). Declining levels of insulin-sensitizing maternal adiponectin (Cseh, 2004, Guelfi, 2017) also contribute *(see background; maternal white adipose tissue*), considering the inverse relation between adiponectin and IR indices (Cseh, 2004, Lacroix, 2013). Exosomes derived from murine pancreatic cancer cells can trigger IR *in vitro* (Wang, 2017) and ST-derived exosomes likely represent novel candidates involved in the induction of the gestational IR state. The multifactorial nature of gestational IR may explain why correlations between maternal inflammatory mediators and various IR indices are modest (Guillemette, 2014, Kirwan, 2002, Melczer, 2002) or even absent (Altinova, 2007, Anim-Nyame, 2004, McLachlan, 2006, Saucedo, 2011). Some question the role of maternal cytokines in gestational IR (Hill, 2018) as opposed to others who believe that cytokines are important contributors (Hauguel-de Mouzon, 2006, Guillemette, 2014, Kirwan, 2002).

Taken together, we argue that the low-grade maternal inflammatory response contributes to the allocation of maternal resources to cover the increased metabolic demands in late gestation as suggested by others (Hauguel-de Mouzon, 2006). Via pro-inflammatory cytokine release, the ST inform the mother on its default metabolic demands, just like immune cells and hard-working muscle. It triggers direct (combined effort with HPA-axis and SNS) or indirect (IR) maternal energy/nutrient re-allocation. An excessive, and/or badly timed pro-inflammatory response, whether originating from the placenta or from maternal WAT, may result in the re-allocation of maternal resources in excess of those actually needed for that particular time in pregnancy.

D. SUMMARY

The ST is a post-mitotic syncytium that cannot dilute/distribute its waste by division. Hence, it primarily relies on its degradation capacity, which includes autophagy. The ST may experience oxidative stress and inflammaging, especially during the last stage of its lifespan when it has to be highly active (fetal supply, steroid synthesis). These events serve a function and are induced by physiological triggers as part of the gestational trajectory. Such forms of stresses may be exploited to optimize placental/fetal supply (maternal re-allocation) and finally prelude the end of pregnancy (**Figure 1**). An aged and exploited placenta at the end causes fetal stress and provides a signal that the baby is ready for existence outside of the womb, perfectly synchronized with other inputs (fetal

maturation, uterine distension) of the integral process of human parturition. Finalization of the gestational trajectory is considered successful when delivery is perfectly timed resulting in the birth of a fully developed newborn.

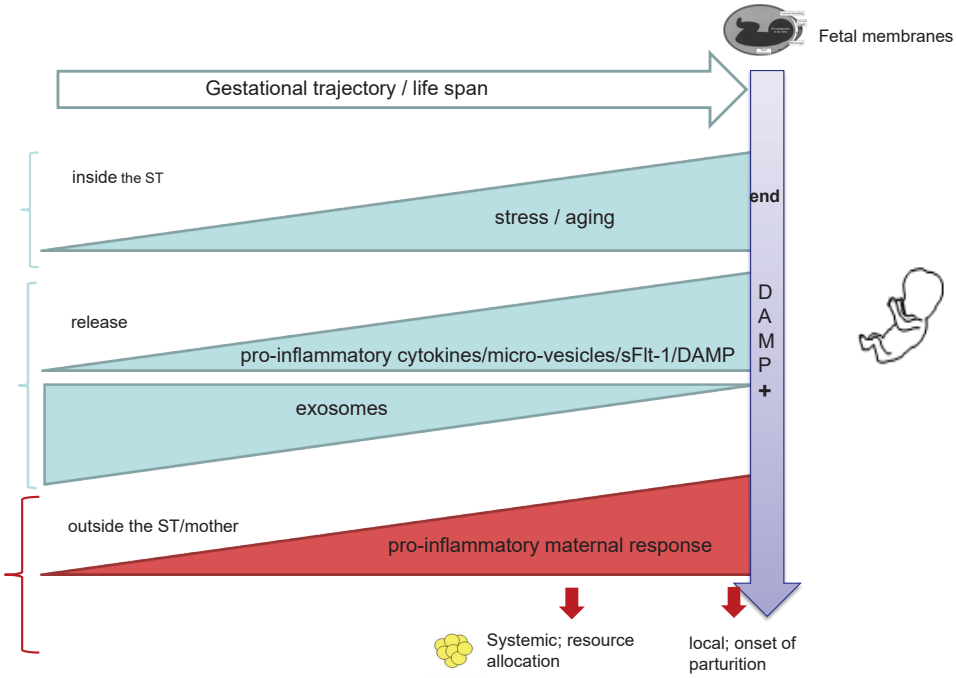

Figure 1. Normal gestational trajectory. The normal gestational stress trajectory of the placenta, which results in an aged, stressed placenta at the end of its lifespan. This may be used to promote maternal resource allocation and to trigger a perfectly timed parturition onset. DAMP, damage-associated molecular pattern; sFlt-1, soluble fms-like tyrosine kinase-1; ST, syncytiotrophoblast.

PREECLAMPSIA

A. PREECLAMPSIA, A HETEROGENEOUS SYNDROME OF PREGNANCY

Preeclampsia is a pregnancy complication affecting about 5-8% of all pregnancies worldwide (Cheng, 2016). The key hallmark is maternal endothelial dysfunction (Roberts, 1989). Preeclampsia is traditionally clustered within one syndrome, diagnosed by the combination of gestational hypertension, with rather arbitrary chosen cut-off levels (Roberts, 2009), in combination with proteinuria. The clinical manifestations of the syndrome, however, form a heterogeneous and at times life-threatening spectrum. The maternal symptoms can vary considerably in time of onset, severity, distribution of endothelial dysfunction and the organs involved. At one extreme, one may find a woman in the beginning of her third trimester, with blood coagulation problems, renal malfunction (gross urinary loss of proteins), liver dysfunction, in a complete hemostatic imbalance with hypovolemia, vascular hyper-permeability, hypo-albuminuria, and uncontrolled severe hypertension. All of which form a threat to her own life and that of her severely growth restricted fetus. You may also find a preeclamptic woman, who begins to seizure (eclampsia), a sign of involvement of the central nervous system. At the other end of the spectrum, one may find an obese mother, in her last weeks of pregnancy, with a normally thriving fetus and a little edema, where upon routine check blood pressure falls within the diagnostic criteria of preeclampsia, as does the urinary protein loss upon further examination. Although both extremes will be clinically diagnosed as preeclampsia, these women are not even close in terms of morbidity and mortality risk, risk of persistent organ damage, long-term health concerns, and the immediate and future health of her offspring. In addition, a preeclamptic variant is the HELLP syndrome, characterized by hemolysis, elevated liver enzymes and low platelet count (abbreviated “HELLP”), with its own distinct clinical presentation. Some women with HELLP do not experience hypertension (12-18%) or proteinuria (13%) (Sibai, 2004). It has become clear that the clinical manifestations are as heterogeneous as the numerous pathophysiological mechanisms that have emerged for this enigmatic maternal syndrome.

Preeclampsia seems to be a placenta-related disorder, as the only definite treatment is removal of the placenta, and because it may also develop in (non-embryonic) complete molar pregnancies (Soto-Wright, 1995). The syndrome can persist, however, after the removal of the placenta, and the onset of preeclampsia may even occur several days after delivery (Yancey, 2011).

In the past, spiral artery remodeling defects, i.e. poor placentation, were thought to be the obligatory first step in preeclampsia development. However, preeclampsia of late-onset (≥ 34 weeks) can occur in the absence of poor placentation (Redman, 2014). In case of poor placentation, preeclamptic onset often occurs before 34 weeks of pregnancy

(i.e. early-onset preeclampsia) and is accompanied by intra-uterine growth retardation (IUGR) (Burton, 2017a, Huppertz, 2008, Myatt, 2015, Steegers, 2010). Poor placentation is, however, shared with other pregnancy complications, including isolated IUGR and preterm delivery, without any maternal involvement (Labarerre, 2017). Thus, poor placentation predisposes to preeclampsia (Steegers, 2010) and preeclampsia has been assigned as a disease primarily relating to the villous ST (Huppertz, 2008), in which a stressed ST hurts the mother. As discussed in the previous section, a stressed ST may 'survive' quite long, as it has to 'await' parturition. And as an in-between organ, dysfunction of the placenta can hurt both the fetus (impaired oxygen/nutrient delivery, not further discussed here) and the mother.

B. PREECLAMPSIA, AN ENDOTHELIAL CELL DISORDER

Generalized maternal endothelial dysfunction is central (the "common denominator") in the syndrome of preeclampsia (Roberts, 1989). The vascular endothelial lining represents the largest "endocrine organ" of the human body. An excessive maternal systemic inflammatory response (Redman, 2005) and angiogenic imbalance are well-documented hallmarks of the maternal syndrome, which are implicated in maternal endothelial dysfunction.

1. Excessive maternal inflammatory response and endothelial dysfunction; a neutrophilic affair?

Preeclampsia is characterized by an excessive maternal systemic inflammatory response. Almost all studies on this topic including ours (Velzing-Aarts, 2002a), reported elevated pro-inflammatory cytokines, such as TNF-α (Conrad, 1998, Johnson, 2002, Kupferminc, 1994, Moreno-Eutimio, 2014, Sharma, 2007, Szarka, 2010, Tosun, 2010, Velzing-Aarts, 2002a, Vince, 1995), IL-6 (Conrad, 1998, Greer, 1994, Johnson, 2002, Jonsson, 2006, Kupferminc, 1996, Luppi, 2006, Madazli, 2003, Moreno-Eutimio, 2014, Pinheiro, 2013, Sharma, 2007, Stallmach, 1995, Szarka, 2010, Tosun, 2010, Vince, 1995) and IL-8 (Johnson, 2002, Jonsson, 2006, Kauma, 2002, Moreno-Eutimio, 2014, Pinheiro, 2013, Sharma, 2007, Stallmach, 1995, Sun, 2016, Szarka, 2010, Tosun, 2010, Velzing-Aarts, 2002a, Yang, 2016, Zhang, 2003) in preeclamptic women. Two meta-analyses confirmed higher TNF-α and IL-6 levels (IL-8 not studied) in preeclampsia (Lau, 2013, Xie, 2011). A recent systematic review also confirmed CRP, TNF-α, IL-6 and IL-8 as prominent pro-inflammatory parameters in preeclampsia from the second trimester and onwards (Black, 2018). In addition, first trimester maternal serum IL-8 levels and the TNF-α/IL-10 ratio were best predictors of subsequent preeclampsia (Salazar Garcia, 2018). Of note, preeclamptic IL-8 levels vary over a wide range, while control IL-8 levels were within a narrow limit (Moreno-Eutimo, 2014, Velzing-Aarts, 2002a, Yang, 2016). IL-8 levels were higher in severe as compared to mild preeclamptic women (Ellis, 2001, Sahin, 2015, Sun, 2016, Tosun, 2010).

Preeclampsia, a neutrophilic affair. Although many maternal immune cells, including monocytes/macrophages (Gervasi, 2001, Haeger, 1992, Luppi, 2006, Sacks, 1998), and lymphocytes (Luppi, 2006, Sacks, 1998) are activated in preeclampsia, neutrophils, in particular, seem to have a prominent role in the exaggerated maternal inflammatory state. Numerous reports found evidence for neutrophil activation as assessed by various methods during preeclampsia (Barden, 1997, Belo, 2003, Gervasi, 2001, Gupta, 2005, Halim, 1996, Kobayashi, 1998, Lee, 2003, Luppi, 2006, Sabatier, 2000, Sacks, 1998, Tsukimori, 2008, 2007, 2005, 1993). Neutrophil activation decreased after delivery (Tsukimori, 2007).

Ex vivo studies report that activated neutrophils infiltrate the intimal space in preeclamptic resistance-sized vessels of subcutaneous fat. In contrast, in vessels of normal pregnancy, neutrophils sporadically adhere and infiltrate (Cadden, 2008, Leik, 2004, Shah, 2007). Neutrophils that migrate into the sub-endothelial space can cause endothelial cell layer permeability, a major event by which our immune system can cause organ damage (Wenceslau, 2016). Once within the sub-endothelial space, neutrophils may prolong their survival and release toxic products, including superoxide, which consumes vasodilatory NO to form peroxynitrite (Cadden, 2008, Lee, 2003, Leik, 2004, Tsukimori, 2008), and thromboxane, which causes vasomotor imbalance (hypertension) (Cadden, 2008).

An excessive maternal inflammatory response characterized by increased levels of pro-inflammatory cytokines such as IL-6 and TNF-α, is a common feature with other pregnancy complications, such as gestational diabetes mellitus (Altinova, 2007, Atègbo, 2006, Briana, 2009, Kirwan, 2002, McLachlan, 2006, Morisset, 2011, Winkler, 2002, Xu, 2014). So, why are neutrophils so prominently involved in preeclamptic pathogenesis?

Preeclampsia, similarities with the systemic inflammatory response syndrome (SIRS).
A classic study explains that the maternal (preeclamptic) inflammatory response has certain features in common with the septic state (Sacks, 1998). Preeclampsia is also characterized by certain similar features as observed with the systemic inflammatory response syndrome (SIRS), a sepsis-like condition with wide-ranging neutrophil activation. SIRS develops when e.g. mitochondrial DAMPs (mitochondrial DNA and *N*-formyl peptides) are released into the circulation after e.g. severe trauma (Gu, 2013, Lam, 2004, Yamanouchi, 2013). It associates with a robust pro-inflammatory cytokine response, complement activation (Hazeldine, 2015), and infiltration of neutrophils into various organs (Zhang, 2010). It can lead to serious, even deadly complications, like multiple organ failure (MOF) and adult respiratory distress syndrome (ARDS). High plasma DAMPs and extracellular vesicles were positively correlated with mortality/morbidity in trauma patients (Eppensteiner, 2018). Mitochondrial DAMPs activate circulating neutrophils, causing the release of for instance IL-8 and render these activated neutrophils refractory to subsequent triggers, like chemotactic ones. Hence, these activated neutrophils do not locate to any danger and/or infection site (Zhang, 2010). Mitochondrial DAMPs increase endothelial cell layer permeability (Sun, 2013, Wenceslau, 2016). This ‘dual’ systemic action on

both neutrophils (activation and loss of responsiveness) and endothelial cells (increased permeability) allows easy access of neutrophils to any location, a key event in SIRS, were neutrophils inappropriately infiltrate distal organs (Pittman, 2013, Zhang, 2010).

Serious (deadly) complications of SIRS, such as MOF and ARDS do develop in severe preeclamptic cases (Duarte, 2014, Rojas-Suarez, 2012, Vasquez, 2015). Mitochondrial DAMPs have been implicated in the pathophysiology of preeclampsia (Goulopoulou, 2012, McCarthy, 2016, Qiu, 2012), but its role has yet to be conclusive confirmed. Preeclampsia does share features with SIRS, although levels of DAMPs may not be that high as compared to levels encountered in patients with severe trauma.

The loss of responsiveness of neutrophils to chemotactic stimuli may explain the susceptibility of SIRS patients for secondary (hospital) infections (Hazeldine, 2015). Similarly, neutrophils may not be responsive to chemotactic stimuli and their 'homing' into gestational tissues. Neutrophil's seem to play a role late in the parturition process (Gomez-Lopez, 2014), first arriving in the cervix to promote dilation (Sakamoto, 2005, Winkler, 1999). If neutrophil activation does contribute to parturition onset, from the mother's perspective, this is the only adequate adaptation in response to her stressed conditions, i.e. the removal of her stress stimulus.

2. Angiogenic imbalance; predominant impact on fenestrated endothelium

It is well recognized that an angiogenic imbalance during preeclamptic pregnancies results in maternal endothelial dysfunction (Chaiworapongsa, 2004, Foidart, 2009, Maynard, 2003, Venkatesha, 2006). Endothelial health which relies on vascular endothelial growth factor (VEGF) (Luttun, 2003) and TGF-β signaling for differentiation, maintenance and survival, seem specifically affected. These endothelia include fenestrated endothelium of renal glomeruli and choroid plexus (Maharaj, 2008), as well as discontinuous hepatic sinusoidal endothelium (Obeidat, 2012). The placenta produces and secretes VEGF and PlGF (shares ~ 50 sequence identity with VEGF) (Christinger, 2004) into the maternal circulation, in order to exert endothelial protection. VEGF signaling up-regulates endothelial nitric oxide synthase (eNOS) (Tanimoto, 2001) causing vasodilatation via increased NO production (Müller-Deile, 2011), and enhanced prostacyclin production (Venkatesha, 2006). The angiogenic imbalance reported in preeclampsia includes an excess of anti-angiogenic soluble receptors relative to their pro-angiogenic ligands VEGF, PlGF, and TGF-β. In brief, the anti-angiogenic receptors soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng) decrease the bioavailability of their respective pro-angiogenic ligands, VEGF and TGF-β, by trapping them into a non-signaling alliance (Palmer, 2017). The role of PlGF is to bind sFlt-1 and as such, indirectly augment levels of unbound, bio-available VEGF.

The angiogenic imbalance; evidenced during and before preeclampsia. Lower levels of PlGF (Crispi, 2006, Levine, 2004a, Livingston, 2000, Masuyama, 2006, 2007, Maynard, 2003, Ohkuchi, 2007, Robinson, 2006, Shibata, 2005, Staff, 2005, Taylor, 2003, Teixeira, 2008, Torry, 1998, Tsatsaris, 2003, Wikström, 2007) and low bio-available VEGF (Lee,

2007, Levine, 2004a, Livingston, 2000, Lyall, 1997, Maynard, 2003) in preeclamptic pregnancies have been frequently reported by others and our group (Reuvekamp, 1999).

Further studies confirmed higher levels of sFlt-1 (Chaiworapongsa, 2004, Crispi, 2006, De Vivo, 2008, Koga, 2003, Lee, 2007, Levine, 2004a, Masuyama, 2006, 2007, Maynard, 2003, Ohkuchi, 2007, Reddy, 2009, Robinson, 2006, Shibata, 2005, Staff, 2005, Tsatsaris, 2003, Wikström, 2007) and sEng (De Vivo 2008, Kim, 2009, Levine, 2006, Masuyama, 2007, Reddy, 2009, Venkatasha, 2006) in preeclampsia.

As changes in PlGF, sFlt-1 and sEng (Erez, 2008, Levine, 2006, 2004a, Lim, 2009, Romero, 2008, Stepan, 2008, Su, 2001, Taylor, 2003, Tidwell, 2001, Tjoa, 2001, Wathén, 2006) were shown to precede clinical manifestations, focus was directed at preeclamptic prediction. Currently, the maternal sFlt-1/PlGF ratio is one of the most promising variables, either in combination with clinical parameters or not, for preeclampsia prediction (Lecarpentier, 2016). However, the major clinical relevance of the sFlt-1/PlGF ratio probably lies in its negative predictive value; the sFlt-1/PlGF ratio has a robust negative predictive value of 99.3% and 99.97% (no preeclampsia within the subsequent week), in both high-risk (Zeisler and Verlohren, 2016) and low-risk populations, respectively (Dragan, 2017), with a respective preeclampsia prevalence of 19% and 2.6%, respectively (Vatish, 2017).

The angiogenic imbalance; proteinuria and hypertension. The net result of the angiogenic imbalance (excess sFlt-1/sEng relative to PlGF/VEGF/TGF-β) in preeclampsia is reduced VEGF-signaling, with additive effects of reduced TGF-β signaling (Venkatesha, 2006). The angiogenic imbalance can induce the two clinical diagnostic criteria, hypertension (glomerular endothelial dysfunction) and proteinuria (glomerular podocyte involvement), of preeclampsia (Maynard, 2003). This is evidenced by the results of various animal experiments (Bergmann, 2010, Eremina, 2003, Maharaj, 2008, Maynard, 2003, Sugimoto, 2003, Venkatesha, 2006, Woods, 2011). In cancer patients receiving anti-VEGF monoclonal antibodies, hypertension and proteinuria are reported side-effects (Hurwitz, 2013, Kabbinavar, 2003, Ranieri, 2006). A recent meta-analysis in cancer patients robustly showed that anti-VEGF therapy increases the risk of (high-grade) hypertension and proteinuria (Zhao, 2017).

A morphological lesion in glomerular endothelial cells as a result of the angiogenic imbalance is known as “glomerular endotheliosis”, characterized by swollen glomerular endothelial cells, reduced fenestration and loss of podocytes (Collino, 2008, Kelder, 2012). Traditionally, glomerular endotheliosis was believed pathognomonic for preeclampsia, but it occasionally develops in hypertensive and even normal pregnancies (Strevens, 2003).

Reaching term in uncomplicated pregnancies, the angiogenic balance moves in a similar direction as seen in preeclampsia. Maternal PlGF increases from early gestation, peaks around GW 30 and then gradually decrease (bell-shape curve) (Romero, 2008, Torry, 1998). sFlt-1 levels are fairly constant up to the third trimester and modestly increase thereafter (Levine, 2004a, Romero, 2008). Hypothetically, if pregnancy would

last long enough (beyond post-term), every pregnant woman would eventually face an angiogenic imbalance and may develop hypertension and proteinuria. Similarly, only a minimally accelerated placental aging trajectory with a minimal early shift in angiogenic balance towards the anti-angiogenic site may induce late term preeclampsia. Women with "vulnerable" endothelium, e.g. diabetic, obese, or hypertensive women (Palmer, 2017), may develop preeclampsia following normal gestational shifts in the angiogenic balance. In contrast, early-onset preeclampsia is associated with aberrant increments in placental sFlt-1 production, which relates to disease severity (Whitehead, 2011). This production involves a dominant placental isoform, i.e. sFlt-1e15a (Jebbink, 2011, Whitehead, 2011).

C. POOR PLACENTATION OR (MULTIPLE) STRESSES ALONG THE WAY

As outlined before, normal human gestation undergoes a timely- and spatially-controlled program. This gestational program includes a well-timed and well-controlled trophoblastic stress trajectory that is communicated and perceived by the mother (and the fetus at the end), all being physiologically planned and highly functional.
Poor placentation (disturbed, less optimal placental development related to defects in spiral artery remodeling) as well as any other insult or even multiple small events, challenge the normal trophoblastic stress trajectory.

1. Poor placentation
Obviously, poor placentation restricts the functional performance from the beginning. The placental developmental trajectory has to deal with (sometimes impressive) setbacks from early on. Poor placentation accounts for most early-onset (i.e. before 34 weeks of gestation), severe (clinical criteria), preeclamptic cases with a growth-restricted fetus (Burton, 2017a, Huppertz, 2008, Myatt, 2015, Steegers, 2010). In theory, a mild form of poor placentation may be associated with late-onset, term preeclampsia (Redman, 2014).

Poor placentation inevitably leads to villous oxidative stress due to hypoxia and/or ischemic-reperfusion events (Burton, 2017a). Oxidative stress is one of the discriminating features of accelerated aging (Terman, 2010); it adversely impacts autophagic degradative capacity due to lysosomal dysfunction, when these organelles are loaded with non-degradable material damaged by oxidation (lipofuscin) (*see background; aging*). Lipofuscin accumulation, used as a marker of aging, may even have an active role in neurodegeneration (Moreno-Garcia, 2018). Previous work reported increased lipofuscin in placental obtained from "gestosis" pregnancies (Volkova, 1991), which was recently confirmed in placentas from early-onset (with IUGR) preeclampsia (Cindrova-Davies, 2018).

Severe fluctuations in oxygenation mimics oxidative stress in villous explants *in vitro*. Under these conditions, stress pathways in the placenta, like p38 mitogen-activated protein kinase signaling, are activated (Cindrova-Davies, 2007a, Yung, 2014). VEGF and in

particular, sFlt-1 protein were increased under *in vitro* oxidative stress (Cindrova-Davies, 2009). ER stress (Yung, 2014) and loss of cytochrome-c from mitochondria, indicative of mitochondrial defects (Hung, 2002), were also documented. Finally, oxidatively-stressed villous explants from term placentas release more cell-free fetal DNA fragments (Tjoa, 2006), and TNF-α (Cindrova-Davies, 2007a). Considering human labor as an *in vivo* ischemic-reperfusion model, the NF-κB pathway was activated, sFlt-1 increased and PlGF decreased in placentas obtained after labor (especially long labor) as compared to those from elective Caesarean sections (Cindrova-Davies, 2007b). Based on these *in vitro* and *in vivo* data, placental oxidative stress accelerates aging processes and associates with local mitochondria and ER stress, which are interconnected (Burton, 2017a). Poor placentation induces many arms of the maternal syndrome of preeclampsia, such as an excessive pro-inflammatory state, increased DAMP release (e.g. cell-free fetal DNA), and perturbed angiogenic balance.

2. Multiple stresses along the way

The ST can be subject to other stress along the way. Stress may be severe or mild/modest due to multiple smaller insults, derives from variable origins, and could result from a range of pathophysiological impacts. To illustrate, obese women (Durst, 2016, Spradley, 2017) and women with systemic lupus erythematosus (SLE) (Duckitt, 2005) have increased risk (i.e. 2.5-fold and 10-fold, respectively) of developing preeclampsia (Duckitt, 2005).

Obesity is one of the few modifiable risk factors in preeclampsia (Vaisbuch, 2017). The first accumulation phase in maternal white adipose tissue (WAT), in which her adipose reserves are build up (*see background; maternal white adipose tissue*), relies heavily on the expansive capacity of this maternal compartment. The risk of 'overloading' an already expanded obese WAT compartment increases progressively until the end of the accumulation phase (around GW 30). If 'overloaded', inflammatory pathways are up-regulated within these adipocytes, designated metabolically-triggered inflammation or "meta-inflammation" (Gregor, 2011, Hotamisligil, 2006), creating local IR. Under these circumstances, the induction of an inflamed and insulin resistant state in maternal WAT, a normal phenomenon in later pregnancy, occurs too early and is inappropriate for that particular time in pregnancy (*see background; maternal white adipose tissue*). This may have several consequences. First, inflamed and insulin resistant WAT releases more pro-inflammatory cytokines and less insulin-sensitizing adiponectin, which leads to an early maternal systemic inflammatory response and insulin resistant state, respectively. Secondly, the maternal WAT compartment switches to the mobilization phase too early, which results in the allocation of energy/nutrients to an extent that exceeds fetal needs at that particular time in pregnancy. Such nutritional abundance may trigger inflammatory cascades ("metaflammation") within the placenta (*see background; insulin resistance*), and accelerate the aging process ("inflammaging"). An unfavorable feed- forward loop then develops; the placenta augments the maternal inflammatory and insulin resistant state, which triggers even more maternal energy/nutrient mobilization, further contributing to the redundant excess in available nutrients.

Obesity is associated with late-onset preeclampsia; out of 10196 overweight, obese, or morbidly obese women, 10.5% delivered before and 89.5% ≥34 weeks (Durst, 2016). The onset of disturbances leading to the 'metabolic scenario' of preeclampsia may thus occur during the later stages of the accumulative phase. Of note, theoretically preeclampsia can develop even without any placental involvement, as both its diagnostic criteria (hypertension and proteinuria) are often observed in non-pregnant obese individuals whose metabolism is affected. This may represent "true maternal" preeclampsia.

Anti-phospholipid antibodies (aPL) present in SLE, may induce mitochondrial stress/damage (Pantham, 2015), which is considered 'atypical' and not seen in the regular trophoblast stress trajectory. aPL are internalized by first trimester villous explants through a receptor-mediated process (Viall, 2013). These treated explants release extracellular vesicles with increased DAMP content, including calreticulin (Pantham, 2015), HMGB1 (Shao, 2016) and mitochondrial DNA (Tong, 2017b). This may be an important link between these events in the placental villi and clinical manifestations in the mother.

D. PLACENTAL STRESS RESPONSES

Any stress experienced during the ST's lifespan can perturb the normal stress trajectory.

At best, the regular trophoblast stress trajectory is maintained, accelerated, with a relatively 'older' ST considering the stage of pregnancy. Maternal manifestations may then be rather uniform, with an exaggerated maternal inflammatory response, angiogenic and hemostatic imbalance (the relative amplification of normal late gestational events), and the onset of proteinuria and hypertension (Redman, 2014).

This is opposed to a grossly disturbed stress trajectory, with intense regular trophoblast stress (e.g. oxidative stress) and the onset of secondary stress (e.g. unresolved ER stress). A grossly disturbed trajectory is particularly harmful to the mother, inducing the severe phenotype, with more heterogeneity of maternal manifestations. Such a scenario is typical for early-onset preeclampsia resulting from poor placentation.

The ST's adaptive stress responses aim to minimize any consequences of the perturbed stress trajectory. Decisive adaptive stress responses include autophagic-lysosomal flux that clears oxidatively damaged cellular constituents and organelles (like mitochondria), and the UPR that aims to remain/restore ER homeostasis (Redman, 2014).

Findings in preeclampsia suggest a challenged/compromised autophagic-lysosomal flux. The first steps in autophagy seem very active (Akcora-Yildiz, 2017, Gao, 2015, Nakashima, 2017, Oh, 2008), but the final step, i.e. degradation of material within the lysosomes, may be compromised (Dimasuay, 2017, Götzl, 2016, Nakashima, 2017, 2015) (*see background; autophagic-lysosomal flux*). This is in line with reported lipofuscin abundance in "gestosis" pregnancies (Volkova, 1991) and early-onset (with IUGR) preeclampsia (Cindrova-Davies, 2018).

In addition, there is a call upon all the three arms of the UPR in preeclamptic placentas (Fu, 2015, Mizuuchi, 2016, Yung, 2014) (*see background; the unfolded protein response,*

UPR). In severe preeclamptic placentas, especially in the early-onset ones, this is accompanied by increased pro-apoptotic CHOP and caspase-12 expression (Fu, 2015). Increased pro-apoptotic CHOP is indicative of a failing UPR that is unable to regain ER homeostasis. Higher CHOP expression was confirmed in preeclamptic placental tissue and proved related to increased ST-derived micro-vesicles in preeclamptic pregnancies (Verma, 2018). In cultured BeWo cells, pharmacologically induced severe (but not mild) ER stress is associated with increased CHOP expression and the release of DAMP-associated micro-vesicles (carrying e.g. HMGB1) (Collett, 2018). This micro-vesicle release was considered a putative link between organelle (ER) stress and maternal preeclamptic manifestations (Collett, 2018); preeclamptic micro-vesicles carrying various DAMPs act both on maternal immune cells and endothelial cells (Collett, 2018, Redman, 2012).

Normally, the loss of ER homeostasis, with expression of pro-apoptotic CHOP, can lead in a regulated death scenario (*see background; the unfolded protein response, UPR*). Within the preeclamptic ST (unable to execute its own apoptotic death), however, high CHOP expression may indicate chronic un-resolved severe ER stress and release of micro-vesicles and/or DAMPs.

Preeclampsia; a stressed ST remains in situ and releases till the end of pregnancy. As suggested previously, stress in the ST even when profound with loss of homeostasis, may not necessarily be synchronized to parturition onset (prolonging the dysfunctional state). This may last until the 'burden' is high enough to trigger onset of parturition. Intriguing is the proposal by Labarerre et al. that those preterm deliveries originating from poor placentation would have developed into preeclampsia if they had lasted longer (Labarrere, 2017). Apparently, these pregnancies undergo the only proper adaptive response, i.e. delivery, before the mother gets involved.

During preeclampsia, however, a stressed ST remains *in situ* and finds itself in a "chronic" stress condition (Redman, 2014). This holds true particularly in early-onset preeclampsia, a time in pregnancy when other triggers to parturition onset do not put much weight yet. The 'resolution' is not to be expected and the pathophysiological process may go on; dangerous feedforward loops may underlie rapid clinical deterioration.

The ST loses functionality and the placenta's secretory/release profile (e.g. gestational hormones, exosomes, PlGF) is no longer adaptive nor protective for the mother. ST stress is communicated to the mother via the release of DAMPs and micro-vesicles, mistimed and/or to an excessive extent for that particular stage of pregnancy. When waste accumulates, a degradative (also referred to as catabolic) component is introduced, with disposal of non-degraded material, like proteo-toxic aggregates, in the maternal circulation.

ST's partner, the cytotrophoblast. The ST is the primary releaser (remaining *in situ* to do the harm) and the instigator of the maternal syndrome (Huppertz, 2008), but the impact of the underlying CT monolayer cannot be ignored.

Unlike the ST, stressed CT cells that do not restore homeostasis, can undergo cell death. CT appear particularly vulnerable to changes in perfusion, based on *in vitro* hypoxia-re-oxygenation experiments (Soleymanlou, 2007). Increased rates of apoptosis in villous tissue were reported in preeclamptic pregnancies (Allaire, 2000, Heazell, 2008, Leung, 2001a). As compared to normal villi, apoptosis was 4- and 7-fold higher in villi of isolated preeclamptic pregnancies and those combined with IUGR, respectively (Longtine, 2012b).

In addition, CT can go into senescence, and then, the pro-inflammatory SASP component may augment the maternal inflammatory burden. Increased rates of CT apoptosis and/or CT senescence indirectly impact ST well-being (additive stress factor) by depriving ST from fresh organelles (Burton, 2017a, Wietrak, 2015).

E. PREECLAMPTIC SECRETORY/RELEASE PROFILE

1. Loss of protection, wrongful maternal adaptations

The ST loses functionality and the placenta's secretory/release profile is no longer protective for the mother, and maternal adaptations are lost or impaired. For instance, the angiogenic imbalance in preeclamptic pregnancies deprives the pregnant woman of the protective effects of VEGF on her endothelium (see angiogenic imbalance; predominant impact on fenestrated endothelium).

Exosomes isolated from second and third trimester preeclamptic women had reduced syncytin-2, and may be less immunosuppressive (Vargas, 2014). Attenuation of immunosuppressive function of ST-derived exosomes may impact the 'education' of maternal NK and cytotoxic T cells, which can contribute to the Th1 dominance often reported in preeclamptic pregnancies (reviewed by Saito, 2003).

Another example is the loss of anti-atherogenic protection mediated by the normal gestational decrease in hepatic lipase activity. Lower hepatic lipase activity in normal pregnant women confers anti-atherogenic protection as it reduces the formation of pro-atherogenic small-dense LDL. Preeclamptic women display increased hepatic lipase activity, which results in a more pro-atherogenic lipid profile (Sattar, 1997). This is likely related to lower 17-β estradiol levels observed in preeclamptic pregnancies (Hertig, 2010).

We reported on an important gestational adaptation in the mother's one-carbon metabolism, which is thought to rely on estrogens. This adaptation allows the mother to prioritize folate to her fetus, because another functional role of folate, the remethylation of homocysteine is partly taken over by betaine (Velzing-Aarts, 2005). Preeclamptic women had a similarly inverse folate-homocysteine relationship as non-pregnant women, indicating that this adaption is lost in preeclamptic pregnancies (Velzing-Aarts, unpublished).

2. The stressed syncytiotrophoblast 'communicates' its stress to the mother

2a. Micro-vesicles. As markers of ST stressed conditions (Tannetta, 2017), increased numbers of micro-vesicles are found in peripheral blood of preeclamptic women (Germain, 2007, Goswani, 2006, Knight, 1998), but not in those with isolated IUGR pregnancies (Goswani, 2006). Preeclamptic micro-particles exhibit compositional changes, including increased tissue factor activity (Gardiner, 2011) and content of anti-angiogenic molecules (Tannetta, 2013, Tong, 2017d). Proteins differentially expressed in micro-vesicles obtained from supernatants of preeclamptic and normal placental explants include annexins (involved in inflammation, coagulation) and DAMPs (e.g. heat shock proteins) (Baig, 2014). *In vitro,* preeclamptic micro-vesicles dose-dependently (mimicking severity of disease) increase superoxide production in neutrophils from male donors (Aly, 2004) and increase IL-1β production by peripheral blood mononuclear cells as compared to control micro-vesicles (Holder, 2012). This confirms that preeclamptic micro-vesicles are more pro-inflammatory, anti-angiogenic, and pro-coagulant (substantial pro-thrombotic stimulus, Gardiner, 2011), as compared to normal pregnancy micro-vesicles (Mincheva-Nilsson, 2014). Median size of EVs obtained from perfusate of preeclamptic placentas is increased compared to those in perfusate of normal placentas, suggesting a shift towards more micro-vesicle release relative to exosomes (Tannetta, 2013).

The surface of preeclamptic micro-vesicles display changes in glycosylation profile (Tannetta, 2017) and integrin expression (Baig, 2014). This may have repercussions with regards to target cell binding, clearance (Tannetta, 2017), and perhaps final tissue destination.

2b. Damage-associated molecular patterns (DAMPs) release. DAMPs can be released as isolated molecules, but also within the confinement of extra-cellular vesicles. Whether the biological consequences differ between these two routes is not known (Collett, 2018). The type of the underlying stress may be a crucial determinant of DAMP signature and may help explain the heterogeneity of the maternal syndrome of preeclampsia. The increase in cell-free fetal DNA is discussed in the next section.

Mitochondrial DAMPs, as discussed before, may be particularly involved in profound neutrophil activation (see excessive maternal inflammatory response and endothelial dysfunction; a neutrophilic affair?). Mitochondrial DNA has been implicated in the pathophysiology of preeclampsia (McCarthy, 2016), but its role has yet to be conclusive confirmed. Pregnant rat dams injected with rat liver mitochondria developed hypertension (Goulopoulou, 2012). One study reports a modest increase in mt-DNA copy number in whole blood of preeclamptic women compared to control women (Qiu, 2012). Conversely, a recent pilot study, reports lower mt-DNA copy numbers in first trimester peripheral blood of women who later developed preeclampsia, especially in those with IUGR (Busnelli, 2018).

A number of studies have investigated HMGB1, calreticulin and extracellular-ATP levels in the context of preeclampsia. The general finding is that HMGB1, calreticulin and extracellular-ATP levels are higher in preeclamptic placentas and the maternal circulation as compared to normal pregnancies.

Most studies reported increments in maternal and placental HMGB1 levels in preeclamptic pregnancies (Chen, 2016, Naruse, 2012, Pradervand, 2014, Xu, 2018, Zhu, 2015a), but not all (Wang, 2011). HMGB1 was primarily located in ST cytoplasm (Chen, 2016) of severe preeclamptic placentas (Zhu, 2015a, Xu, 2018), characterized by a higher cytoplasmic/nuclear ratio (Xu, 2018), with HMBG release from its organelle confinement during severe preeclampsia. In contrast, HMGB1 concentrates within the nuclei in control placentas (Zhu, 2015a, Xu, 2018). In BeWo cells, severe ER stress with increased CHOP expression induced the release of HMGB1 within the confinement of micro-vesicles (Collett, 2018). Given the short half-life of HMGB1 and the persistence of HMGB1 levels two days after delivery, a placental source for the maternal HMGB1 was considered unlikely (Pradervand, 2014). *In vitro*, HMGB1 can adversely impact endothelial cells via RAGE (receptor for advanced glycation end products) (Shao, 2016). Sera from severe preeclamptic women containing high levels of HMGB1 induce hyper-permeability in glomerular vascular endothelial cells (Xu, 2018) providing a link between high circulating levels of HMBG1 and proteinuria in severe preeclampsia (Xu, 2018).

Circulating levels of calreticulin were higher in preeclamptic women compared to control women (Gu, 2008). One study reported higher placental calreticulin mRNA and protein levels in preeclamptic pregnancies that correlated with disease severity (Shi, 2012), while Gu et al. reported similar mRNA and protein levels in preeclamptic and normal pregnant placentas (Gu, 2008).

Plasma levels of extracellular-ATP and adenosine were higher in preeclamptic women, with an elevated ATP/adenosine ratio, suggesting a vaso-constrictive and pro-inflammatory impact (Bakker, 2007). Indeed, administration of ATP in pregnant rats resulted in the development of preeclampsia-like symptoms (Faas, 2010).

Cancer cells that experience profound ER stress with excessive ROS generation and severe proteo-toxicity release several DAMPs (HMGB1, calreticulin, extracellular-ATP), in a strictly controlled (temporal) manner (Garg, 2014, Land, 2016). This DAMP signature induces immogenic cell death by mounting anti-tumor T cell responses (*see background; damage-associated molecular patterns, DAMPs).* Although some DAMPs are individually linked to preeclamptic manifestations, whether they are released in such a coordinated manner to inflict a break in fetal tolerance is unknown. Such a break in fetal tolerance (i.e. the presentation of fetal alloantigens in the presence of danger) may especially intensify preeclamptic decidual inflammation and the promotion of acute atherosis (see later). Anti-fetal T cells can encounter fetal allo-antigens at the decidual interface. In support, these atherosclerotic decidual lesions resemble lesions within rejected grafts (Pijnenborg, 2006).

3. Failing degradative capacity, a catabolic component in preeclamptic pathophysiology

Terminally differentiated ST are unable to dilute its cellular waste by division. Clearance of ST relies heavily on its degradative machinery (Chifenti, 2013), including proteasome activity, autophagic-lysosomal flux and the UPR. The result of a failing autophagic-

lysosomal flux (notably the last lysosomal step) and UPR as reported in preeclampsia, is that that damaged organelles, such as mitochondria, effete/aged nuclei, and (toxic) protein aggregates are not properly cleared and end up in the maternal circulation. Some evidence points to a catabolic component in preeclampsia, when waste is disposed to the mother.

First, a compromised autophagic-lysosomal flux may explain the increased numbers of syncytial knots in preeclamptic placentas (Buurma, 2013, Fogarty, 2013), with a "knotting index" that relates to disease severity (Fogarty, 2013). Lipofuscin staining in early-onset, (with IUGR) preeclamptic placentas revealed "nuclear localization" (Cindrova-Davies, 2018) indicating that autophagic-lysosomal flux is impaired. Compared to normal pregnancy, preeclampsia is associated with an increased release of macro-vesicular trophoblastic material (Buurma, 2013, Johansen, 1999) with more syncytial knot-like structures as compared to normal pregnancy (Burton, 2009c). Nevertheless, even in preeclampsia, structures with the appearance of syncytial sprouts (tear-drop) are more often found in the uterine vein as compared to syncytial knots (Johansen, 1999).

Second, instead of full degradation of nuclear DNA within the ST's lysosomes, cell-free fetal DNA fragments may be released into the maternal circulation contributing to the higher levels observed in preeclamptic women (Lo, 1999, Swinkels, 2002, Zhong, 2001). Elevated maternal levels of cell-free fetal DNA have been demonstrated prior to the onset of symptoms (Leung, 2001b, Levine, 2004b, Zhong, 2002) and acknowledged as a disease marker (Martin, 2014).

Third, proteo-toxicity can evolve from a compromised capacity to clear misfolded proteins and (toxic) protein aggregates, which is a joined effort by the UPR and autophagy (then termed aggre-phagy) (*see background; proteo-toxicity*). Certain functional proteins have the tendency to aggregate and form cytotoxic oligomers/fibrils in their misfolded state, collectively referred to as amyloids (Borland, 2017). Some of these proteins, such as amyloid precursor protein (Buhimschi, 2014) and transthyretin (Kalkunte, 2013) are expressed in the human placenta. There is evidence for the presence of amyloid assemblies in placentas (Buhimschi, 2014, Tong, 2017e), in serum (Buhimschi, 2014, Cheng, unpublished, 2016), and in the urine (Buhimschi, 2014, Millen, 2018) of severe preeclamptic women. Amyloid assemblies in urine were observed prior to the clinical onset of preeclampsia (Buhimschi, 2014). Collectively, these findings may reflect the accumulation of amyloids in the preeclamptic placenta and their disposal into the maternal circulation (Tong, 2017e). This may be a result of a compromised ST degradative clearing capacity (Buhimschi, 2014). Accumulation of amyloid aggregates in the ST can induce serious feed forward loops (Cheng, 2016). These aggregates induce ER- and mitochondrial stress and further impair autophagic-lysosomal flux (Borland, 2017). Brain-derived exosomes in Alzheimer's disease carry more neurotoxic amyloid β-oligomers (Sardar Sinha, 2018). Likewise, nano-sized vesicles (not specified into exosomes) prepared from severe preeclamptic placentas carry more aggregated transthyretin compared to those from control placentas and may be pathogenic (e.g. causing ER stress) in their target tissues (Tong, 2017e, 2017c). In the maternal circulation, amyloid deposits have been

associated with vascular dysfunction (Millen, 2018). Preeclampsia incidence is higher in diseases characterized by an increased load of aggregate-prone misfolded proteins, like renal disease, and chronic autoimmune diseases (Buhimschi, 2014). In these women, the placenta is exposed to higher levels of misfolded proteins, i.e. an 'atypical' stressor, that challenge the clearing degradative capacity and thereby predispose to preeclampsia.

4. Excessive maternal inflammatory response, metabolic consequences

The excessive maternal inflammatory response may, in addition to endothelial cell dysfunction, induce 'metabolic upset'. The mother prioritizes excessively more nutrients than necessary to meet with fetal needs for that particular time of pregnancy and even more so under conditions of impaired placental uptake. Preeclamptic women exhibit exaggerated insulin resistance (Anim-Nyame, 2004, Ghosh, 2017, Kaaja, 1999, Masuyama, 2011), increased free fatty acids (Kaaja, 1999, Villa, 2009), and an atherogenic hyperlipidemia (Spracklen, 2014). It is noteworthy that preeclampsia has been referred to as the insulin resistance syndrome (Kaaja, 1999, Parretti, 2006, Seely, 2003) or metabolic syndrome (Rodie, 2004) of pregnancy.

Acute atherosis. An atherogenic hyperlipidemia, a preeclamptic decidual inflammatory background and an atherosclerosis-prone jet stream flow in un-remodeled spiral arteries are a dangerous cocktail for acute atherosis (Staff, 2014). Acute atherosis is a lesion alike atherosclerotic lesions in the coronary arteries (Staff, 2014) or in rejected transplants (Pijnenborg, 2006). Macrophages and adaptive immune cells (T cells) are implicated in the atherosclerotic changes of acute atherosis (Staff, 2014). Acute atherosis is not restricted to preeclamptic pregnancies and can even be found observed in uneventful pregnancies, often localized to those spiral arteries that have escaped (full) remodeling (Staff, 2014). In preeclampsia, acute atherosis relates to disease severity (Stevens, 2013) and placental infarction is a plausible downstream result of acute atherosis (Brosens, 1972).

F. THE ULTIMATE OUTCOME, THE MATERNAL SYNDROME OF PREECLAMPSIA

The ST experiences stress during its lifespan, even in an uneventful uncomplicated pregnancy. These stresses are communicated and perceived by the mother. Any insult or even multiple smaller events can interfere with this normal gestational stress trajectory. The timing, severity, and type of stress (atypical), and their handling by the placental ST (genetic component) determines the 'preeclamptic release profile' and the 'burden' for the mother.

The way the mother copes ultimately determines the final preeclamptic phenotype (**Figure 2**). A well coping mother is not able to prevent preeclamptic maternal manifestations if experiencing a very disturbed placental stress trajectory, but nevertheless can minimize maternal consequences. In contrast, a poorly coping mother will occasionally develop

a life-threatening preeclamptic phenotype if subject to a very disturbed placental stress trajectory. Even, a highly unfit mother cannot cope with the normal gestational trajectory of late trophoblastic stress (designated "maternal" preeclampsia, Myatt, 2015, Steegers, 2010) and are likely candidates to develop term preeclampsia (Yung, 2014). For example, women with "vulnerable" endothelium, as observed with diabetes, obesity and hypertension (Palmer, 2017), may develop preeclampsia following normal gestational shifts in the angiogenic balance.

Maternal coping efficiency is the sum of many factors, either of intrinsic, extrinsic (lifestyle), or racial nature. Every (adverse) factor, even if small, has impact by adding to an overall coping (in)-efficiency. For instance, women in our study population are all inhabitants of the island of Curaçao and are predominantly of West African descent. Sub-Saharan women frequently lack expression of the erythrocyte Duffy antigen, i.e. the

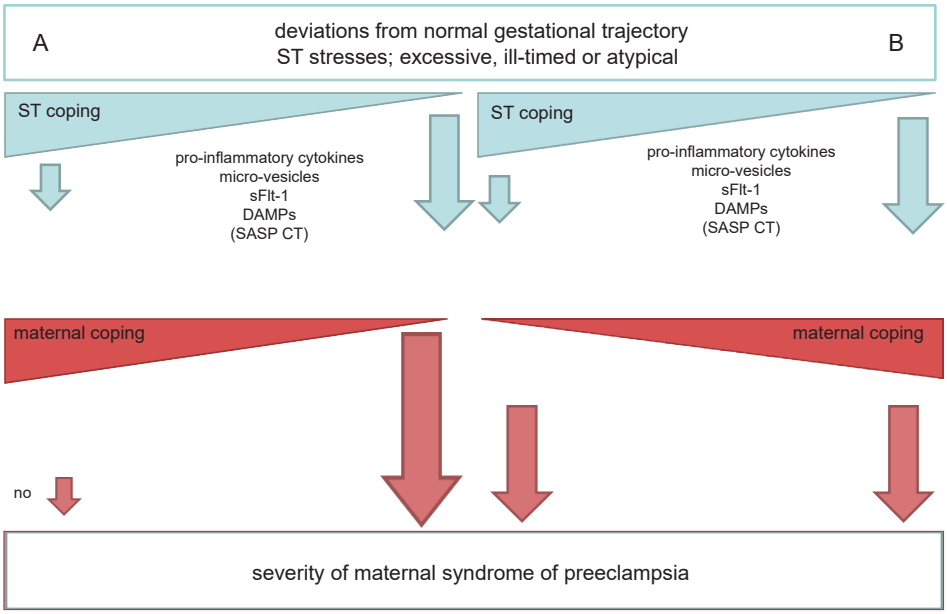

Figure 2. The ST (CT) and maternal coping efficiency and clinical syndrome. The ST (CT) and maternal coping efficiency determine final maternal disease. **A.** A perfectly coping placenta and mother may minimize or even prevent a final clinical picture. A poorly coping placenta and mother may result in severe, occasionally life-threatening, preeclampsia. **B**. The severity of the clinical picture may be similar in the presence of a poorly coping placenta and a well coping mother and *vice versa*, if one assumes that placental and maternal coping contribute equally. CT, cytotrophoblasts; DAMPs, damage-associated molecular patterns; SASP, senescent-associated secretory phenotype; sFlt-1, soluble fms-like tyrosine kinase-1; ST syncytiotrophoblast.

entry site of the malaria parasite *Plasmodium vivax*. The Duffy antigen turned out to be a promiscuous non-signaling receptor for > 20 chemokines, including IL-8, and hence, was designated Duffy antigen receptor for chemokines (DARC) (Chaudhuri, 1994, Horuk, 1993, Peiper, 1995). DARC, now renamed atypical chemokine receptor-1 (ACKR-1), binds its cognate chemokines like IL-8 when circulating levels are high, but in a saturable manner (Darbonne, 1991). Erythrocyte DARC has been assigned a role as chemokine reservoir (Darbonne, 1991), and is seemingly more important during mild than severe inflammation (Zarbock, 2010). In our small sample sized study on pro-inflammatory cytokines in preeclampsia, 9/10 of preeclamptic women lacked erythrocyte DARC compared to 3/11 in matched controls (Velzing-Aarts, 2002a). The one preeclamptic subject that expressed erythrocyte DARC had IL-8 levels in the lower preeclamptic range (Velzing-Aarts, 2002a). It prompted us to investigate the association of the DARC phenotype with preeclampsia in a larger sample size. In a retrospective study design, we did find a clear association between the Duffy negative phenotype and women with a history of preeclampsia; odds ratio 2.98 (95%CI 1.40-6.23, p=0.004) (Velzing-Aarts, 2002b).

Discriminating between the relative maternal and placental contribution is useful to address future health risks for both mother and child. Likewise, as a fetal tissue, coping performances of the ST/CT, may be an indication of how preeclamptic offspring may address future challenges.

G. CONCLUSIONS

Processes that can easily be labeled as "adverse", including the pro-inflammatory response, (oxidative) stress in the presence of waning adaptive stress responses, release of danger molecules, induction of an allogenic response, and even caspase activity, have carefully timed functional roles in the remarkable gestational trajectory that ultimately leads to a perfectly timed birth of a well-grown baby to a mother with sufficient resources for the upcoming nurturing period.

All of these processes, with their ingenious roles in normal pregnancy, need to be critically controlled. If not (i.e. unplanned, badly timed), adverse downsides will emerge with the potential induction of the syndrome of preeclampsia.

A true danger of preeclampsia lies in the fact that the stressed multinucleated syncytium, failing to maintain/restore homeostasis, cannot undergo cell death as other 'regular' cells do, for the sake of the whole organism (mother, fetus). Instead, the ST ends its life at the final stage of the human parturition process. Some types of ST stress and (associated) pathophysiological cascades may be more potent in causing a pregnancy ending. Other pathophysiological cascades may not play primary roles in the onset of parturition and then the dysfunctional state will be prolonged until the 'burden' is high enough that it can inflict parturition. Until that time, the mother suffers from the maternal manifestations of preeclampsia. Indeed, delivery is the sole solution.

Background; maternal white adipose tissue. The release of numerous adipokines, including pro-inflammatory cytokines, assigns white adipose tissue (WAT) an endocrine role (Trayhurn, 2005) able to influence systemic (metabolic) processes. The maternal WAT compartment may follow a developmental program of its own. During the first anabolic phase of pregnancy, with a trend for increased insulin sensitivity (Kirwan, 2002), maternal WAT accumulates extra fat up until the end of the second trimester (Pipe, 1979). This extra fat can be mobilized thereafter, at a time of increasing metabolic (fetal) demands (Catalano, 2015, Haggarty, 2010). Processes within the WAT itself may be responsible for its own IR state; inflammatory pathways are up-regulated in early pregnancy (Resi, 2012), which may be amplified at the end of the accumulation phase to induce local IR (Catalano, 2015, Resi, 2012). Late gestational 'inflamed, IR' maternal WAT releases less insulin-sensitizing adiponectin and more pro-inflammatory cytokines, which contributes to the maternal systemic IR and adds to the maternal low-grade inflammatory state, respectively.

Background; cellular death. Normally, a stressed cell that cannot restore cellular homeostasis makes arrangements to die for the higher sake of organismal wellbeing (Galluzzi, 2016a, 2012). Damaged/stressed cells that lose functional capacity are better be replaced by new ones. If dys-homeostatic stressed cells remain alive, they confer a potential hazard, e.g. because DNA damage and associated risk of neoplastic transformation, or persistent release of DAMP. Cell death is inevitable when energy levels (e.g. ATP) drop below a critical level (Galluzzi, 2015, Green, 2014), which coincides with a grossly disturbed redox balance, which inhibits enzymes and damages cellular organelles and membranes (Galluzzi, 2015). These processes act in concert with the central role of mitochondria in regulated cell death.

Stress-induced cell death is mediated by well-defined molecular pathways/machineries (regulated cell death, RCD). It can be either apoptotic, necrotic (morpho-types), or have both apoptotic and necrotic features (Kroemer, 2009). Molecular machineries are just the executioners of RCD, and when inhibited, cellular demise is not prevented but proceeds with different kinetics and a different morphological outcome (Galluzzi, 2015).

Background; functional progesterone withdrawal. Unique to human and primate species is that parturition is not triggered by a decline in systematic progesterone levels as in most mammals, but by functional progesterone withdrawal (Menon, 2016b, Nadeem, 2016). Maternal progesterone levels drops when parturition is completed (Menon, 2016b). This functional progesterone withdrawal may be mediated by a so called "progesterone receptor switch". In the human myometrium, two progesterone receptor isoforms (PR-A and PR–B) are present (Menon, 2016b, Nadeem, 2016), with PR-A repressing the transcriptional activity of PR-B (Menon, 2016b). Throughout pregnancy, the PR-B isoform dominates, which signals the anti-inflammatory actions of progesterone, and represses transcription of so-called "labor" genes and keeps the myometrium in a quiescent state (Menon, 2016b, Nadeem, 2016). In labor, there is abundance of PR-A over PR-B, which induces the repression of the transcriptional activity of PR-B (Menon, 2016b). PR-A abundance (Menon, 2016b) and an increase in local progesterone metabolization (Nadeem, 2016) reverse this repression, with the activation of "labor" genes (Menon, 2016b, Nadeem, 2016). This unlocks a laboring phenotype in the myometrium (rhythmic contractions) and cervix (shortening, dilating) (Menon, 2016a, 2016b, Nadeem, 2016). Inflammatory cytokines and DAMPs increase PR-A abundance, suggesting that inflammation induces the functional progesterone withdrawal (Menon, 2016b). The loss of PR-B-mediated anti-inflammatory actions of progesterone amplifies the pro-inflammatory environment stimulating the recruitment to maternal immune cells in the gestational tissues and prolonging of labor (Menon, 2016b).

Background; cellular senescence. Senescence is a state of permanent cell cycle arrest used by proliferating cells to avoid replication. Unrepaired DNA damage is an important drive for cells to go into senescence, as it prevents the risk of transmitting a neoplastic transformation (Galluzzi, 2016b). Other stresses and aging can induce a senescent state as well (Burton, 2017a, Land, 2016, Wiley, 2016). Senescent cells are resistant to apoptosis and permanently display a so-called senescent-associated secretory phenotype (SASP), which is characterized by the secretion of several cytokines, chemokines, and proteases (Wiley, 2016). Cellular senescence is implicated in many physiological processes, including wound healing and tissue repair (Campisi, 2014), as well as in ST formation (Chuprin, 2013). Fetal membrane senescence is considered to be a primary instigator of human parturition (Menon, 2016a, 2016b). In addition, senescence is identified as an evolutionary conserved principle of mammalian developmental processes (Muñoz-Espín, 2013, Storer, 2013). The different senescent states employ different molecular pathways; e.g. p21 versus p16/p53 in embryonic and non-embryonic senescence, respectively (Campisi, 2014). Because of their resistance to apoptosis and permanent secretory SASP, senescent cells can induce sustainable systemic effects in their adjacent environment (Zhu, 2015b). This is a positive characteristic of physiological senescence, providing a permanent supportive secretory profile. But at the same time, stress-, age- and onco-suppressive senescence can profoundly increase the body's permanent pro-inflammatory burden (Rajagopalan, 2014, Zhu, 2015b). The proportion of senescent cells in the whole body maybe rather low (Zhu, 2015b), but only a small number of senescent cells may be required to really make a difference (Wiley 2016). Indeed, cellular senescence has been identified as a contributor to "inflammaging" and is implicated in cardiovascular disease (Bochenek, 2016), inflamed obese WAT (Newsholme, 2014, Tchkonia, 2013), and non-alcoholic fatty liver disease (Ogrodnik, 2017).

Background; mitochondrial-centered death pathways. Intrinsic apoptosis is cell death as a consequence of (un-repaired) intrinsic perturbations, e.g. oxidative stress. It always occurs via permeabilization of the outer mitochondrial membrane (so-called mitochondrial outer membrane permeabilization, MOMP), which is due to the pore-forming activity of the inserted pro-apoptotic proteins and results in the dissipation of the mitochondrial membrane potential and hence, loss of ATP generation. At the same time, enzymes that are normally confined to the inter-membrane space, but are toxic when in the cytosol, are released and can activate the "apoptosome", with ensuing caspase activation (cytochrome-c, cyt-c), or induce caspase-independent DNA fragmentation (endonuclease G, ENDOG) (Galluzzi, 2012). Loss of cyt-c hampers the electron transport chain and augments ROS generation (Galluzzi, 2012). Extrinsic apoptosis is cell death induced by extra-cellular stress (signals), via ligand binding of death receptors (Fas, TRAILR1/2, TNFR1) or by critically low ligand availability of dependence receptors. It can proceed via MOMP (e.g. pancreatic β-cells, hepatocytes), or is independent of mitochondria (e.g. lymphocytes) dependent on the cell type (Galluzzi, 2012). A form of regulated necrosis due to oxidative stress or calcium overload occurs via sudden pore-forming in the inner mitochondrial membrane, called the membrane permeabilization transition (MPT), via a multi-molecular complex without the participation of caspases (Galluzzi, 2015). It leads to the dissipation of the mitochondrial trans-membrane potential ($\Delta\psi m$), the mitochondrial matrix is swollen (influx of water/ions) and the outer membrane eventually disrupts (Green, 2014, Kroemer, 2010).

Background; autophagic-lysosomal flux. Macro-autophagy (further referred to as autophagy) is central to many adaptive stress responses due to its degradative capacity, which can serve dual roles. First, it can eliminate damaged structures. Second, it can provide internal energy under conditions of stress (e.g. starvation) by recycling intracellular components. Hence, autophagy is induced by a broad variety of stressors, including hypoxia, oxidative stress, and DNA damage, directly or via

other stress pathways (Kroemer, 2010). Autophagy eliminates damaged structures in collaboration with lysosomes and enzymes therein; damaged structures are tagged (e.g. ubiquitin) and shuttled into an autophagosome (e.g. via p62), which finally fuses with the lysosomal membrane followed by degradation of its contents (Sica, 2015). For the degradation of potentially toxic proteins, autophagy works together with the protease system and shares its mechanism to tag intracellular "garbage" with ubiquitin moieties as a way to identify structures subject to elimination (Kroemer, 2010, Terman, 2010). Even, basal levels of autophagy (constitutive autophagy) have been proposed to keep cells clean from cellular "waste" that accumulated as a result of routine daily cellular activities (Galluzzi, 2016a, Sica, 2015), as suggested for pancreatic-β cells and hepatocytes (Czaja, 2011). Cellular waste includes defective organelles, like mitochondria and peroxisomes, as well as modified and/or aggregated protein complexes. Moreover, when cells are exposed to more general stress, such as nutritional deprivation or growth factor depletion, autophagy is important for cell survival by breaking down the cells "assets" (endogenous sources) (Zechner, 2009). Recycling of organelles, proteins, lipid droplets, and membranes provides building blocks and maintains cellular energy homeostasis (Czaja, 2011, Galluzzi, 2015, Kroemer, 2010, Mizushima, 2008, Sica, 2015). Of note, this recycling may not be entirely random and potentially target minimally damaged organelles (e.g. mitochondria) and proteins (aggregates) that contributes to the wellbeing of the cell.

Autophagic-lysosomal flux in preeclampsia. Several markers of autophagy (beclin-1, LC3-II and STQM/p62) are up-regulated in preeclamptic villous tissue (Akcora-Yildiz, 2017) in early onset (Gao, 2015) and severe (Oh, 2008) preeclampsia. This coincides with decreased expression of the lysosomal-associated membrane protein-2 (LAMP-2; related to the numbers of lysosomes) and the lysosomal transcription factor EB (TFEB) (Nakashima, 2015). Together, these data indicate that in preeclamptic placentas, the selection procedure of autophagy is (highly) operational (increased beclin-1, LC3-II), but the flux is compromised. Especially the final degradation of sequestered material within lysosomes might be affected, based on decreased LAMP-2, TFEB and higher p62, which is normally degraded within the lysosomes (Dimasuay, 2017, Götzl, 2016, Nakashima, 2017). Such lysosomal dysfunction and 'catabolic' defect has been implicated in neurodegenerative diseases as well (Redmann, 2016).

Background; the unfolded protein response (UPR). Most cellular proteins are synthesized in the ER and then used internally or secreted. The UPR is an ancient stress response that detects unfolded/misfolded proteins within the ER lumen during periods of extensive metabolism or downstream ER stress (Burton, 2017a, Collett, 2018). The UPR works together with other mechanisms (such as autophagy) to resolve any ensuing accumulation of these dysfunctional proteins. Three UPR arms can be called upon. First, protein kinase RNA-like ER kinase (PERK)-mediated eIF2α phosphorylation induces a transient arrest of mRNA translation, with the exception of some selective genes, including those associated with the UPR and autophagy (Hotamisligil, 2010). Second, inositol requiring enzyme-1 (IRE-1) degrades unfolded/misfolded proteins and together with stimulated autophagy immediately relieves the protein load (Sica, 2015, Hetz, 2012). Third, activating transcriptional factor 6 (ATF6) and IRE-1-spliced XBP-1 (X-box binding protein-1) transactivate genes encoding molecular chaperones and augment protein-folding capacity (Sica, 2015). When the UPR fails to restore protein homeostasis, the cell may succumb in demise. Activated ATF4 and CHOP (transcription factor C/EBP homologous protein) can induce apoptosis (Burton, 2017a, Green, 2014), with caspase-12 implicated as the executor of ER-mediated apoptosis (Nakagawa, 2000).

The unfolded protein response in pregnancy and preeclampsia. There was no difference in UPR activation between second trimester and term control placentas, which suggests that increased ER stress is not part of the normal gestational trajectory (Yung, 2014). Findings in preeclamptic villous trophoblast (phosphorylation of eIF2α and IRE-α, increased XBP-1, ATF6) indicate that

there is involvement of the three arms UPR (Fu, 2015, Mizuuchi, 2016, Yung, 2014). The degree of placental UPR activation seems to relate to the timing of preeclampsia onset; early-onset placentas showing higher activation, placentas from late-onset (≥ GW 34) preeclamptic and control pregnancies had similar UPR responses (Yung, 2014). However, in another study, late-onset severe preeclamptic placentas express higher CHOP and caspase-12 expression as compared to control placentas (Fu, 2015).

CHOP content was elevated in preeclamptic placentas with IUGR, but not in isolated IUGR placentas (Yung, 2008). A concept that moderate ER stress induces an IUGR phenotype and severe ER stress provokes IUGR with maternal preeclamptic manifestations is emerging (Burton, 2011). Recent experimental evidence supports this model (Collett, 2018); only severe (not mild) ER stress, which is accompanied with CHOP expression, triggers the release of DAMP-associated micro-vesicles in BeWo cells.

Background; aging. Aging can be defined as "loss of cell/organismal adaptability, followed by functional loss/morbidity and cellular death/mortality" (Terman, 2010). Cellular aging may depend on challenges experienced over the cellular lifespan (e.g. type of stress), the efficiency of adaptive stress responses and the efficacy of waste elimination (catabolic degradative capacity). Oxidative stress may be one of the discriminating features of accelerated aging (Terman, 2010), and lysosomes may get overloaded with non-degradable oxidatively damaged material (e.g. protein aggregates) (Terman, 2010). Especially oxidatively damaged mitochondria trigger lipofuscin formation (i.e. age pigment, a rather slow process) within lysosomes, which renders them dysfunctional (Terman, 2010). Lipofuscin accumulation has been addressed in neurodegeneration (Moreno-Garcia, 2018). Cells that divide can redistribute their waste over two daughter cells, and delay the aging process. Post-mitotic cells lack this ability and long-lived post-mitotic cells, especially those with high oxidative capacity (like cardiac myocytes and cortical neurons) (Redmann, 2016), have to rely heavily on their catabolic degradative capacity.

Background; damage-associated molecular patterns (DAMPs). The rationale of DAMP release is to identify and act upon a site of "endogenous danger". DAMPs are recognized by pattern recognition receptors (PPRs), like Toll-like receptors (TLRs) and receptors for advanced glycation end products (RAGEs). PPRs were first appreciated for their detection of pathogen-associated molecular patterns (PAMPs), with downstream pro-inflammatory responses. These help innate immune cells to recognize and act upon microbial invaders (first line of immune defense, innate immunity), and inflict an anti-microbial T cell response if necessary (adaptive immunity). The same PPRs recognize DAMPs, albeit downstream responses are less vigorous, which enables (innate) immune cells to recognize and migrate to an "endogenous danger site" (stress, injury) with the intention to restore tissue homeostasis by processes such as cellular debris clearance and tissue repair (Giaglis, 2016, Pittmann, 2013, Tan, 2017).

Programmed apoptotic cell death, as part of normal cellular turnover, coincides with minimal release of DAMPs, as these remain sequestered within apoptotic bodies. These bodies are neatly engulfed by professional phagocytes, particularly macrophages, and their content degraded within their lysosomes (Fadok, 2000, Kawane, 2014, Peter, 2008). Any (parts of) antigen presented will instigate tolerating Treg cell responses "in absence of danger". This provides one rationale by which apoptotic sperm cells (unprotected intercourse) and apoptotic extra-villous trophoblasts (at the decidual interface) during normal cellular turnover are involved in generating tolerance against paternal and fetal alloantigens, respectively. In stark contrast, cells dying of severe insults (e.g. trauma, ischemia) termed accidental death, release substantial DAMPs, due to excessive, uncontrolled membrane disintegration with the free release of danger molecules (Galluzzi, 2016a, 2012).

Cells dying of stress in a regulated way do release DAMPs and it has been suggested that the underlying type of stress causing cell death of the cells is an important determinant of the DAMP signature. Cancer cells that undergo apoptosis due to a few specific treatments have been found to release death-associated DAMPs (Casares, 2005) that instigate anti-cancer T cell responses (Kepp, 2014). Identifying such an "immunogenic" DAMP release is important for onco-immunologists as it can improve cancer prognosis (Kepp, 2014). Indeed, several DAMPs have been identified that need to be released in a strictly controlled (temporal) manner (HMGB1, calreticulin, extracellular ATP), with the release of dys-homeostatic DAMPs (e.g. calreticulin) prior to the cell death (Garg, 2014, Land, 2016). The underlying stress causing the cellular demise may be decisive (Land, 2016) and was believed to be excessive ROS generation by the ER, associated with severe proteotoxicity (Garg, 2014, Land, 2016). Similar DAMP release has been documented in *in vivo* settings of ischemic reperfusion injury in solid graft transplants (Land, 2016). It could be relevant to human pregnancy, as an stressed ST can release DAMPs for a prolonged period of time.

Background; circulating cell-free DNA. Under homeostatic conditions, a small amount of cell-free human adult DNA circulates in the blood in a fragmented form, due to the numerous, particularly blood-born cells that normally die every day (Basak, 2016, Mittra, 2012, Thierry, 2016). In apoptotic cells, caspase-activated DNAse cleaves DNA (at linker DNA between nucleosomes), which yields specific-sized fragments or multiples thereof (Kawane, 2014, Pisetsky, 2012). When macrophages engulf apoptotic bodies, the DNA is further degraded into its end-products, i.e. nucleic acids by DNAse-II in the macrophage's lysosome. This process is not 100% perfect and some fragmented DNA will appear in the circulation as a normal by-product of daily cellular turnover (Mittra, 2012). Under physiological conditions, the amount of DNA is not sufficient to trigger endosomal TLR9 activation (Mittra, 2012) and does not serve as a 'danger signal'. Similarly, maternal macrophages that take up nuclear trophoblastic material (sprouts, knots, apoptotic CT) may spill over cell-free fetal DNA into the maternal circulation.

Cell-free fetal DNA in the maternal circulation may thus be derived from apoptotic bodies (apoptotic CT, transported through the ST) and multi-nucleated macro-vesicular material (sprouts, knots) released by the ST. In addition, the ST itself maybe a direct source of cell-free fetal DNA, primarily released as isolated fragments (Gupta, 2004), although it can be released in micro-vesicles as well, in accordance with *in vivo* findings (Bischoff, 2005).

Background; insulin resistance. As insulin controls the daily clearance of dietary glucose and lipids by skeletal muscle and WAT, the creation of resistance to this action enables the body to redistribution these incoming nutrients. Tissues that do not rely on insulin for uptake, like immune cells and human villous trophoblast, are beneficiaries of the redistribution of dietary nutrients and can use them to cover their extra needs. Furthermore, IR results in mobilization of nutrients from tissue stores (WAT lipolysis) and stimulates *de novo* production of glucose (e.g. hepatic gluconeogenesis), as these processes are suppressed by insulin. At the molecular level, inflammatory mediators, like TNF-α, activate inflammatory kinases that trigger inhibitory serine phosphorylation of substrates of the insulin signaling cascades (Hotamisligil, 1996). Hence, metabolic cells, like adipocytes and hepatocytes, are able to 'perceive' an inflammatory state, such as an infection or the low-grade inflammation of human pregnancy, and make metabolic adaptations accordingly. If allocated/prioritized nutrients are effectively consumed, IR is a well-adapted physiological metabolic state allowing the body to cope with (temporary) extra demands.

This is in contrast to the IR state that develops in a subpopulation of obese individuals, notably the visceral obese, where nutritional surplus seems to activate similar inflammatory kinases within metabolic cells (Gregor, 2011). This may be directly induced by the nutrient itself or via cellular stress (Gregor, 2011). As a metabolic trigger induces the inflammatory cascade, this phenomenon

was addressed as "metaflammation" (Hotamisligil, 2006). The net result is energy/nutritional allocation in absence of extra metabolic demands, even in the presence of a positive energy balance; a complete misperceived metabolic adaptation. Dietary glucose and lipids are not cleared in their appropriate tissues, are even liberated or newly formed. Lipids can end up in extra-adipose tissues, like the liver and the pancreas, with limited storage capacity, a phenomenon referred to as ectopic fat accumulation. In order to maintain glucose homeostasis, compensatory hyperinsulinemia may lead to further metabolic disturbances as it impacts lipid homeostasis (surplus glucose can be converted into a fatty acid via *de novo* lipogenesis); it has even been labeled a "mixed blessing" (Reaven, 2005). Metabolic aberrations include elevated triglycerides, an atherogenic lipoprotein profile (low HDL-c, increased small dense LDL), endothelial dysfunction, hypertension, all clustered in the term insulin resistance syndrome (Reaven, 2005). Obese individuals with such metabolic derangements meet the criteria of the metabolic syndrome and are at increased risk for atherogenic cardiovascular diseases as well as diabetes mellitus type 2, when glucose homeostasis is no longer maintained (pancreatic β-cells failure, Leahy, 2005). Obesity, especially visceral obesity, is often accompanied by proteinuria and increases the risk for renal diseases (Amann, 2013).

Background; proteo-toxicity. Certain proteins, like amyloid precursor protein and transthyretin, have the tendency to form non-functional aggregates (oligomers) under conditions of environmental stresses. This process starts with the rare assembly of some misfolded monomers of the protein (Borland, 2017). This so-called "nucleus" triggers a pathological cascade of oligomerization and fibril formation of other monomers (Borland, 2017). These assemblies are collectively termed amyloids. In an attempt to relieve cytotoxic burden, neurons try to dispose of these amyloid seeds/aggregates into the extracellular space (Borland, 2017). Although preventing cytotoxicity this may simultaneously "spread" these toxic aggregates among other neuronal cells, triggering similar pathological cascades (Borland, 2017). To date, 36 proteins that can form extracellular amyloid fibrils have been identified (Sipe, 2016). The loss of glomerular barrier function in preeclampsia-induced proteinuria may have the beneficial effect of eliminating large toxic aggregates from the circulation (Buhimschi, 2014). Next to the cytotoxicity of amyloid aggregates, the consumption of protein monomers for aggregate formation results in the loss of the protein monomer to exerts its functional role. For example, transthyretin binds sEng and reduced maternal levels observed in preeclamptic pregnancies can adversely impact the angiogenic balance (Kalkunte, 2013). Different species of amyloid aggregates may be present, which can contribute to clinical variation in preeclamptic manifestations (Millen, 2018).

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Scope of the thesis

SCOPE OF THE THESIS

Maternal preeclamptic manifestations reflect the manner by which the mother copes with preeclamptic placental stress that originate from a combination of genetic and environmental risk factors. Following a general introduction on the (patho)physiology of normal and preeclamptic pregnancy, this thesis concentrates on genetic, immunological and nutritional factors that may be involved in the etiology of preeclampsia. The study population was composed of women living in the island of Curaçao, who are predominantly of West-African descent with ample Caucasian admixture.

Preeclamptic women may carry genetic risk factors. We investigated three single nucleotide polymorphisms (SNPs) for their association with preeclampsia. One of the SNPs, the Duffy-null phenotype) (a SNP that conveys resistant to malaria; Chapter 2.1) is prevalent in our study population, whereas the other two SNPs, i.e. MTHFR C677T (involved in one-carbon (folate) metabolism; Chapter 2.2) and HFE C282Y (involved in iron transport; Chapter 2.3) are particularly common in Caucasian populations.

Preeclampsia is clearly characterized by an inflammatory response. We evaluated whether the activation of neutrophils by interleukin-8 (IL-8) may be involved in the underlying immunological response (Chapter 3).

Preeclampsia is characterized by reduced placental nutrient transfer and a maladapted hormonal milieu. In Chapter 4, we studied the potential involvement of: long-chain polyunsaturated fatty acids (LCP), nutrients involved in one-carbon metabolism (folate, vitamin B_{12}, choline, betaine) and a parameter of iron status (soluble transferrin receptor). LCP, notably arachidonic acid (AA), eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), are, amongst many other functions, important for the balance between inflammatory and anti-inflammatory reactions, and for fetal growth and brain development. Apart from a case-control study investigating the LCP content of umbilical vessels of preeclamptic and normal pregnancies (Chapter 4.1.1), we conducted an intervention study with the fish oil fatty acids EPA+DHA status in healthy pregnant women (Chapter 4.1.2). The aim was to improve their EPA+DHA status without affecting the AA status. Such an outcome may cause a switch to a less pro-inflammatory and pro-coagulant state, prominent features of preeclampsia. One-carbon metabolism is involved in numerous methylation reactions. Its adequate functioning is dependent on vitamins (folate, vitamins B_{12}, vitamin B_6) and hormones (e.g. estrogens). Inadequate functioning of one-carbon metabolism, reflected by circulating homocysteine has been implicated in many pathologies, such as a pro-coagulant state. We investigated the determinants of circulating homocysteine in healthy pregnant women (Chapter 4.2.1) and preeclamptic counterparts (Chapter 4.2.2). Iron is best known as an essential constituent of hemoglobin, but an iron surplus may cause oxidative stress. An abnormally high (maladapted) hemoglobin and oxidative stress are features of preeclampsia. We investigated the value of the circulating soluble transfer

receptor concentration as a tool for the diagnosis of iron abnormalities in healthy pregnant women (Chapter 4.3).

Chapter 2

Genetics

The association of pre-eclampsia with the Duffy negative phenotype in women of West African descent

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ABSTRACT

Objective To investigate whether pre-eclampsia in Curaçao is associated with the Duffy negative phenotype.
Design Retrospective study.
Setting Population Methods
Results Women with a history of pre-eclampsia had a higher Duffy negative phenotype frequency compared with women with a history of uncomplicated pregnancies (52.8 *vs* 27.3%, respectively; odds ratio 2.98; 95% CI 1.40 – 6.32; $P = 0.004$).
Conclusions Pre-eclampsia is associated with the Duffy negative phenotype in women of West-African descent in the island of Curaçao.

INTRODUCTION

The Duffy blood group antigens Fy^a and Fy^b are the expression products of the alleles FY*A and FY*B. Phenotypes Fy(a+,b-), Fy(a-,b+) and Fy(a+;b+) derive from the genotypes FY*A/FY*A, FY*B/FY*B and FY*A/FY*B, respectively. Most West Africans (>95%) do not express Fy^a and Fy^b on their red blood cells. This condition is designated as the Duffy negative phenotype and denoted Fy(a-,b-)[1]. Tournamille *et al.*[2] demonstrated that Duffy negative individuals from West African descent carry a FY*B allele with normal coding sequences, but with a single T to C substitution at nucleotide 46. The mutation disrupts a binding site of the erythroid transcription factor and thereby abolishes the erythroid-specific expression of the Duffy antigen. The expression on (subsets of) endothelial cells remains preserved[3].

Chemokines are chemoattractant cytokines that can be subdivided into various families that each recruit and activate different leucocyte subsets[4]. The effects of chemokines are mediated by binding to specific high affinity receptors and a second class of receptors with lower affinity that bind the same family members. Darbonne *et al.*[5] discovered a third receptor on red blood cells membranes, that is able to bind chemokines of different families at high affinity. This receptor proved identical to the Duffy blood group antigen and is designated Duffy Antigen Receptor of Chemokines (DARC)[6]. Interleukin-8 (IL-8) is the most intensively investigated ligand of erythroid DARC. IL-8 is known for its ability to attract and activate neutrophils. Following its introduction in the circulation, IL-8 is rapidly and efficiently bound to erythroid DARC[5,7,8]. Duffy negative red blood cells, by the absence of erythoid DARC, bind IL-8 minimally [9].

Stallmach *et al.*[10] reported increased serum IL-8 in 13 pre-eclamptic women compared with 20 normotensive women. In a preliminary study, we determined serum IL-8 levels and the Duffy phenotype in 13 pre-eclamptic women with 13 gestational age- and parity-matched controls living in the island of the Netherlands Antilles (mainly Curaçao). These islands are inhabited by a population of West African descent, with an estimated frequency of the Duffy negative phenotype of 37% (as derived from unpublished data from 70 consecutive blood donors). We found significantly higher serum IL-8 and higher frequency of the Duffy negative phenotype in pre-eclampsia[11]. In the present investigation we elaborated on the association between the Duffy negative phenotype and pre-eclampsia in a retrospective study design. The numbers of women with pre-eclampsia and controls were upscaled to 72 and 55, respectively.

METHODS

Hypertensive pregnant women were retrospectively recruited from the records of the St Elisabeth Hospital, which is the only hospital in Curaçao to which pre-eclamptic women are referred. They qualified for participation when they had a diastolic blood pressure of ≥90 mm Hg (measured at two consecutive occasions), in combination with a urinary

protein excretion of ≥0.3 g/L (two consecutive occasions), or ≥0.3 g/24h (one occasion)[12]. The survey yielded a total of 188 pre-eclamptic women in the period 1994 to 1996. Of these, 72 responded upon our request to donate EDTA-blood for Duffy phenotyping. All responders had pre-eclamptic singleton pregnancies and all were of West-African descent.

The controls were recruited from a study on clinical chemical and haematological reference values of pregnant women in the period 1997 to 2000. This study encompassed 193 singleton pregnant women. Sixty-one volunteered to donate EDTA-blood for Duffy phenotyping on request. Six were excluded because of caucasian ethnicity. The remaining 55 had uncomplicated normotensive pregnancies and no history of pre-eclampsia and all of them gave birth at term to apparently healthy newborns. The study protocol was in agreement with local ethical standards and the Helsinki declaration of 1975 as revised in 1989.

Duffy phenotypes were determined with an indirect antiglobulin test according to the manufacturer's instructions (CLB, The Netherlands). The Duffy positive phenotypes were Fy(a+,b-), Fy(a-,b+) and Fy(a+,b+) and the Duffy negative phenotype was Fy(a-,b-).

Statistics

Between-group differences in clinical characteristics were analysed with the Student's *t* test (maternal age, gestational age at delivery, and birthweight) or the Fisher's exact test (parity). Between-group differences in Duffy phenotype frequencies were investigated with the Fisher's exact test. $P < 0.05$ was considered significant[13].

RESULTS

The characteristics of the 55 women with normotensive uncomplicated pregnancies and the 72 women with pre-eclamptic pregnancies are presented in Table 1. The pre-eclamptic pregnancies ended at lower gestational age and the newborns had lower birthweights. Women with a history of pre-eclampsia had a higher Duffy negative phenotype frequency compared with women with a history of uncomplicated pregnancies (52.8 *vs* 27.3%, respectively; odds ratio 2.98; 95% CI 1.40 – 6.32; $P = 0.004$).

DISCUSSION

Using a retrospective study design we compared the Duffy negative phenotype frequencies in 72 black women with an obstetric history of pre-eclampsia and 55 racematched normotensive controls with a history of uncomplicated pregnancies. Our results show that women with a history of pre-eclampsia had a higher Duffy negative phenotype frequency compared with women with a history of uncomplicated pregnancies. It must be noted that the present number of patients is relatively small, but that the results point

to an association between the Duffy negative phenotype and pre-eclampsia in this black population. As the Duffy negative phenotype among caucasians is rare[14], it is unlikely to play a significant role in pre-eclampsia of the white population.

Duffy negative individuals do not express erythroid DARC, but DARC expression remains preserved on endothelial cells. The exact physiologic significance of erythroid DARC and endothelial DARC remains to be established, despite sizable knowledge on the tissue distribution and structure-function relationships of these receptors[1]. Erythroid DARC can not internalise its ligands and there is no signal transduction upon ligand binding[1,5]. Darbonne *et al.*[5] showed that IL-8 bound to erythroid DARC is incapable of activating neutrophils and in this way erythroid DARC may act as a 'sink' for IL-8 (and other chemokines) that have been released into the circulation. Absence of erythroid DARC in Duffy negative individuals consequently reduces red blood cells IL-8 binding capacity[9]. This may be particularly important during processes with higher production of IL-8 (as proposed in pre-eclampsia[10]) resulting in increased IL-8 accessibility for binding to non-erythroid receptors, such as those located on neutrophils, monocytes and lymphocytes[4] and endothelial DARC. Especially binding to endothelial DARC may be relevant to the pre-eclampsia pathophysiological cascade. Due to its capacity to internalise IL-8[3], endothelial DARC is proposed to be an IL-8 presenting receptor, participating in the attraction and activation of neutrophils[1,15,16]. The latter process is implicated in the endothelial damage of pre-eclampsia[17 – 19].

In conclusion, women with a history of pre-eclampsia have a higher Duffy negative phenotype frequency compared with women with a history of uncomplicated pregnancies. The present finding would be in line with the growing body of evidence that pre-eclampsia is an excessive maternal inflammatory response to pregnancy[20]. Prospective studies with larger subject numbers should establish whether the Duffy negative phenotype constitutes a risk factor for pre-eclampsia in populations with high frequencies of Duffy negative phenotypes.

Table 1. Clinical characteristics and Duffy phenotype of pre-eclamptic women and controls. The Duffy positive phenotypes are Fy(a+,b-), Fy(a-,b+) and Fy(a+,b+), the Duffy negative phenotype is Fy(a-,b-).

Variables	Controls (*n*=55)	Pre-eclampsia (*n*=72)
Mean [SD] maternal age (years) Parity	28.3 [6.2]	28.6 [6.5]
(nulliparae/multiparae), *n*	16/39	29/43
Mean [SD] gestational age at delivery (wks)	39.8 [1.0]	35.3 [4.0]*
Mean [SD] birthweight (g)	3367 [418]	2194 [882]*
Duffy phenotype		
Negative *n* (%)	15 (27.3)	38 (52.8)**
Positive *n* (%)	40 (72.7)	34 (47.2)

* $P < 0.001$, Student's *t*-test.
** $P < 0.01$, Fisher's exact test.

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Maternal and infant methylenetetrahydrofolate reductase C677T genotypes of Afro-Caribbean women with preeclampsia

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ABSTRACT

Objective and Study Design: We established in a retrospective study design the methylene tetrahydrofolate reductase (MTHFR) C677T genotypes and T-allele frequencies of 89 preeclamptic Afro-Caribbean women living in Curaçao (Netherlands Antilles), 89 race and parity-matched normotensive controls with uncomplicated pregnancies, 49 children born to the preeclamptic women during their preeclamptic pregnancy and 49 children born to the controls.

Results: There were no significant case-control differences in MTHFR C677T genotype frequencies and T-allele frequencies of the mothers, children and the mother/child combinations.

Conclusion: We conclude that MTHFR C677T polymorphism of mother, child and mother/child combination does not constitute a preeclampsia risk factor in Curaçao.

INTRODUCTION

Preeclampsia is considered to be an endothelial cell disorder and high circulating homocysteine is a recently identified risk factor for endothelial damage.[1] Several investigators have reported increased homocysteine in preeclampsia.[2-4] Powers *et al* reported a positive relation between plasma homocysteine and fibronectin, which is a marker of endothelial damage.[3]

Plasma homocysteine is dependent on genetic factors, environmental factors and other factors including renal function.[5] Proper functioning of enzymes involved in homocysteine metabolic clearance is dependent on their cofactors, vitamin B_{12} and vitamin B_6, and their methyl donors betaine and notably 5-methyl tetrahydrofolate (5-methyl THF). The latter is the predominant form of plasma folate, and is formed from 5,10-methylene THF by methylene tetrahydrofolate reductase (MTHFR). Low folate status may especially give rise to hyperhomocysteinemia when it occurs in subjects who carry a common MTHFR isoform caused by a C to T mutation at nucleotide 677 (C677T). The mutation may involve the folate binding region of the enzyme[6] and the so-called MTHFR thermolabile variant may have reduced folate affinity.[7] Subjects with the 677 TT genotype exhibit an exaggerated elevation of plasma homocysteine in response to folate depletion. This results in moderately elevated plasma homocysteine, notably at lower folate status.[8] The prevalence of the MTHFR isoforms differs among various ethnic populations with high prevalence among Caucasians and low prevalence among blacks.[9]

The MTHFR C677T mutation could be involved in high circulating homocysteine reported in preeclampsia[2-4] and the incidence of the MTHFR C677T mutation in preeclampsia was consequently investigated in various ethnic groups. Some investigators reported higher incidence[10-12] while others found no differences.[13-15] Systematic reviews of Zusterzeel *et al.*[15] and Ray and Laskin[16] identified the MTHFR thermolabile variant as a moderate risk factor for preeclampsia with an odds ratio of 1.9 (95% CI: 1.4-2.6) and 2.6 (95% CI: 1.4-5.1), respectively.

Homocysteine is capable of crossing the placenta. The fetus is therefore likely to participate in homocysteine homeostasis during pregnancy.[17] Maternal homocysteine levels may thus be critically dependent on folate status, maternal MTHFR C677T genotype, but possibly also fetal MTHFR C677T genotype.

We investigated in a retrospective study design whether preeclamptic pregnancies are characterized by higher frequencies of MTHFR C677T in the mothers, children and the mother/child combinations. For this we genotyped 89 Afro-Caribbean women who had a history of preeclampsia and compared the outcome with that of 89 race and parity-matched controls with uncomplicated pregnancies. We also genotyped 49 children born to the preeclamptic women during their preeclamptic pregnancies and 49 children born to the controls. All women live in the island of Curaçao (The Netherlands Antilles). Curaçao is inhabited by about 150,000 subjects of predominantly West-African (Ghana) origin. There has been ample interracial mixture, notably with Caucasians. Preeclampsia is the most severe complication encountered in obstetric care in Curaçao.

MATERIAL AND METHODS

Study group

Hypertensive pregnant women were retrospectively recruited from the records of the St. Elisabeth Hospital in Curaçao. They qualified for participation when they had a diastolic blood pressure of ≥90 mm Hg (measured at two consecutive occasions), together with a urinary protein excretion of ≥0.3 g/l (two consecutive occasions), or ≥0.3 g/24 h (one occasion).[18] Severe preeclampsia was defined as a diastolic blood pressure of ≥120 mm Hg, an urinary protein excretion of ≥5 g/l (two occasions) or ≥5 g/24 h (one occasion), occurrence of the HELLP syndrome (i.e. platelet count less than $100x10^{-9}$/l, ASAT >48 U/l and LDH >168 U/l) or eclampsia.[13,18] The survey yielded a total of 188 preeclamptic women in the period of 1994-1996. Upon our request 89 donated EDTA-blood for MTHFR C677T genotyping. We were also able to obtain EDTA-blood for genotyping from 49 infants who were liveborn to these preeclamptic women during their singleton preeclamptic pregnancy. Preeclamptic index pregnancies were divided in nulliparous (parity 0), parity 1 to 3 and parity ≥4 to allow selection of a parity-matched control group.

Controls were recruited from a study on the incidence of hemoglobinopathies in Curaçao. This study was composed of 326 consecutive deliveries that took place in the St. Elisabeth Hospital or the Maternity Clinic "Rio Canario" in the period 1992-1993. Women of whom the buffy coats of both maternal and cord EDTA-blood were available to us for analysis, who had singleton pregnancies, who had no history of preeclampsia and were normotensive during the index-pregnancy were selected. From the total of 167 who met these criteria we selected 89 parity-matched mothers to serve as maternal controls. From their 89 children we randomly selected 49 to serve as infant controls. The matching criterion was based on parity of their mothers.

The study protocol was in agreement with local ethical standards and the Helsinki declaration of 1975, as revised in 1989.

MTHFR C677T genotyping

Genomic DNA was isolated from buffy coats that had been stored at -70° C. MTHFR C677T genotypes were analyzed by restriction fragment length polymorphism[6] in the Laboratory of Pediatrics and Neurology of the University Hospital Nijmegen, The Netherlands.

Statistics

Between-group differences of pregnancy characteristics were analyzed with the Student's T-test for paired samples (controls versus preeclampsia) or Student's T-test for independent samples (severe versus mild preeclampsia) corrected for type-1 errors (Bonferroni). Between-group differences in MTHFR C677T frequencies and T-allele frequencies were investigated with the Pearson's chi-square test.[19] $p<0.05$ was considered significant.

RESULTS

Preeclampsia versus controls

The characteristics of the preeclamptic and control pregnancies are presented in Table 1, together with the corresponding maternal, infant and mother/child MTHFR C677T genotypes and T-allele frequencies. The preeclamptic women proved older. They had higher maximum diastolic blood pressure (by definition), their children had lower birth weights and they had lower gestational age at delivery. There were no differences between the maternal, infant and mother/child MTHFR C677T genotypes and T-allele frequencies of preeclamptic women and controls.

Mild versus severe preeclampsia

Preeclamptic pregnancies were subdivided according to severity into mild and severe preeclamptic pregnancies. Mild and severe preeclamptic pregnancies were compared to their race- and parity matched controls. The characteristics of these control pregnancies and the mild and severe preeclamptic pregnancies are presented in Table 2, together with the corresponding maternal, infant and mother/child MTHFR C677T genotypes and T-allele frequencies. Women with mild preeclampsia proved older, had higher maximum diastolic blood pressure, their children had lower birth weights and they had lower gestational age at delivery compared to controls. Women with severe preeclampsia had higher maximum diastolic blood pressure, their children had lower birth weights and they had lower gestational age at delivery compared to controls and compared to women with mild preeclampsia.

Maternal MTHFR genotype distribution differed among women with mild preeclampsia compared to women with severe preeclampsia (heterozygous MTHFR C677T genotype 34.5 versus 9.7% and homozygous MTHFR C677T genotype 3.4 versus 0.0%, respectively; p=0.039; odds ratio of 5.7 (95% CI: 1.5-21.0) for the heterozygous- and homozygous MTHFR C677T genotype relative to the wild type genotype). This resulted in a higher T-allele frequency for women with mild preeclampsia compared with severe preeclampsia (20.7% versus 4.8%, respectively; p=0.014; odds ratio 5.1 (95%CI: 1.5-17.8). There were no such differences between women with mild preeclampsia and controls and between women with severe preeclampsia and controls. There were also no differences in infant and mother/child MTHFR C677T genotypes and T-allele frequencies for mild and severe preeclamptic pregnancies.

Table 1. Pregnancy characteristics together with maternal, infant and mother/child MTHFR C677T genotypes and T-allele frequencies for preeclamptic and control pregnancies.

	controls	preeclampsia
Pregnancy characteristics		
number	89	89
maternal age (mean+SD; years)	25.8±6.5	28.0±6.0[1]
parity [number (%)]		
para 0	38 (42.7)	38 (42.7)
para 1-3	51 (52.8)	51 (52.8)
para ≥4	4 (4.5)	4 (4.5)
gestational age (weeks)	39.2±2.6	35.0±3.8[1]
birth weight (g)	3280±495	2121±830[1]
diastolic blood pressure (mm Hg)	72±8	108±14[1]
Maternal MTHFR genotypes [number (%)]		
number	89	89
CC	68 (76.4)	64 (71.9)
CT	20 (22.5)	23 (25.8)
TT	1 (1.1)	2 (2.2)
T-allele frequency (%)	12.4	15.2
Infant MTHFR genotypes [number (%)]		
number	49	49
CC	37 (75.7)	37 (75.7)
CT	11 (22.4)	11 (22.4)
TT	1 (2.0)	1 (2.0)
T-allele frequency (%)	13.3	13.3
MTHFR genotypes of mother/child combination [number (%)]		
number	49	49
CC/CC	33 (67.3)	27 (55.1)
CC/CT or CT/CC	9 (18.4)	15 (30.6)
CC/CT	5 (10.2)	5 (10.2)
CT/CC	4 (8.2)	10 (20.4)
CT/CT	5 (10.2)	5 (10.2)
CT/TT or TT/CT	2 (4.1)	2 (4.0)
CT/TT	1 (2.0)	1 (2.0)
TT/CT	1 (2.0)	1 (2.0)
T-allele frequency (%)	12.8	15.8

[1]Statistically different from controls by Student's T-test for paired samples at $p<0.001$. The maternal, infant and mother/child MTHFR C677T genotype distributions and T-allele frequencies of preeclamptic women and controls were not statistically different by Pearson's chi-square test.

Table 2. Pregnancy characteristics together with maternal, infant and mother/child MTHFR C677T genotypes and T-allele frequencies for pregnancies with mild and severe preeclampsia and their parity-matched controls.

Pregnancy characteristics	controls	mild preeclampsia	controls	severe preeclampsia
number maternal/infant MTHFR genotype	58/39	58/39	31/10	31/10
mother/child MTHFR genotype	39	39	10	10
maternal age (mean±SD; years)	25.9±6.7	28.2±5.9[1]	25.7±6.3	27.9±6.1[1]
parity [number (%)]				
para 0	23 (39.6)	23 (39.6)	15 (48.4)	15 (48.4)
para 1-3	31 (53.4)	31 (53.4)	16 (51.6)	16 (51.6)
para ≥4	4 (6.9)	4 (6.9)	0 (0.0)	0 (0.0)
gestational age (weeks)	39.2±3.1	36.1±3.1[1]	39.2±1.4	32.9±4.2[1,2]
birth weight (g)	3274±535	2374±775[1]	3293±415	1615±704[1,3]
diastolic blood pressure (mm Hg)	73±8	105±12[1]	71±7	114±15[1,3]
Maternal MTHFR genotype [number (%)]				
CC	43 (74.1)	36 (62.1)	25 (80.6)	28 (90.3)[4]
CT	15 (25.9)	20 (34.5)	5 (16.1)	3 (9.7)
TT	0 (0.0)	2 (3.4)	1 (3.2)	0 (0.0)
T-allele frequency (%)	12.9	20.7	11.3	4.8[4]
Infant MTHFR genotype [number (%)]				
CC	31 (75.5)	29 (74.3)	6 (60.0)	8 (80.0)
CT	7 (22.4)	9 (23.1)	4 (40.0)	2 (20.0)
TT	1 (2.0)	1 (2.6)	0 (0.0)	0 (0.0)
T-allele frequency (%)	11.5	14.1	20.0	10.0
MTHFR genotypes of mother/child combination [number (%)]				
CC/CC	27 (69.2)	20 (51.3)	6 (60.0)	7 (70.0)
CC/CT or CT/CC	6 (15.4)	12 (30.8)	3 (30.0)	3 (30.0)
CC/CT	2 (10.2)	3 (7.7)	3 (30.0)	2 (20.0)
CT/CC	4 (8.2)	9 (23.1)	0 (0.0)	1 (10.0)
CT/CT	5 (12.8)	5 (12.8)	0 (0.0)	0 (0.0)
CT/TT or TT/CT	1 (2.6)	2 (5.1)	1 (10.0)	0 (0.0)
CT/TT	1 (2.6)	1 (2.6)	0 (0.0)	0 (0.0)
TT/CT	0 (0.0)	1 (2.6)	1 (2.0)	0 (0.0)
T-alleles frequency (%)	12.2	17.9	15.0	7.5

Statistically different from controls by Student's T-test for paired samples at [1]$p<0.001$ and statistically different from mild preeclampsia by Student's T-test for independent samples at [2]$p<0.05$ and [3]$p<0.001$ corrected for Bonferroni. Maternal MTHFR C677T genotype distribution and maternal T-allele frequencies statistically different from mild preeclampsia by Pearson's chi-square test at [4]$p<0.05$.

DISCUSSION

We established in a retrospective study design the MTHFR C677T genotypes and T-allele frequencies of 89 preeclamptic black Afro-Caribbean women, 89 race and parity-matched normotensive controls with uncomplicated pregnancies, 49 children born to the preeclamptic women during their preeclamptic pregnancy and 49 children born to the controls. We consider the selected preeclamptic mothers and the subset of their liveborn infants as representative for the preeclamptic pregnancies that occurred in Curaçao in the period of 1994-1996**.** There were no significant case-control differences in MTHFR C677T genotype frequencies and T-allele frequencies of the mothers, infants and the mother/child combinations (Table 1).

There is controversy regarding the influence of MTHFR C677T polymorphism in the etiology of preeclampsia. Higher frequencies of the homozygous genotype in preeclamptic women were reported for Japanese,[10] Jewish1[11] and European populations,[12] but similar frequencies were found in 99 white Americans,[13] 167 Dutch Caucasian women[15] and 171 African blacks.[14] Disparities could not be explained by different prevalence of the MTHFR C677T mutation in the various ethnic populations, since similar frequencies of the homozygous genotype among controls and preeclamptic women were found in populations composed of white Caucasian women with known high MTHFR C677T prevalence (T-allele frequency 34%)[13,15] and black African women with known low prevalence (T-allele frequency 4%).[14] Our study population was composed of black women from West-African descent and ample mixture with Caucasians, who are carrying a T-allele frequency of about 12%.

With the exception of the study of Powers *et al.*[13], none of the above studies determined the MTHFR C677T genotypes of children born to preeclamptic women. Powers *et al.* reported no difference in the frequency of T677 homozygosity in 15 children born to heterozygous and homozygous preeclamptic mothers when compared with 20 children born to controls. Accordingly, our results show that preeclampsia is not accompanied by higher fetal MTHFR C677T or T-allele frequencies, and in addition shows that this is also not the case when one considers the mother-child combination. It seems therefore unlikely that the fetal genotype is responsible for the conflicting results regarding the influence of the MTHFR C677T mutation in preeclampsia.

There is growing evidence that preeclampsia is a disorder of heterogeneous causes.[20] Differences in time of onset, disease severity and organ involvement suggest different underlying etiologies precipitating into a single syndrome characterized by hypertension and proteinuria.[21] Identification of one conclusive genetic disorder is not to be expected, and multiple risk factors of genetic, environmental and mixed nature are likely to become identified. Different mixtures of underlying etiologies may consequently explain the apparent discrepancies in the association between MTHFR C677T and preeclampsia. One of these may be the ratio between mild and severe preeclampsia, as suggested by the coincidental finding that women with mild preeclampsia have higher frequencies of heterozygous and homozygous MTHFR C677T genotype and higher T-allele frequency, compared with women with severe preeclampsia (Table 2).

We conclude that MTHFR C677T polymorphism of mother, child and mother-child combination does not constitute a preeclampsia risk factor in black women living in Curaçao.

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HFE C282Y heterozygosity and preeclampsia in Afro-Caribbean women

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TO THE EDITORS:

Homozygosity for the common C282Y mutation in the HFE gene explains approximately 85% of cases with hereditary hemochromatosis (HH). C282Y heterozygous individuals do not usually develop clinical manifestations of HH, but their body iron stores are high. C282Y heterozygosity may therefore be a genetic marker of lifelong moderate iron overload.[1]

Moderate excessive iron may participate in the formation of highly reactive free radicals[1] and iron-mediated lipid (low-density-lipoprotein; LDL)-oxidation is e.g. suggested to promote atherogenesis. Studies on the association between C282Y heterozygosity and coronary heart disease (CHD) have, however, produced inconsistent results. One large prospective population-based cohort study showed an association of C282Y heterozygosity with cardiovascular death in Dutch women.[1] A more recent case-control study in Curaçao revealed higher C282Y heterozygosity in patients with CHD compared with controls, 9.6% versus 1.2%, respectively.[2] Preeclampsia shares risk factors with atherosclerosis. LDL-oxidation is proposed to play a role in its pathogenesis and increased serum iron, ferritin and percentage transferring saturation have been reported. We have investigated in a retrospective study design whether preeclampsia is associated with C282Y heterozygosity.

The C282Y genotype prevalence of women with previous preeclampsia was compared to that of controls with a history of uncomplicated pregnancies. Participating women lived in the island of Curaçao, which is inhabited by a population of West-African (mostly Ghana) descent with ample Caucasian mixture. Preeclampsia was defined as a diastolic blood pressure ≥ 90 mm Hg in combination with proteinuria (≥0.3 g/l or ≥0.3 g/24 h). A total of 188 preeclamptic women were recruited from records of the main referral hospital. Of these, 74 donated a blood sample. Control women (n=84) were participants on a study on clinical chemical references values in pregnancy. PCR amplification with sequence-specific primers (PCR-SSP) was used to determine C282Y genotypes. The H63D genotype was determined in C282Y heterozygotes.

C282Y heterozygosity amounted 4.1% (3/74) in women with previous preeclampsia and 2.4% (2/84) in controls (Fisher's exact test p=0.66). There were no C282Y homozygotes or compound C282Y/H63D heterozygotes. The relative low C282Y heterozygosity frequency is in agreement with an admixture of a population from Ghana (0% C282Y prevalence;[3]) and Caucasians, notably Dutch (7.2% C282Y heterozygosity;[1]). It compares reasonably well with the C282Y carrier frequency of our previous study in Curaçao.[2] The presently encountered low heterozygosity prevalence in a relatively small study groups precludes any firm conclusions. We encourage other investigators to study the association between C282Y and preeclampsia in population with well-known high heterozygosity frequency.

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Chapter 3

Immunology

High serum interleukin-8 levels in Afro-Caribbean women with pre-eclampsia. Relations with tumor necrosis factor-α, Duffy negative phenotype and von Willebrand factor

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ABSTRACT

Problem: Pre-eclampsia is characterized by neutrophil activation. Interleukin-8 (IL-8) is a strong neutrophil chemo-attractant and activator.
Method of Study: We measured serum IL-8 in 13 pre-eclamptic Afro-Caribbean women and 13 gestational age-, race- and parity- matched normotensive and non-proteinuric controls. We also determined serum tumor necrosis factor-α (TNF-α), the phenotypes of the IL-8 binding Duffy blood group antigen receptor and the von Willebrand factor (vWF) plasma levels.
Results: Serum IL-8, TNF-α, Duffy negative phenotype frequency and plasma vWF were higher in pre-eclamptic women compared with controls. IL-8 correlated positively with both TNF-α and vWF in the entire study group.
Conclusions: Higher IL-8 levels in pre-eclampsia may result from increased production (secondary to increased TNF-α levels) and/or reduced clearance (related to a high frequency of Duffy negative phenotype).

Key words:
Duffy blood group antigen; receptor of chemokines; neutrophil activation

INTRODUCTION

Pre-eclampsia is characterized by endothelial cell dysfunction and neutrophil activation.[1–4] The underlying mechanism of neutrophil activation is as yet unclear. A conceivable candidate to participate in the process is interleukin-8 (IL-8), because this chemokine is considered to be a strong attractant and activator of neutrophils.[5] IL-8 can be produced by a variety of cells, notably by activated endothelial cells. Tumor necrosis factor-α (TNF-α) is capable of inducing cellular IL-8 production. In the circulation, IL-8 is rapidly and efficiently bound to a high affinity multispecific chemokine receptor located on red blood cells (RBCs). This receptor proved identical to the Duffy blood group antigen[6] and was designated Duffy blood group antigen receptor of chemokine (DARC). IL-8 bound to RBCs is unable to activate neutrophils, and RBC DARC was proposed to function as a sink for excess IL-8 in the circulation.[7] Most West Africans do not express DARC on their RBC, giving rise to the Duffy negative phenotype. In Caucasians, the Duffy negative phenotype is rare.[8] In contrast to Duffy positive RBC, Duffy negative RBC bind IL-8 minimally.[9]

We determined serum IL-8 levels in a group of 13 pre-eclamptic Afro-Caribbean women and 13 gestational age-, race- and parity-matched normotensive and non-proteinuric controls. Concomitantly, we investigated factors that determine serum IL-8 levels; TNF-α by its involvement in IL-8 production and the Duffy phenotype by its involvement in RBC IL-8 binding and subsequent clearance. The von Willebrand Factor (vWF) was determined in plasma as a general marker of endothelial cell dysfunction.[10] All women lived in the island of Curaçao (the Netherlands Antilles) and were predominantly of West African descent. The Curaçao population has an estimated frequency of the Duffy negative phenotype of 37%, as obtained from a study of 70 consecutive blood donors (unpublished).

MATERIALS AND METHODS

Pre-eclampsia was defined as a diastolic blood pressure of ≥90 mmHg (measured at two consecutive occasions) together with a urinary protein excretion of ≥0.3 g/L (two occasions) or ≥0.3 g per 24 hr (one occasion).[11] Apparently, healthy normotensive and non-proteinuric outclinic pregnant Afro-Caribbean women served as controls and were matched for gestational age at the time of blood sampling (±1 week) and parity (nullipara versus multipara). None of the women had preexisting hypertension, preexisting renal disorders and clinical signs of infections. None of them were at labor at the time of blood sampling. All women gave their informed consent. The study was in agreement with local ethical standards and the Helsinki declaration of 1975, as revised in 1989. Serum IL-8 (CLB, The Netherlands), serum TNF-α (Medgenix, Belgium) and plasma vWF (Dakopatts, Denmark) were determined by enzyme-linked immunosorbent assay, as described by the manufacturer. Upon our request, ten women in the pre-eclampsia group

and 11 control women donated ethylenediaminetetraacetic acid blood at least 6 weeks postpartum for the analysis of the Duffy blood group antigens Fy^a and Fy^b by the indirect antiglobulin test (CLB, The Netherlands). Between-group differences of age, gestational age at delivery, diastolic blood pressure and birth weight were analyzed with Student's *t*-test for paired samples at $P < 0.05$, and between-group differences in IL-8, TNF-α and vWF were analyzed with the Wilcoxon matched pairs signed rank test at $P < 0.05$. The between-group difference in Duffy phenotype frequency was analyzed with Fischer's exact test at $P < 0.05$. Correlations were determined with the Spearman rank test at $P < 0.05$.

RESULTS

Serum IL-8 levels were increased in pre-eclampsia ($P = 0.0033$) with each pre-eclamptic woman exhibiting higher levels compared with her matched counterpart. Also, serum TNF-α ($P = 0.0067$) levels and plasma vWF ($P = 0.019$) were increased in pre-ec-lampsia compared with controls (Table I). There were positive correlations between serum IL-8 levels and TNF-α levels ($r = 0.58$; $P = 0.004$) and between serum IL-8 and plasma vWF ($r = 0.45$; $P = 0.048$) for the entire study group. The Duffy negative phenotype frequency was higher in pre-eclampsia compared with the control group (9/10 and 3/11, respectively; $P = 0.0037$). Fig. 1 shows serum IL-8 levels for both pre-eclamptic and control women, as subdivided according to their Duffy phenotype.

Table 1. Subject Characteristics Together with Serum IL-8, Serum TNF-α, Plasma vWF and Duffy Negative Phenotype for Pre-eclamptic Women and their Matched Controls

	Controls	Pre-eclampsia
Number	13	13
Maternal age (years)	26.0 ± 7.3	29.2 ± 7.6*
Parity (nullipara/multipara)	6/7	6/7
Socioeconomic class	12/1	12/1
Birth weight (g)	3322 ± 698	1754 ± 885**
Gestational age at blood sampling (weeks)	32.7 ± 4.4	33.0 ± 4.6
Gestational age at delivery (weeks)	39.4 ± 2.4	33.4 ± 4.5**
Diastolic blood pressure (mmHg)	72 ± 6	102 ± 8**
Serum IL-8 (ng/L)	13 (7–21)	61 (13–251)****
Serum TNF-α (ng/L)	27 (9–34)	42 (16–95)****
Plasma vWF (u/dL)	157 (67–339)	314 (149–600)***
Duffy negative phenotype (fraction)	3/11	9/10*****

Data represent mean ± S.D. or median (range). Statistically different from controls by Student's t-test for matched samples at *P < 0.05, **P < 0.001 and by Wilcoxon matched pairs analysis at ***P < 0.05, ****P < 0.01 and by Fischer's exact test at *****P < 0.01. Socioeconomic class derives from type of health insurance, indicating the number of subjects with low-income insurance/ medium- and higher-income insurance. Duffy negative phenotype Fy(a–b–) for controls (n = 11) and pre-eclampsia (n = 10).

DISCUSSION

We found higher serum IL-8 levels in 13 pre-eclamptic Afro-Caribbean women compared with 13 gestational age-, race- and parity-matched controls. It is important to note that blood sampling of both groups occurred at similar gestational age. Higher serum IL-8 levels are in agreement with Stallmach et al.[12] who reported increased serum IL-8 levels in 13 pre-eclamptic women compared with 20 controls. The authors explained the high IL-8 levels by a reactive phenomenon in the mother. Concomitantly, we found elevated TNF-α levels in the pre-eclamptic women and a positive correlation between serum IL-8 and TNF-α levels. This suggests that the high serum IL-8 levels may result from increased production induced by TNF-α. Elevated TNF-α levels in pre-eclampsia has been reported quite consistently by many other investigators.[13–15] In contrast, Greer et al.[16] reported no higher TNF-α levels in pre-eclamptic women. Remarkably, they found no higher IL-8 levels either. Next to increased production, the higher serum IL-8 levels may also result from reduced IL-8 clearance as suggested by the higher frequency of the Duffy negative phenotype in our study group.

It is noteworthy to emphasize that serum IL-8 levels do not simply reflect IL-8 functional involvement in neutrophil activation. The role of IL-8 in the neutrophil inflammatory response is orchestrated through the interaction of IL-8 and its receptor at the appropriate

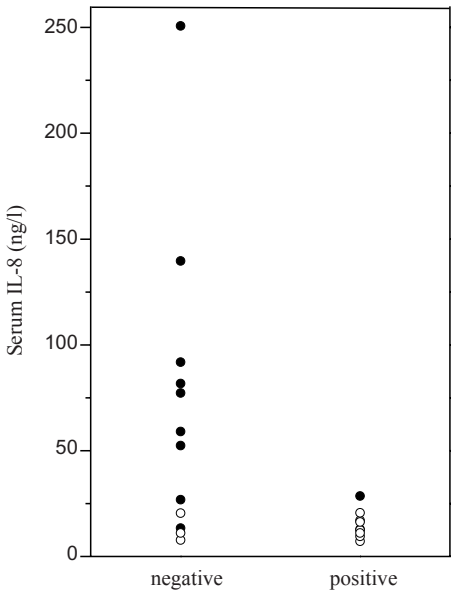

Figure 1. Serum IL-8 for ten patients with pre-eclampsia and 11 matched controls subdivided according to the Duffy negative [Fy(a–b–)] and positive [Fy(a–b+), Fy(a+b–) or Fy(a+b+)] phenotypes.
Patients with pre-eclampsia were gestational age, race and parity matched. ●, patients with pre-eclampsia; ○, controls.

place at the appropriate time.[17] This is a complex and coordinated interaction influenced by many modulating factors. Therefore, the interpretation of the high serum IL-8 levels has its restrictions. However, we found a positive correlation between serum IL-8 and vWF, i.e. a marker of endothelial dysfunction[10] indicating that high serum IL-8 levels in pregnancy do not seem favorable.

CONCLUSIONS

Our findings suggest that the encountered higher serum IL-8 levels in pre-eclamptic Afro-Caribbean women is unfavorable and that it might result from increased production, reduced RBC binding capacity, or both. The Duffy negative phenotype is being further investigated as a possible pre-eclampsia risk factor in populations with high frequencies of the Duffy negative phenotype.

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Chapter 4

Nutrition

Umbilical vessels of preeclamptic women have low contents of both n-3 and n-6 long-chain polyunsaturated fatty acids[1]

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ABSTRACT

Background: Preeclampsia is characterized by enhanced platelet aggregation and vasoconstriction and is related to an elevated ratio of thromboxane A_2 to prostacyclin I_2.
Objective: We investigated whether altered eicosanoid production in preeclamptic women could be explained by the fatty acid composition of umbilical vessel walls and platelets.
Design: The fatty acid composition of maternal and umbilical platelets and of umbilical arteries and veins in 27 preeclamptic women and 24 normotensive women was determined. Between-group differences were analyzed with linear discriminant analysis, the Kruskal-Wallis test, or analysis of covariance with gestational age as the covariate.
Results: Platelets of preeclamptic women contained lower amounts of 20:5n-3 and a higher ratio of 20:4n-6 to 20:5n-3 than did platelets of normotensive women. Additionally, linear discriminant analysis revealed higher amounts of 20:4n-6 in platelets of preeclamptic women. Umbilical arteries and veins in preeclamptic women contained lower amounts of long-chain polyunsaturated fatty acids (PUFAs) of the n-3 series, n-6 long-chain PUFAs, and 20:3n-6 than did umbilical arteries and veins of normotensive women. Umbilical arteries also had lower amounts of 20:4n-6, higher amounts of 20:3n-9, and a higher ratio of 20:3n-9 to 20:4n-6.
Conclusions: Low amounts of long-chain n-3 and n-6 PUFAs in umbilical vessels of preeclamptic women with adequate n-6 status may indicate insufficient transplacental transfer of long-chain PUFAs. The low amounts of 20:4n-6, high amounts of 20:3n-9, and high ratio of 20:3n-9 to 20:4n-6 in umbilical arteries may unfavorably affect local prostacyclin production. Low amounts of 20:3n-6 in umbilical arteries and veins and low amounts of 20:5n-3 in maternal platelets may contribute to the dominance of eicosanoids derived from 20:4n-6. *Am J Clin Nutr* 1999;69:293–8.

Key words: Preeclampsia, fatty acids, umbilical vessels, platelets, eicosanoids, long-chain polyunsaturated fatty acids, women, pregnancy, n-3 fatty acids, n-6 fatty acids

INTRODUCTION

The pathogenesis of preeclampsia is unknown, but may involve genetic, immunologic, and dietary factors. Endothelial dysfunction seems to be a common denominator (1). Preeclampsia is characterized by hypertension and proteinuria and is often associated with high perinatal mortality as a result of fetal growth retardation and (induced) early delivery (2). It is also associated with enhanced platelet aggregation and vasoconstriction (3). The ensuing hypertensive and prothrombotic state may at least partly be caused by abnormal eicosanoid production, notably the elevated ratio of thromboxane A_2 (TxA_2) to prostacyclin I_2 (PGI_2) in maternal plasma and placental tissue (4, 5). Dietary influences are therefore conceivable because eicosanoid production is influenced by the type of dietary fat and, more specifically, the status of long-chain polyunsaturated fatty acids (PUFAs).

Long-chain PUFAs are fatty acids with ≥20 carbon atoms and ≥3 double bonds. They are derived from the parent essential fatty acids linoleic (18:2n-6) and a-linolenic (18:3n-3) acids by alternating desaturation and chain elongation (6). Long-chain PUFAs are structural components of membrane phospholipids and are precursors of eicosanoids (prostaglandins, thromboxanes, and leukotrienes). The quantitatively most important long-chain PUFA from 18:2n-6 is arachidonic acid (20:4n-6), whereas 18:3n-3 is converted into eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids. The fatty acids 20:4n-6 and 20:5n-3 are precursors of TxA_2 and TxA_3 in platelets and of PGI_2 and PGI_3 in vascular endothelium, respectively. TxA_2 is a potent vasoconstrictor and platelet aggregator, whereas TxA_3 is less potent (7). PGI_2 and PGI_3 are equipotent vasodilators and inhibitors of platelet aggregation. The dietary intake of n-3 and n-6 fatty acids determines to a large extent the ratio of 20:4n-6 to 20:5n-3, and can thereby influence eicosanoid-mediated vasoactive effects. The present Western diet, characterized by a high intake of n-6 fatty acids (notably 18:2n-6) and a low intake of n-3 fatty acids (from 18:3n-3 and fish), may promote platelet aggregation and vasoconstriction (8).

Fetuses have a high long-chain PUFA requirement, eg, for development of the brain. It is widely recognized today that the fetal rate of long-chain PUFA synthesis from the parent essential fatty acids is insufficient to cover the fetus's long-chain PUFA needs (9). A maternal supply is therefore essential; insufficient fetal long-chain PUFA accrual may cause growth retardation and influence gestational age. For instance, prenatal long-chain PUFA status, notably that of 20:4n-6 and 22:6n-3, is related to birth weight (10–12), head circumference (13–16), and abdominal circumference (15) and 22:6n-3 is correlated with length of gestation (11). Studies have shown that umbilical wall 22:6n-3 concentrations correlate with weight, length, and head circumference at birth (16, 17). Fetal long-chain PUFA status seems marginal even under normal conditions because of the lower long-chain PUFA status in umbilical arteries than in umbilical veins (13). It is possible that abnormalities in maternal essential fatty acid status, in transplacental long-chain PUFA transport, or in essential fatty acid handling by the fetus accentuate the already low long-chain PUFA status of the umbilical arteries under normal conditions and thereby induce a local state of platelet aggregation and vasoconstriction.

In the present study, the fatty acid compositions of platelets, umbilical veins, and umbilical arteries of 27 preeclamptic women in Curaçao (Netherlands Antilles) were investigated. Results were compared with those in 24 normotensive, nonproteinuric, pregnant women.

SUBJECTS AND METHODS

Patients and control subjects

Fifty-one pregnant women were included in the study. All women were from Curaçao, a Caribbean island inhabited mostly by people of West African descent. The dietary habits of the people are essentially Western. All women delivered their infants in the main obstetric facilities in Curaçao, ie, the obstetric wards of the St Elisabeth Hospital and the maternity clinic Rio Canario. Preeclampsia was clinically diagnosed in 27 women. Preeclampsia was defined as a diastolic blood pressure ≥90 mm Hg on ≥2 consecutive occasions ≥4 h apart (or as a diastolic blood pressure ≥110 mm Hg on any 1 occasion) in previously normotensive women in combination with proteinuria (one 24-h urine collection with a total protein excretion ≥0.3 g or 2 specimens of urine collected ≥4 h apart with ≥0.3 g albumin/L) (18). Twentyfour normotensive, nonproteinuric pregnant women served as control subjects. The characteristics of the study population are given in **Table 1**. Informed consent was obtained from all participants and the study was conducted in agreement with local ethical standards and the Helsinki Declaration of 1975, as revised in 1989.

Samples, transport, and fatty acid analysis

Maternal venous EDTA-treated blood was collected in an undefined metabolic condition either during delivery or within 2 h after birth. Umbilical EDTA-treated blood was

Table 1. Characteristics of the study population

	Control women (n = 24)	Preeclamptic women (n = 27)
Maternal age (y)	27.8 ± 6.5[1]	27.1 ± 6.0
Nullipara (%)	21 [5][2]	52 [14]
Diastolic blood pressure (max, mm Hg)	74 ± 8	104 ± 10[3]
Cesarean delivery (%)	4 [1]	41 [11][3]
Gestational age (wk)	40 ± 1	36 ± 3[3]
Birth weight (g)	3357 ± 546	2354 ± 932[3]
SGA (%)	21 [5]	33 [9]

[1] $\bar{x}$ ± SD. SGA, small for gestational age: birth weight below the 10th percentile for gestational age, according to Kloosterman (19).
[2] n in brackets.
[3] Significantly different from control women, $P < 0.0001$.

collected immediately after birth. The EDTA-treated blood was subsequently centrifuged at 800 × *g* for 10 min at 4 °C and the supernate (platelet-rich plasma) was collected. Platelets were isolated from 2 mL platelet-rich plasma by centrifugation at 2500 × *g* for 10 min at 4 °C. The platelet pellet was washed twice, without resuspension, with 5 mL of a 0.9%-NaCl solution. Each wash was followed by centrifugation at 800 × *g* for 10 min at 4 °C. Two milliliters of methanol:HCl (5:1, by vol; 6 mol HCl/L) containing 1 mg butylated hydroxytoluene (antioxidant) and 25 µg 17:0 methyl ester (internal quantification standard) was added for immediate preservation and later transmethylation.

Immediately after delivery, an 7-cm sample of the umbilical cord located at the most proximal site to the placenta was removed. The sample was immediately placed in an ice-cold 0.9%-NaCl solution until further processing. The 2 umbilical arteries and the umbilical vein were subsequently dissected from the surrounding tissue, thoroughly washed with an ice-cold 0.9%-NaCl solution, and dried on paper tissue. Umbilical arteries and veins were weighed and subsequently preserved by adding 2 mL methanol:HCl (5:1, by vol; 6 mol HCl/L) that contained 5 mg butylated hydroxytoluene.

All samples were stored at -20 °C until transported on dry ice to the Central Laboratory for Clinical Chemistry (University Hospital Groningen). The long-chain fatty acid compositions of platelets, umbilical arteries, and umbilical veins were determined after transmethylation by capillary gas chromatography with split injection and flame-ionization detection (20). Results are expressed as relative amounts (mol%).

Statistics

Between-group comparisons of clinical characteristics were done with Student's *t* test at $P < 0.05$ (21). Differences in the fatty acid contents of umbilical arteries and veins were tested for both groups with the Kruskal-Wallis test at $P < 0.05$. In the analysis of between-group fatty acid differences, we first tested whether each of the individual fatty acids or ratios correlated with gestational age in preeclamptic women and control women separately by using the Spearman rank coefficient at $P < 0.05$. A finding of insignificant correlations in both subgroups was followed by use of the Kruskal-Wallis test at $P < 0.05$ for analysis of between-group differences. The finding of significant correlations for control women, preeclamptic women, or both was followed by analysis of covariance (ANCOVA) with gestational age as the covariate at $P < 0.05$ (21). Ratios of each of the fatty acids in umbilical arteries to umbilical veins were tested by using the same approach. Between-group comparisons of gestational age and fatty acid data were also investigated with linear discriminant analysis (21) by using the raw data. With stepwise variable selection, this method identified those variables that contributed to the discrimination between preeclamptic and control women.

Table 2. Fatty acid compositions of maternal and umbilical cord platelets from normotensive (control) and preeclamptic women[1]

	Control women (n = 18)		Preeclamptic women (n = 25)	
Fatty acid	Maternal	Umbilical	Maternal	Umbilical
		mol%		
16:0	21.89 ± 1.54	22.04 ± 1.82	22.19 ± 1.13	22.83 ± 1.40[2]
18:0	17.31 ± 1.79	18.99 ± 1.05	17.55 ± 1.20	18.47 ± 1.20
18:3n-3	0.27 ± 0.10	0.14 ± 0.10	0.22 ± 0.11	0.21 ± 0.11
20:5n-3	0.29 ± 0.14	0.16 ± 0.07	0.21 ± 0.07[2]	0.17 ± 0.07
22:5n-3	1.13 ± 0.25	0.45 ± 0.12	1.01 ± 0.31	0.51 ± 0.23
22:6n-3	2.03 ± 0.62	2.33 ± 0.58	2.16 ± 0.93	1.97 ± 0.30
Total n-3	3.70 ± 0.73	3.06 ± 0.71	3.59 ± 1.10	2.77 ± 0.29
LC n-3 PUFAs	3.45 ± 0.72	2.93 ± 0.66	3.37 ± 1.06	2.60 ± 0.30
18:2n-6	9.66 ± 2.75	3.73 ± 0.76	7.02 ± 1.91[3]	4.16 ± 1.51
20:2n-6	0.46 ± 0.08	0.28 ± 0.09	0.47 ± 0.19	0.26 ± 0.10
20:3n-6	1.38 ± 0.26	1.69 ± 0.17	1.33 ± 0.19	1.52 ± 0.37
20:4n-6	19.05 ± 3.12	23.32 ± 2.80	20.48 ± 2.58	22.20 ± 2.66
22:4n-6	1.86 ± 0.42	2.27 ± 0.26	1.97 ± 0.23	2.20 ± 0.29
22:5n-6	0.45 ± 0.11	0.76 ± 0.23	0.56 ± 0.18[2]	0.67 ± 0.29
Total n-6	32.87 ± 1.40	32.02 ± 2.30	31.82 ± 1.52[2]	31.00 ± 2.08
LC n-6 PUFAs	23.21 ± 3.14	28.29 ± 2.56	24.81 ± 2.53	26.84 ± 2.92
LC n-3 + n-6 PUFAs	26.66 ± 2.94	31.22 ± 2.36	28.18 ± 2.34	29.44 ± 2.94
18:1n-9	15.02 ± 1.59	12.46 ± 1.56	15.07 ± 2.35	13.93 ± 2.82
20:3n-9	0.32 ± 0.12	0.53 ± 0.18	0.31 ± 0.10	0.45 ± 0.16
Total n-9	17.32 ± 1.70	15.17 ± 1.91	17.41 ± 2.43	16.33 ± 2.90
SFAs	44.48 ± 1.65	48.01 ± 1.40	45.33 ± 1.86	48.17 ± 2.06[4]
MUFAs	18.59 ± 1.81	16.23 ± 1.98	18.87 ± 2.59	17.54 ± 3.13
PUFAs	36.93 ± 1.17	35.76 ± 1.96	35.81 ± 1.38	34.29 ± 2.11
20:3n-6 to 18:2n-6	0.15 ± 0.03	0.46 ± 0.08	0.21 ± 0.88[5]	0.41 ± 0.15
20:4n-6 to 20:5n-3	78.13 ± 33.1	181.87 ± 91.2	109.13 ± 38.2[2]	163.48 ± 77.5
22:6n-3 to 20:5n-3	7.82 ± 2.89	17.41 ± 8.73	11.00 ± 3.73[5]	14.10 ± 6.85

[1] $\bar{x}$ ± SD. LC, long-chain (≥ 20 carbon atoms); PUFAs, polyunsaturated fatty acids (≥ 3 double bonds); SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids

[2–5] Significantly different from control: [2] $P < 0.05$ (Kruskal-Wallis), [3] $P < 0.001$ (Kruskal-Wallis), [4] $P < 0.05$ (ANCOVA), [5] $P < 0.01$ (Kruskal-Wallis).

RESULTS

Study group

Characteristics of the study groups are given in **Table 1**. Preeclamptic women had higher maximum diastolic blood pressures (by definition) and percentages of cesarean deliveries than did control women. Newborns of preeclamptic women had significantly lower birth weights and gestational ages at delivery. There were no significant differences in the number of small-for-gestational-age infants when birth weights of this mostly AfroCaribbean study group were evaluated according to Dutch intrauterine growth curves by sex and parity (19).

Fatty acid compositions of maternal and umbilical cord platelets

The fatty acid compositions of maternal and umbilical cord platelets are presented in **Table 2**. The following significant relations were found between the fatty acids and gestational age. There were positive relations with gestational age for 18:1n-9, total n-9 fatty acids, and monounsaturated fatty acids (MUFAs) in maternal platelets of control women and for 20:3n-6 in maternal platelets of preeclamptic women. There were negative relations with gestational age for 18:1n-9, total n-9 fatty acids, and MUFAs in maternal platelets of preeclamptic women. In umbilical platelets of preeclamptic women there were positive relations with gestational age for 18:0, 22:6n-3, 20:4n-6, 22:5n-6, total n-6 fatty acids, long-chain n-6 PUFAs, long-chain n-3 and n-6 PUFAs, saturated fatty acids (SFAs), PUFAs, and the ratios of 20:3n-6 to 18:2n-6, 20:4n-6 to 20:5n-3, and 22:6n-3 to 20:5n-3. In umbilical platelets of preeclamptic women there were negative relations with gestational age for 18:3n-3, 20:5n-3, 18:2n-6, 20:2n-6, 18:1n-9, total n-9 fatty acids, and MUFAs.

Platelets of preeclamptic women contained lower amounts of 20:5n-3, 18:2n-6, and total n-6 fatty acids and higher amounts of 22:5n-6 than did maternal platelets of control women. Ratios of 20:3n-6 to 18:2n-6, 20:4n-6 to 20:5n-3, and 22:6n-3 to 20:5n-3 were higher in platelets of preeclamptic women than in maternal platelets of control women. Umbilical cord platelets of preeclamptic women had higher amounts of 16:0 and SFAs than did those of control women.

Fatty acid compositions of umbilical venous and arterial vessels

The fatty acid compositions of umbilical veins and arteries are presented in **Table 3**. The following significant correlations with gestational age were found. Amounts of 22:6n-3 and 22:5n-6 in umbilical veins and amounts of 18:3n-3 in umbilical arteries were positively correlated with gestational age in control women, whereas amounts of 22:4n-6 in umbilical veins and the ratio of 20:3n-6 to 18:2n-6 in umbilical arteries were positively correlated with gestational age in preeclamptic women. 18:1n-9, total n-9 fatty acids, MUFAs, and the ratio of 20:3n-9 to 20:4n-6 in umbilical veins and amounts of 18:1n-9 and MUFAs in umbilical arteries were negatively correlated with gestational age in preeclamptic women.

Table 3. Fatty acid compositions of umbilical veins (UV) and umbilical arteries (UA) from normotensive (control) and preeclamptic women[1]

	Control women (n = 22)		Preeclamptic women (n = 27)	
	UV	UA	UV	UA
	mol%			
16:0	24.86 ± 1.25	23.19 ± 0.73	25.98 ± 2.07[2]	23.78 ± 1.79
18:0	19.52 ± 2.40	19.35 ± 1.04	19.67 ± 3.15	20.11 ± 2.67
18:3n-3	0.10 ± 0.05	0.10 ± 0.04	0.11 ± 0.05	0.10 ± 0.06
20:5n-3	0.09 ± 0.04	0.09 ± 0.03	0.07 ± 0.02	0.06 ± 0.03[3]
22:5n-3	0.30 ± 0.11	0.22 ± 0.06	0.23 ± 0.08[2]	0.17 ± 0.06[3]
22:6n-3	4.26 ± 0.85	4.83 ± 0.76	3.35 ± 0.96	3.73 ± 1.03[4]
Total n-3	4.72 ± 0.93	5.23 ± 0.81	3.73 ± 1.00[4]	4.03 ± 1.09[4]
LC n-3 PUFAs	4.62 ± 0.92	5.13 ± 0.81	3.63 ± 1.02[4]	3.94 ± 1.09[4]
18:2n-6	2.69 ± 0.44	1.87 ± 0.39	2.89 ± 0.56	1.74 ± 0.75
20:2n-6	0.42 ± 0.10	0.24 ± 0.06	0.40 ± 0.09	0.21 ± 0.06
20:3n-6	1.94 ± 0.33	1.53 ± 0.36	1.64 ± 0.31[3]	1.25 ± 0.24[3]
20:4n-6	14.67 ± 2.52	12.63 ± 2.49	13.44 ± 3.35	10.38 ± 2.74[3]
22:4n-6	5.09 ± 1.26	2.95 ± 0.77	3.72 ± 1.21	2.16 ± 0.58[3]
22:5n-6	2.58 ± 0.66	3.14 ± 0.57	2.51 ± 0.79	3.22 ± 0.68
Total n-6	27.40 ± 3.85	22.35 ± 3.58	24.84 ± 4.48[2]	18.96 ± 4.08[3]
LC n-6 PUFAs	24.71 ± 3.73	20.48 ± 3.33	21.70 ± 4.77[3]	17.22 ± 3.80[3]
LC n-6 + n-3 PUFAs	29.33 ± 4.38	25.61 ± 3.93	25.33 ± 5.70[3]	21.16 ± 4.78[3]
18:1n-9	11.17 ± 1.15	13.35 ± 2.09	12.52 ± 3.16	15.11 ± 2.29
20:3n-9	0.70 ± 0.39	2.76 ± 1.11	0.85 ± 0.45	3.42 ± 0.96[2]
Total n-9	16.37 ± 1.72	21.60 ± 3.91	18.20 ± 3.35	24.49 ± 3.24[3]
SFAs	48.72 ± 4.20	47.90 ± 1.59	50.38 ± 5.50	49.52 ± 4.57
MUFAs	17.85 ± 1.56	20.40 ± 2.82	19.50 ± 3.28	22.60 ± 2.65
PUFAs	33.42 ± 4.54	31.70 ± 2.96	30.11 ± 5.38[3]	27.89 ± 4.81[3]
20:3n-9 to 20:4n-6	0.05 ± 0.03	0.24 ± 0.15	0.07 ± 0.04	0.37 ± 0.19[3]
20:3n-6 to 18:2n-6	0.73 ± 0.15	0.83 ± 0.15	0.59 ± 0.13[3]	0.78 ± 0.20
22:5n-6 to 22:4n-6	0.54 ± 0.23	1.16 ± 0.44	0.73 ± 0.03[2]	1.58 ± 0.48[3]
20:4n-6 to 22:6n-3	3.50 ± 0.54	2.62 ± 0.40	4.10 ± 0.57[4]	2.81 ± 0.40
22:6n-3 to 22:5n-6	1.72 ± 0.43	1.59 ± 0.16	1.34 ± 0.47[3]	1.20 ± 0.01[3]
n-3 + n-6 to n-7 + n-9	1.71 ± 0.33	1.18 ± 0.34	1.38 ± 0.38[3]	0.87 ± 0.26[3]

[1] $\bar{x}$± SD. LC, long chain (≥ 20 carbon atoms); PUFAs, polyunsaturated fatty acids (≥ 3 double bonds); SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; ratio of 20:3n-9 to 20:4n-6; essential fatty acid (EFA) deficiency index; ratio of 20:3n-6 to 18:2n-6, expresses Δ6 desaturation and elongation of n-6-series; ratio of 22:5n-6 to 22:4n-6, 22:6n-3 deficiency index; ratio of 20:4n-6 to 22:6n-3, ratio of the major LC PUFAs of the n-6 and n-3 series; ratio of 22:6n-3 to 22:5n-6, 22:6n-3 sufficiency index; ratio of n-3 + n-6 to n-7 + n-9, EFA status index. There were no significant differences by ANCOVA.

[2–4] Significantly different from control (Kruskal-Wallis): [2] $P < 0.05$, [3] $P < 0.01$, [4] $P < 0.001$.

All n-6 fatty acids in umbilical arteries of both preeclamptic and control women were significantly lower than in umbilical veins ($P < 0.001$), except for higher amounts of 22:5n-6 in umbilical arteries ($P < 0.01$). Umbilical arteries also contained higher amounts of n-9 fatty acids than did umbilical veins in both groups ($P < 0.0001$). Umbilical arteries contained 4.53 ±1.62 and 4.68 ±1.85 times as much 20:3n-9 than did umbilical veins in control and preeclamptic women, respectively ($P < 0.0001$). The umbilical arteries of control women contained higher amounts of 22:6n-3 ($P = 0.0411$) and lower amounts of 22:5n-3 ($P = 0.0045$) than did umbilical veins, whereas in preeclamptic women, only 22:5n-3 was lower in umbilical arteries than in umbilical veins ($P = 0.0023$).

Amounts of 22:5n-3, total n-3 fatty acids, long-chain n-3 PUFAs, 20:3n-6, total n-6 fatty acids, long-chain n-6 PUFAs, long-chain n-3 and n-6 PUFAs, and PUFAs were lower in umbilical arteries and veins of preeclamptic women than in control women. In addition, 20:5n-3, 22:6n-3, 20:4n-6, and 22:4n-6 were lower and 20:3n-9 and total n-9 fatty acids were higher in umbilical arteries of preeclamptic women than in control women; 16:0 was higher in umbilical veins of preeclamptic women than in control women. Ratios of 22:5n-6 to 22:4n-6 were higher and ratios of 22:6n-3 to 22:5n-6 and of n-3 + n-6 to n-7 + n-9 fatty acids were lower in umbilical arteries and veins of preeclamptic women than in control women. The ratio of 20:3n-9 to 20:4n-6 was higher in umbilical arteries of preeclamptic women than in control women, whereas the ratio of 20:3n-6 to 18:2n-6 was lower and that of 20:4n-6 to 22:6n-3 was higher in umbilical veins of preeclamptic women than in control women. Only the umbilical artery-to-vein ratio of 18:2n-6 was lower in preeclamptic women than in control women.

Linear discriminant analysis

Linear discriminant analysis revealed that gestational age and amounts of 20:5n-3, 22:4n-6, and 22:5n-3 contributed to the discrimination between preeclamptic and control women for umbilical artery fatty acids. For umbilical vein fatty acids these variables were gestational age and amounts of 22:4n-6, 20:5n-3, 20:3n-6, 22:5n-3, and 22:6n-3; for maternal platelet fatty acids they were gestational age and amounts of 22:5n-6 and 20:4n-6; and for umbilical platelet fatty acids they were gestational age and amounts of 20:2n-6.

DISCUSSION

We studied the fatty acid compositions of maternal and umbilical platelets and umbilical arteries and veins of 27 preeclamptic women and 24 normotensive control women. The women lived on the island of Curaçao; they were mostly of West African descent and their dietary habits were essentially Western. The use of a well-matched control group for preeclamptic women is important because the fatty acid composition of fetal organs, including umbilical vessels (22), is known to be subject to change, especially during the last trimester. Matching for gestational age, however, is virtually impossible because it requires selection of women with uncomplicated pregnancies who deliver before term.

We therefore first tested whether the fatty acids or ratios correlated with gestational age. An insignificant finding was tested further by use of the Kruskal-Wallis test, whereas a significant finding was tested further by ANCOVA with gestational age as the covariate. Few differences in maternal and umbilical platelet fatty acid compositions were found by this approach, but striking differences for some fatty acids in umbilical veins and arteries were identified. These results were confirmed and extended by multivariate data analysis. Taken together, our data show that umbilical veins and especially umbilical arteries of preeclamptic women contain lower percentages of essential fatty acids of both the n-3 and n-6 series than do veins and arteries of control women, suggesting that abnormal fetal deposition of these fatty acids is a feature of preeclampsia.

The lower long-chain n-3 and n-6 PUFA status of umbilical arteries especially may reflect impaired fetal long-chain PUFA accrual in preeclampsia, with insufficient amounts of long-chain PUFAs remaining for incorporation into the fetal tissues located most distal from the placental supply. Insufficient fetal long-chain PUFA deposition derives, theoretically, from abnormal fetal handling of essential fatty acids or an insufficient fetal long-chain PUFA supply. An insufficient supply may be the result of marginal maternal long-chain PUFA status, insufficient transplacental transport, or both. From a percentage point of view, the most striking abnormality in preeclampsia seemed to be the lower amounts of long-chain n-3 PUFAs in both umbilical arteries (-23%; Table 3) and umbilical veins (-21%). The quantitatively most important long-chain n-3 PUFA in these compartments is 22:6n-3. Part of the difference in 22:6n-3 between preeclamptic and control women may be explained by the gestational age dependency of 22:6n-3. The marginal 22:6n-3 status in umbilical arteries and veins of preeclamptic women was confirmed by the lower ratio of 22:6n-3 to 22:5n-6 and higher ratio of 22:5n-6 to 22:4n-6, both of which are indicators of functional 22:6n-3 deficiency (23). Note that the dietary intake of n-3 fatty acids (8) from vegetable oils and fish in many Western societies, including Curaçao (24), is low compared with the intake of the competing essential fatty acids of the n-6 series (notably 18:2n-6). On the other hand, amounts of 20:4n-6 in umbilical arteries (-18%) and umbilical veins (-9%; NS) were also low. Lower amounts of 20:4n-6 may be of special interest because this finding is related to lower birth weight (12). Low fetal n-3 and n-6 status in mothers with adequate n-6 status argues in favor of insufficient transplacental transport. Future maternal long-chain PUFA supplementation studies may indicate whether fetal long-chain PUFA shortages are indeed causally related to the pathogenesis of preeclampsia.

Interpreting our findings in terms of eicosanoid production is difficult because our fatty acid data were derived from whole tissue, not from those subcellular compartments and lipid classes that exclusively add to the local production of eicosanoids. Moreover, many factors influence the subsequent relation between eicosanoid production and the long-chain PUFA contents of the subcellular phospholipids that actually serve as eicosanoid precursors. However, preeclamptic women did have lower platelet contents of the precursor of TxA_3 (ie, 20:5n-3) together with unaltered contents of the precursor of TxA_2 (ie, 20:4n-6) and a higher ratio of 20:4n-6 to 20:5n-3 than control women (Table

2). Higher amounts of 20:4n-6 in platelets of preeclamptic women became apparent from the linear discriminant analysis. Taken together, these results may be consistent with TxA_2 dominance, but it must be pointed out that amounts of 20:5n-3 in platelets were extremely low compared with those of 20:4n-6 and that the encountered differences were small.

Fatty acid differences between preeclamptic and control women in the umbilical vessels **(Table 3)** seemed more pronounced than the differences in platelets. This result may be in line with the finding that umbilical arteries of preeclamptic women produce less prostacyclin (25) than umbilical arteries of normotensive women and that prostacyclin production is inversely related to the ratio of 20:3n-9 to 20:4n-6 in umbilical arteries (26). Umbilical arteries in our study had lower contents of 20:4n-6, higher contents of 20:3n-9, and a higher ratio of 20:3n-9 to 20:4n-6 than did umbilical veins. Crawford et al (14) found that umbilical arteries of babies with the lowest birth weights had the highest 20:3n-9 contents. It is as yet unclear whether the 4 times higher 20:3n-9 content in umbilical arteries than in umbilical veins reflects a local essential fatty acid deficiency. Classically, essential fatty acid deficiency results in the production of 20:3n-9 from 18:1n-9 by Δ6 desaturation and chain elongation because of the local lack of the preferred Δ6 desaturase substrates 18:3n-3 and 18:2n-6. Umbilical arteries and veins do not seem to differ much, however, in the contents of these substrates, which may argue in favor of a derivation of 20:3n-9 from the maternal circulation. Feeding rats with a 20:3n-9-rich oil was found to cause incorporation of this fatty acid into various plasma lipid classes and into the phospholipids of all investigated organs (27). It is possible that 20:3n-9 undergoes similar preferential transplacental transport as long-chain PUFAs of the n-3 and n-6 series by the poorly understood process of biomagnification (10), but is subsequently deposited downstream as a second-choice long-chain PUFA because of the local limited availability of long-chain PUFAs of the n-3 and n-6 series.

The lower amount of 20:3n-6 in umbilical veins and arteries, the lower ratio of 20:3n-6 to 18:2n-6 in umbilical veins **(Table 3)**, and the lower amount of 20:3n-6 in umbilical veins identified by linear discriminant analysis in preeclamptic women might be consistent with lower Δ6 desaturation activity because neither 18:3n-6 (the intermediate Δ6 desaturase product in the conversion of 18:2n-6 to 20:3n-6) nor 20:3n-6 belong to the usual arsenal of dietary fatty acids. Dihomo-γ-linolenic acid (20:3n-6) is the precursor of eicosanoids of the 1-series (28). These eicosanoids share much of the properties of those of the 3-series (ie, from 20:5n-3) and may therefore counteract the vasoconstrictive and platelet-aggregating effects of eicosanoids of the 2-series (from 20:4n-6). Lower amounts of 20:3n-6 and a lower ratio of 20:3n-6 to 18:2n-6 also suggest that the production of 20:3n-9 is unlikely to be by synthesis from 18:1n-9 via Δ6 desaturation.

In conclusion, preeclampsia is characterized by low amounts of long-chain PUFAs of the n-3 and n-6 series in umbilical veins and most notably in umbilical arteries. The underlying cause may be insufficient transplacental transfer of long-chain PUFAs because the women had normal n-6 status. Platelets of preeclamptic women had lower amounts of 20:5n-3 and a higher ratio of 20:4n-6 to 20:5n-3. These differences, however, were small and it is therefore questionable whether they contributed much to the TxA_2 dominance.

Umbilical arteries of preeclamptic women had lower amounts of 20:4n-6, higher amounts of 20:3n-9, and a higher ratio of 20:3n-9 to 20:4n-6, which may unfavorably affect local prostacyclin production and cause other adverse effects related to 20:3n-9. It is possible that the high amounts of 20:3n-9 in umbilical arteries were not derived merely from local synthesis, but originated from the maternal circulation. Umbilical veins and arteries of preeclamptic women also had low amounts of 20:3n-6, which together with low 20:5n-3 in maternal platelets may contribute to the dominance of 20:4n-6-derived eicosanoids in preeclampsia.

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Effect of three low-dose fish oil supplements, administered during pregnancy, on neonatal long-chain polyunsaturated fatty acid status at birth

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SUMMARY

Adequate long-chain polyunsaturated fattyacid (LCP) status during pregnancy is important. We studied the effect of three low-dose fish oil supplements, administered during uncomplicated pregnancy, on neonatal LCP status at term delivery.

Supplements were administered from the second trimester to delivery, either as fish oil capsules ('fish-1': 336 mg LCPω3, n-15; and'fish-3':1,008 mg LCPω3, n=20) or milk-based supplement ('Mum': 528 mg LCPω3, n=24). Fifty-seven untreated women served as controls. Fatty acids of umbilical veins (UV) and arteries (UA) were measured.The fish-1 group showed no differences, compared to controls.The Mum group had higher 20:5ω3, 22:5ω3, 22:6ω3, LCPω3 and 22:6ω3/22:5ω6 in UV and UA. The fish-3 group had higher 22:5ω3 and 22:6ω3 (UA), LCPω3 and 22:6ω3/22:5ω6 (UV and UA) and 20:3ω6 (UV). A 500-1000 mg daily LCPω3 supplement, taken either as a milk-based supplement or fish oilcapsules, effectively increases fetal LCPω3 status, without affecting LCPω6 status.

INTRODUCTION

Adequate long-chain polyunsaturated fatty acid (LCP) status during pregnancy is important.[1,2] Arachidonic- (20:4ω6), adrenic- (22:4ω6) and docosahexaenoic (22:6ω3) acids are among the quantitatively most important fatty acids in brain[3,4] and their levels in fetus and newborn brain increase steadily up to a postnatal age of at least 2 years.[3] There is little evidence that fetal LCP synthesis is sufficient to cover the high intrauterine needs for fetal (brain) growth and development. Cord plasma[5] and erythrocyte[6] lipids contain higher LCP contents compared with corresponding maternal compartments. This so-called biomagnification suggests the presence of a highly active and specific transplacental LCP transport system that may be driven by a placental fatty acid binding protein,[7] the high LCP affinity of fetal α-fetoprotein,[8] and a fetal RBC membrane exchange protein.[1] Several investigators reported a relation between birth weight and 20:4ω6 contents in cord plasma and umbilical arteries (UA), suggesting that intrauterine 20:4ω6 serves as a growth factor.[1,9–11] Fetal 22:6ω3 status has predominantly been associated with head circumference and gestational age.[10,12] LCP may also be involved in certain pregnancy-associated diseases. Umbilical veins (UV) and UA of preeclamptic pregnancies have lower LCP contents compared with normotensive controls,[13] but such differences were not found in pregnancy induced hypertension.[14]

The maternal plasma phospholipid essential fatty acid concentration increases during pregnancy due to the hyperlipidemia of pregnancy.[15] However, on the basis of deteriorating essential fatty acid-status index [(ω3+ω6)/(ω7+ω9)] and 22:6ω3-status index (22:6ω3/22:5ω6), it has been questioned whether present maternal LCP status, notably that of LCPω3, is sufficient to prevent maternal LCP depletion.[2] Slow recovery after delivery,[16] notably when followed by lactation,[17] multiple births and short interpregnancy interval may add to the depth of the depletion. Various authors demonstrated relationships between maternal LCP intake, and maternal and neonatal LCP status.[18–21] LCP supplementation studies, notably with high-dose LCPω3, have been conducted to influence maternal and neonatal LCP status,[22,23] maternal and neonatal eicosanoid balance,[24,25] maternal angiotensin II refractoriness[26] and clinical outcomes such as perinatal mortality, pregnancy-induced hypertension, preeclampsia, blood pressure, birth weight, intrauterine growth retardation and pregnancy duration.[12,27–30] Supplementation of 2.2 g[22] or 2.6 g[23] LCPω3 in the third trimester of pregnancy improved neonatal LCPω3 status. However, it also lowered selective LCPω6 in maternal and cord plasma phospholipids and in UA and UV phospholipids.[22] This is generally considered to be undesirable and may theoretically be prevented either by using lower dosages or a combination of fish oil and 20:4ω6.

We studied the effect of three low-dose LCPω3 supplements, administered during uncomplicated pregnancy, on neonatal LCP status at term delivery. The supplements were administered from the second trimester to delivery either as fish oil capsules at two dosages (336 and 1008 mg LCPω3) or a milk-based supplement marketed for pregnant and lactating women (528 mg LCPω3). Untreated pregnant women served as controls.

Fetal LCP status was assessed by measurement of the UV and UA fatty acid compositions. The study was performed in the island of Curaçao (The Netherlands Antilles), which is predominantly inhabited by an Afro-Caribbean population with essentially Western dietary habits.

MATERIALS AND METHODS

Subjects

Pregnant women were recruited from October 1995 till July 1996 from the main antenatal clinics in the island of Curaçao (The Netherlands Antilles), i.e. the antenal clinics 'Rio Canario' and 'de Savaan', the antenatal clinic of the'St Elisabeth Hospital' and several private antenatal clinics. Apparently healthy pregnant women with uneventful singleton pregnancies and between 18–23 weeks of gestation were eligible to participate in the study. Women with histories of complications during previous pregnancies or those using medication on a regular basis were excluded. Informed consent was obtained from all participants. The study was in agreement with local ethical standards and the Helsinki declaration of 1975, as revised in 1989.

Study design

The women were assigned to one of the following four groups. The first ('Mum group') received a milk-based multivitamin and mineral supplement powder designed for pregnant and lactating women (Frisomum©, Friesland Nutrition, Leeuwarden, The Netherlands). The women were instructed to take 60 g Frisomum© daily, dissolved in water or other fluids, and not to take other supplements. Their daily supplemental intakes amounted to 528 mg LCPω3 (i.e. 293 mg 20:5ω3 and 185 mg 22:6ω3; as determined by us, Table 1) and 4 mg dl-α-tocopherol acetate (as stated by the manufacturer). The second group (fish-1) received a daily gelatin capsule with 1 g purified fish oil (Omega©, Propharma a/s, Havdrup, Denmark). Their daily intakes corresponded with 336 mg LCPω3 (177 mg 20:5ω3 and 123 mg 22:6ω3; as determined by us, Table 1) and 2 mg D-α-tocopherol (manufacturer). The third group (fish-3) took three fish oil capsules daily, corresponding with daily intakes of 1008 mg LCPω3 (531 mg 20:5ω3 and 369 mg 22:6ω3 and 6 mg D-α-tocopherol. the final group (controls) did not receive supplements.

The women were at the first visit asked about their fish consumption frequency. All of them were instructed to continue their usual dietary habits. Women in the Mum-group received the number of Frisomum© tins necessary until their next visit. Those in the fish-1 group received all capsules at once and those in the fish-3 group received their supplements in two portions. During their regular antenatal visits they were asked about intake regimen and side-effects. All supplemented women were requested to return unused Frisomum© and fish oil capsules at the end of the study.

Samples, storage and fatty acid analysis

An about 7 cm sample of the umbilical cord, located at the most proximal site to the placenta, was collected immediately after delivery. The sample was immediately placed in ice cold 0.9% sodium chloride solution, until further processing. The two UAs and the UV were subsequently dissected from the surrounding tissue, thoroughly washed with ice cold 0.9% sodium chloride solution and dried on paper tissue. The samples were weighed and subsequently preserved by the addition of 2 ml methanol:6 mol/L HCL (5:1, v/v), which contained 5 mg butylated hydroxytoluene. All samples were stored at -20ºC until further processing. Long chain fatty acid compositions of UA and UV were determined in Curaçao after transmethylation, using our previously described capillary gas chromatographic analysis with split injection and flame-ionization detection.[31]

Data evaluation and statistics

Results were expressed as relative amounts (mol%) or ratios (mol/mol). Differences in fatty acid contents between UV and UA (UV minus UA in mol%) were calculated. Only data of women who took calculated amounts of at least 75% of the prescribed dosages of Frisomum© or fish oil capsules were included in the final evaluation. Between-group comparisons of clinical characteristics, fish consumption, UA and UV fatty acid compositions and UV-UA fatty acid differences were done with the Mann-Whitney *U*-test, corrected for type-1 errors (Bonferroni) at $P<0.05$. Correlations between clinical characteristics and fatty acid composition were analyzed with the Spearman rank coefficient at $P<0.05$.[32]

RESULTS

Study group

A total of 202 women agreed to participate in the study. Fourteen dropped out because of the development of complications during the study. These were: hypertension (2 controls, 2 Mum, 1 fish-1 and 1 fish-3), fetal death (2 controls), gestational diabetes (2 controls) or miscellaneous complications (1 control, 1 fish-1 and 2 fish-3). Nine women (4 controls, 2 Mum, 1 fish-1 and 2 fish-3) were excluded because of delivery prior to 37 weeks of gestation. Three women (1 control, 1 Mum, 1 fish-3) withdrew for personal reasons. There were 32 missing values: 24 (6 controls, 5 Mum, 8 fish-1 and 5 fish-3) due to failure of sample collecting at delivery and 8 (1 control, 3 Mum, 2 fish-1 and 2 fish-3) because of analytical imperfections. Twenty-eight women (23 Mum, 1 fish-1 and 4 fish-3) did not reach the minimum intake of 75% of the prescribed dose. The distribution of the remaining 116 women among the subgroups was: 57 controls, 15 fish-1, 24 Mum and 20 fish-3. The majority of them were of West-African descent. Their characteristics are given in Table 1. There were no between-group differences in maternal age, parity, gestational age at delivery, birth weight and estimated frequency of fish consumption at the beginning of the study. The most frequently reported side-effects were metal taste (Frisomum©) and belching (fish oil capsules).

Between-group differences for UV and UA fatty acids

Tables 2 and 3 show selected fatty acid compositions (in mol%) and ratios (mol/mol) of the UV (Table 2) and UA (Table 3) for women in the control, fish-1, Mum and fish-3 groups. Comparison of data of the three LCPω3 supplemented groups with those of the control group gave the following significant results.

UV fatty acids. The Mum group had higher 20:5ω3, 22:5ω3, 22:6ω3, ω3, LCPω3 and 22:6ω3/22:5ω6 ratio. The fish-3 group had higher ω3, LCPω3, 20:3ω6 and 22:6ω3/22:5ω6 ratio and lower 20:4ω6/20:3ω6 ratio. The fish-1 group showed no significant differences.

UA fatty acids. The Mum group had higher 20:5ω3, 22:5ω3, 22:6ω3, ω3, LCPω3 and 22:6ω3/22:5ω6 ratio. The fish-3 group had higher 22:5ω3, 22:6ω3, ω3, LCPω3 and 22:6ω3/22:5ω6 ratio. The fish-1 group showed no significant differences.

UV-UA difference. The UV-UA differences (data not shown) of the fish-1 and Mum groups did not differ significantly from those of the control group. The fish-3 group had a higher UV-UA difference for 20:3ω6, compared with that of the control group (fish-3: mean 0.81 and control: 0.52 mol%).

Table 1. Study group characteristics, daily PUFA supplement dosages and supplementation duration

	Controls	**Fish-1**	**Mum**	**Fish-3**
Maternal age (years)	28.4±5.3	29.1±4.7	29.1±6.6	30.8±6.4
Parity	1.0 (0-5)	1.0 (0-5)	0.0 (0-6)	1.0 (0-6)
Gestational age (weeks)	39.5±1.3	39.8±1.3	39.2±1.3	39.4±1.3
Birth weight (g)	3296±399	3523±519	3412±442	3423±605
Fish consumption (times/month)*	3.3 (1-10)	4.0 (1-16)	4.0 (1-30)	2.0 (1-4)
Daily supplements (mg/day)				
18:2ω6 (LA)	0	14	839	43
18:3ω3 (ALA)	0	8	171	23
20:4ω6 (AA)	0	11	18	34
20:5ω3 (EPA)	0	177	293	531
22:5ω3 (DPA)	0	28	35	84
22:6ω3 (DHA)	0	123	185	369
LCPω3	0	336	528	1,008
ω3	0	367	744	1,100
LCPω6	0	19	32	58
ω6	0	38	882	115
Supplement duration (weeks)	-	20±2	19±2	18±2

Data are means ± SD or medians (range). Statistical analysis by Mann-Whitney *U*-test, corrected for Bonferroni at $P<0.05$. There were no between-group statistical differences in clinical characteristics and supplement duration in completed weeks.

* Data only available 32 controls, 12 fish-1, 16 Mum and 14 fish-3.Fish consumption in frequency per month. For fatty acid abbreviations see legend of Table 2.

Correlations between LCPω3 dose, and UV and UA fatty acids

Correlation of the daily LCPω3 dosages (see Table 1) with the fatty acid compositions of UV and UA gave the following significant results. LCPω3 dosage correlated positively with UV 20:5ω3 (r =0.35, P<0.0001), 22:5ω3 (r =0.32, P<0.0001), 22:6ω3 (r =0.39, P<0.0001), LCPω3 (r =0.40, P<0.0001) and 20:3ω6 (r =0.26, P=0.004), and negatively with UV 22:5ω6 (r = – 0.24, P=0.010). LCPω3 dosage correlated positively with UA 20:5ω3 (r =0.24, P=0.009), 22:5ω3 (r =0.33, P<0.0001), 22:6ω3 (r =0.39, P<.0001) and LCPω3 (r =0.40, P<0.0001), and negatively with UA 22:4ω6 (r = – 0.19, P=0.036) and 22:5ω6 (r= – 0.19, P=0.044).

Table 2. Fatty acids composition of umbilical veins (UV) for controls and women receiving fish oil supplements

Fatty acid	Controls (n=57)	Fish-1 (n=14)	Mum (n=24)	Fish-3 (n =20)
16:0	23.44±1.79	23.22±1.44	22.97±0.98	22.93±1.15
18:0	21.46±2.96	21.80±1.75	21.63±0.80	21.96±1.07
18:3ω3	0.01±0.01	0.01±0.02	0.01±0.01	0.01±0.02
20:5ω3	0.04±0.07	0.04±0.02	0.05±0.02[2]	0.05±0.03
22:5ω3	0.18±0.09	0.23±0.12	0.32±0.17[2]	0.28±0.16
22:6ω3	4.73±0.89	5.32±0.80	5.75±0.78[3]	5.39±0.98
ω3	4.96±0.95	5.60±0.88	6.13±0.87[3]	5.73±1.13[1]
LCPω3	4.96±0.94	5.59±0.89	6.12±0.87[3]	5.72±1.13[1]
18:2ω6	2.71±0.55	2.93±0.54	2.71±0.42	2.94±0.56
20:2ω6	0.68±0.35	0.80±0.26	0.77±0.37	0.72±0.23
20:3ω6	2.26±0.41	2.37±0.58	2.38±0.46	2.67±0.48[2]
20:4ω6	16.53±1.89	16.28±1.56	16.81±1.37	16.49±1.89
22:4ω6	5.54±1.19	5.54±0.79	5.37±0.87	5.38±0.87
22:5ω6	2.81±0.63	2.67±0.72	2.51±0.62	2.45±0.65
ω6	30.53±2.92	30.59±1.98	30.55±1.86	30.66±2.54
LCPω6	27.82±2.71	27.65±1.98	27.84±1.62	27.72±2.31
LCPω6ω3	32.78±3.29	33.24±2.58	33.96±2.11	33.44±3.12
18 :1ω9	11.15±1.49	10.90±1.24	11.12±1.48	11.32±1.34
20:3ω9	1.05±0.75	0.76±0.31	0.96±0.63	0.84±0.48
ω9	14.28±2.45	13.65±1.33	13.97±2.38	13.84±2.14
SAFA	46.87±2.73	46.93±2.16	46.16±1.37	46.40±1.76
MUFA	15.93±2.07	15.53±1.36	15.50±1.86	15.81±2.05
PUFA	37.21±2.89	37.54±2.46	38.34±1.71	37.80±2.73
20:4ω6/20:3ω6	7.54±1.47	7.27±1.93	7.24±1.10	6.37±1.31[1]
22:6ω3/22:5ω6	1.76±0.49	2.15±0.75	2.46±0.77[2]	2.37±0.86[1]

Data are means ±SD in mol% or mol/mol. Statistical analysis: significantly different from control by Mann-Whitney U-test, two-sided, corrected for Bonferroni,[1]P<0.05, [2]P<0.01 and [3]P<0.001. Supplementation of LCω3: controlsnosupplements, fish-1336 mg/day,Mum 528 mg/day and fish-31008 mg/day, ω3, ω6, ω9, total of ω3-, ω6- or ω9 fatty acids respectively; LCP, long chain poly unsaturated fatty acids with ≥20 carbon atoms and ≥3 cis double bonds; LCP ω3, -ω6, -ω3ω6,LCP of ω3-series, LCP of ω6-series and sum of LCP of the ω3- and ω6 series, respectively; SAFA, saturated fatty acids; MUFA, mono unsaturated fatty acids; PUFA, poly unsaturated fatty acids. 22:6ω3/22:5ω6; 22:6ω3 sufficiency index.

Correlations of UV and UA fatty acids with birth weight and gestational age Birth weight of the entire study group correlated negatively with UV 18:2ω6 (r = – 0.30, P=0.002) and positively with UV 20:3ω6/18:2ω6 (r =0.37, P=0.0001). There was no significant correlation between birth weight and LCPω3 in UV or UA. Analyzing each group separately it was found that birth weight correlated positively with UV 20:3ω6/18:2ω6 in controls (r =0.458, P=0.001). UV and UA fatty acids did not correlate with gestational age.

Table 3. Fatty acids composition of umbilical arteries (UA) for controls and women receiving fish oil supplements

Fatty acid	Controls (n=57)	Fish-1 (n=15)	Mum (n=24)	Fish-3
16:0	22.00±1.77	21.30±1.46	21.51±1.77	21.52±1.28
18:0	22.76±1.64	22.88±1.79	22.68±1.34	22.68±1.61
18:3ω3	0.02±0.02	0.03±0.02	0.04±0.03	0.03±0.02
20:5ω3	0.05±0.06	0.06±0.07	0.06±0.05[2]	0.05±0.03
22:5ω3	0.11±0.07	0.13±0.10	0.17±0.09[1]	0.20±0.19[1]
22:6ω3	4.95±0.95	5.37±0.59	5.92±1.16[2]	5.76±0.74[2]
ω3	5.13±1.01	5.59±0.63	6.20±1.22[2]	6.04±0.81[2]
LCPω3	5.10±1.01	5.56±0.64	6.16±1.22[2]	6.01±0.82[2]
18:2ω6	1.85±0.40	2.16±0.61	1.79±0.41	1.89±0.49
20:2ω6	0.66±0.54	0.95±1.02	0.65±0.55	0.60±0.41
20:3ω6	1.74±0.29	1.82±0.35	1.78±0.40	1.80±0.32
20:4ω6	13.83±2.16	13.28±2.19	13.10±2.47	13.46±1.25
22:4ω6	3.56±0.92	3.19±0.68	3.04±0.80	3.25±0.60
22:5ω6	3.33±0.69	3.26±0.59	3.03±0.70	3.10±0.42
ω6	24.96±3.34	24.64±3.48	23.39±3.76	24.11±1.93
LCPω6	23.12±3.08	22.49±3.12	21.59±3.46	22.22±1.71
LCPω6ω3	28.22±3.75	28.05±3.47	27.76±4.44	28.22±2.15
18:1ω9	13.69±2.12	13.94±2.16	14.46±2.48	14.42±1.45
20:3ω9	2.87±1.06	2.90±0.80	3.40±1.26	3.00±0.63
ω9	19.31±3.45	19.85±1.33	20.74±4.32	20.04±2.24
SAFA	46.93±2.00	46.56±1.78	45.93±1.88	46.15±2.05
MUFA	18.65±2.61	18.93±2.62	19.40±3.20	19.27±1.63
PUFA	34.42±2.98	34.51±3.10	34.67±3.36	34.58±2.11
20:4ω6/20:3ω6	8.09±1.54	7.54±1.70	7.55±1.44	7.66±1.34
22:6ω3/22:5ω6	1.55±0.46	1.71±0.42	2.03±0.52[2]	1.90±0.45[2]

Data are means+SD in mol% or mol/mol. Statistical analysis: significantly different from control by Mann-Whitney U-test, two-sided, corrected for Bonferroni,[1]P<0.05, [2]P<0.01 and [3]P<0.001.
For other details see legend toTable 2.

DISCUSSION AND CONCLUSIONS

We investigated in an open trial design the effect of low-dose LCPω3 supplementation of healthy pregnant women on newborn LCP status. UV and UA LCP contents at birth served as markers of neonatal LCP status. The study population was composed of African-Caribbean women who live in the island of Curaçao (The Netherlands Antilles). They had uneventful pregnancies and delivered healthy babies with normal birth weights at term. The mean duration of the LCPω3 supplementation was 19 weeks (from the second trimester to birth). The supplements were given either asish oil capsules or a commercially available milk-based product that is marketed for pregnant and lactating women, giving rise to daily LCPω3 intakes of about 336 (fish-1), 528 (Mum) and 1008 (fish-3) mg. The most important outcome of the present study is that daily supplementation with 528 and 1008 mg LCPω3, starting from the second trimester, significantly augments fetal LCPω3 status without affecting fetal LCPω6 status to an appreciable extent. A 336 mg LCPω3 supplement did not show significant effects.

The present UV and UA fatty acid compositions (Tables 2 and 3) are in reasonable agreement with data for UV and UA phospholipid fatty acids in The Netherlands and Denmark, as reported by Al et al.[15] and Van Houwelingen et al.,[22] respectively. They are in excellent agreement with our previously reported data for the Curaçao population.[13] The influence of LCPω3 supplementation on UV and UA LCPω3 contents proved highly comparable with data that were previously reported by Van Houwelingen et al.[22] Their 2200 mg LCPω3 dose (1280 mg 20:5ω3 and 920 mg 22:6ω3) was, however, about 6.5, 4.2 and 2.2 times higher than the present dosages of the fish-1, Mum and fish-3 groups, respectively. Their supplement caused about 0.97 g% higher UV phospholipid 22:6ω3 (5.62 versus 6.59 g%) and about 0.89 g% higher UA phospholipid 22:6ω3 (5.48 versus 6.37 g%), compared with 15 controls. These 22:6ω3 differences are remarkably similar to those observed in the present Mum and fish-3 groups. Present differences amounted to 0.59, 1.02 and 0.66 mol% in UV and 0.42, 0.97 and 0.81 mol% in UA for the fish-1, Mum and fish-3 groups, respectively (Tables 2 and 3). Similar incorporation efficiencies were noted for 20:5ω3, 22:5ω3 and LCPω3 in the present study (not shown). Incorporation of LCPω3 in umbilical vessel walls seems consequently to be dose-dependent, but to reach a steady state from a daily supplemental intake of about 500 mg LCPω3 on top of the present dietary background. With respect to the latter, it should be noted that dietary LCPω3 intake in Curaçao is conceivably somewhat lower than that of the Danish women studied by Van Houwelingen et al.[22] Both the UV and UA of the Danish control population contained higher LCPω3, compared with that of Curaçao counterparts.

Neither of the present LCPω3 supplements affected the UV and UA total LCPω6 contents to a statistically significant extent. Taking all data together there was, however, a negative correlation between LCPω3 dose and 22:5ω6 in UV and UA, and between LCPω3 dose and 22:4ω6 in UA. This finding is in line with the previously noted[22] lower UV and UA phospholipid 22:5ω6 contents in women receiving 2200 mg LCPω3, compared with controls. It suggests that in the higher dose range, supplemental LCPω3

does not only increase fetal LCPω3 status in an highly ineffective manner, but that it may also lower fetal LCPω6 status, notably that of 22:4ω6 and 22:5ω6. The consequences of lower 22:4ω6 and 22:5ω6 are unknown. Most of the attention is drawn at 20:4ω6, but it should be noted that, next to 20:4ω6, 22:4ω6 is the quantitatively second most important LCPω6 in brain with 22:5ω6 holding the third position.[3]

The 1008 mg LCPω3 supplement of the fish-3 group did, to our surprise, increase UV 20:3ω6 and decrease UV 20:4ω6/20:3ω6, giving rise to a higher UV-UA 20:3ω6 difference. We have no explanation for this finding, but there might be a connection between 20:3ω6, LCPω3 and growth and development. Felton et al.33 found that UV 20:3ω6 was the best indicator for intra-uterine fetal growth and we found lower 20:3ω6 in UA and UV of preeclamptic pregnancies.[13] It has also been pointed out that the high LCPω3 status of Inuits exists in the context of a concomitantly higher 20:3ω6 status.[34] Many favorable features are attributed to the series-1 eicosanoids derived from 20:3ω6, which share many of the properties of those of the series-3 deriving from 20:5ω3.[34] Their relation with pregnancy remains, however, as yet obscure.

Except for 18:2ω6 none of the investigated PUFA related to birth weight. The encountered negative relation between UV 18:2ω6 and birth weight is in agreement with previously reported negative correlation between maternal 18:2ω6 intake and fetal growth in 372 pregnant women.[35]

The Mum group exhibited statistically insignificant higher UA and UV 22:6ω3, compared with that of the fish-3 group (statistics not shown). This difference was unexpected, because of the lower supplemental LCPω3 of the Mum group, compared with the fish-3 group. It is possible that the daily 171 mg 18:3ω3 co-supplementation of the Mum group contributed to the tendency of higher LCPω3 status. A 171 mg 18:3ω3 supplement, taken in conjunction with 839 mg 18:2ω6, is relatively high compared with the 1.2 and 17.8 g estimated daily intakes of 18:3ω3 and 18:2ω6, respectively, from a typical Western diet.[36] This supplement would decrease the average dietary 18:2ω6/18:3ω3 ratio from 14.8 to 13.6. Moreover, enhanced, e.g. hormone-driven, conversion of 18:3ω3 to 22:6ω3 during pregnancy is conceivable, and might be at the basis of the increase of plasma phospholipid 22:6ω3 from early pregnancy till delivery.[17] Whatever the cause of this tendency, present data show that a milk-based supplement containing both 18:3ω3 and low-dose LCPω3 is at least as effective in increasing newborn LCPω3 status as fish oil capsules containing similar LCPω3 contents.

We conclude that a 500–1000 mg daily LCPω3 supplement during pregnancy, taken from the second trimester either as a milk-based supplement or as fish oil capsules, effectively increases newborn LCPω3 status, without affecting LCPω6 status.

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Plasma choline and betaine and their relation to plasma homocysteine in normal pregnancy[1]

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ABSTRACT

Background: Plasma concentrations of total homocysteine (tHcy) decrease during pregnancy. This reduction has been investigated in relation to folate status, but no study has addressed the possible role of betaine and its precursor choline.

Objective: We investigated the courses of plasma choline and betaine during normal human pregnancy and their relations to plasma tHcy.

Design: Blood samples were obtained monthly; the initial samples were taken at gestational week (GW) 9, and the last samples were taken ≈ mo postpartum. The study population comprised 50 women of West African descent. Most of the subjects took folic acid irregularly.

Results: Plasma choline (geometric $\bar{x}$; 95% reference interval) increased continuously during pregnancy, from 6.6 (4.5, 9.7) µmol/L at GW 9 to 10.8 (7.4, 15.6) µmol/L at GW 36. Plasma betaine decreased in the first half of pregnancy, from 16.3 (8.6, 30.8) µmol/L at GW 9 to 10.3 (6.6, 16.2) µmol/L at GW 20 and remained constant thereafter. We confirmed a reduction in plasma tHcy, and the lowest concentration was found in the second trimester. From GW 16 onward, an inverse relation between plasma tHcy and betaine was observed. Multiple regression analysis showed that plasma betaine was a strong predictor of plasma tHcy from GW 20 onward.

Conclusions: The steady increase in choline throughout gestation may ensure choline availability for placental transfer with subsequent use by the growing fetus. Betaine becomes a strong predictor of tHcy during the course of pregnancy. Both of these findings emphasize the importance of choline and betaine status during normal human pregnancy.

Key words: Homocysteine, folate, choline, betaine, pregnancy

INTRODUCTION

Choline is considered to be an essential nutrient because the de novo synthesis via sequential methylation of phosphatidylethanolamine (PE) (**Figure 1**) is not sufficient to meet the metabolic demands when humans are deprived of dietary choline (1). Choline is needed for the normal function of cells (2). It is incorporated into membrane phospholipids and plays a crucial role in membrane integrity and signal transduction. It is converted to the neurotransmitter acetylcholine or oxidized to betaine and provides a source of methyl groups (2).

Human diets are rich in choline, particularly phosphatidylcholine (PC), but under conditions of high nutritional requirements, eg, pregnancy, dietary intake could be a limiting factor (3). Experimental studies show that pregnant rats are more vulnerable to dietary choline deficiency than are nonpregnant female rats (4). Large amounts of choline are transported to the embryo (5), which may deplete maternal choline stores (1). The 60% lower hepatic choline content in late-pregnant rats than in nonpregnant rats is in line with this notion (6). The insight that prenatal choline availability is crucial for rat brain development with lifelong lasting effects (7) warrants attention. The Institute of Medicine (IOM) recommends a higher intake of choline for pregnant than for nonpregnant women (450 and 400 mg/d, respectively) (8). The IOM cautions, however, that insufficient data are available to establish estimated average requirements for different populations. Two cross-sectional studies of free choline concentrations during human pregnancy have shown conflicting results (9, 10). Longitudinal data are not available.

Betaine, which is mostly generated via the hepatic oxidation of choline, donates its methyl group directly to homocysteine (Figure 1). Betaine:homocysteine methyltransferase (BHMT) catalyzes this reaction and resides at the intersection of choline, methionine, and folate metabolism. The sole alternate route of homocysteine remethylation uses 5-methyltetrahydrofolate as a methyl donor and is catalyzed by methionine synthase. In this reaction, vitamin B-12 serves as a cofactor (11). Folate-dependent methionine synthase is present in most tissues, whereas betaine-dependent BHMT is confined to human liver and kidney. Under normal conditions, plasma folate is the main determinant of plasma total homocysteine (tHcy) concentrations (12). Plasma betaine is related to fasting tHcy concentrations in healthy subjects (13) and in patients with cardiovascular disease (14).

Cross-sectional (15–17) and longitudinal (18 –23) studies have investigated plasma tHcy during pregnancy. Plasma tHcy is 30 – 60% lower in pregnant women than in nonpregnant women, and the lowest tHcy concentrations are observed in the second trimester. Malinow et al (24) noted that low folate in pregnant women does not result in high plasma tHcy, as is observed in nonpregnant subjects. This observation suggests the presence of an effective tHcy-lowering mechanism in pregnant women. Nutritional and hormonal factors have been shown to affect betaine-dependent homocysteine remethylation in experimental animals (25, 26). Conceivably, the higher nutritional demands and alterations in hormonal environment during pregnancy may have similar effects in humans.

We investigated the changes in plasma choline and plasma betaine during normal human pregnancy and their relations to plasma tHcy. Plasma choline, plasma betaine, and plasma dimethylglycine (DMG; the demethylated product of betaine) together with plasma tHcy, plasma folate, and plasma vitamin B-12 were measured in 50 women with uneventful pregnancies. Samples were obtained monthly during pregnancy. The initial sampling was at gestational week (GW) 9 and the last sampling at 14.5 wk postpartum.

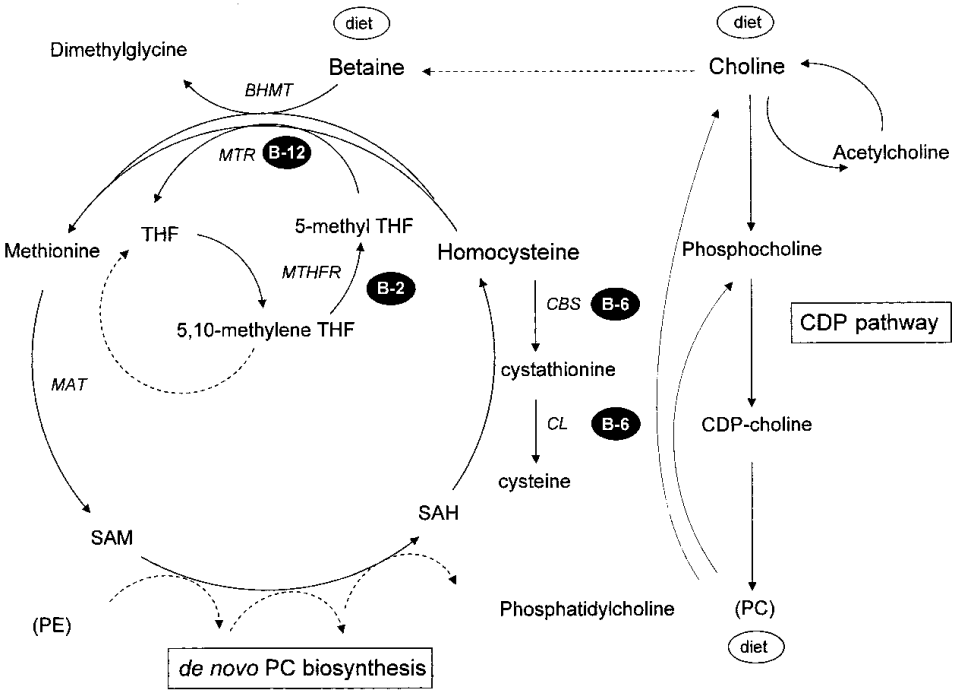

Figure 1. Choline, betaine, and one-carbon metabolism. Choline is provided by food or can be formed de novo via sequential *S*-adenosylmethionine (SAM)– dependent methylation of phosphatidylethanolamine (PE) to form phosphatidylcholine (PC), which in turn can be converted back to choline. Choline serves as a precursor in the synthesis of the neurotransmitter acetylcholine or is oxidized to betaine. The latter compound serves as a methyl donor in the betaine:homocysteine methyltransferase (BHMT) reaction, which converts homocysteine to methionine. This conversion is also catalyzed by methionine synthase (MTR), which requires 5-methyltetrahydrofolate (THF) as methyl donor and couples the choline-betaine pathway to folate metabolism. The solid arrows represent single enzymatic reactions; the dashed arrows represent multiple enzyme reactions. CDP, cytidine 5'-diphosphate; MTHFR, 5,10-methylenetetrahydrofolate reductase; MAT, methionine adenosyltransferase; SAH, *S*-adenosylhomocysteine; CBS, cystathionine-β-synthase; CL, γ-cystathioninase.

SUBJECTS AND METHODS

Study population and protocol

The study participants were recruited from a larger study on clinical chemical reference values of pregnant women in the island of Curaçao, Netherlands Antilles. Curaçao is predominantly inhabited by a population of West African descent with ample Caucasian mixture. The dietary habits of this population are essentially Western. Women were asked to participate at their first prenatal visit. On agreement, singleton pregnancy was confirmed by ultrasonic examination with assessment of gestational age (all before 20 wk of gestation). Thereafter, the visits were monthly. At each visit, blood samples were taken for immediate determination of routine clinical chemical indexes, whereas aliquots of plasma were stored. In addition, women were asked about their use of prescribed medication and over-the-counter prenatal vitamin supplements. All participants gave their written consent. The study followed local ethical standards and the Helsinki Declaration of 1975, as revised in 1989.

Of the 194 women who entered the original study, 108 experienced uneventful pregnancies and delivered healthy term babies weighing >2500 g. Fifty of these women were randomly selected to have B vitamins and metabolites measured in their stored plasma samples. The mean and median (range) gestational ages at the initial visit were 9 and 9.5 (10.2) wk, respectively. This first sampling date is thus referred to as GW 9. Postpartum samples were available for 40 women. The postpartum samples were collected on average 14.5 wk after delivery.

Most women reported taking folic acid irregularly, whereas 7 women reported taking no supplements containing folic acid during pregnancy. Of the 43 women who reported using supplements containing folic acid, 15 used Fero-Folic (800 µg folic acid/d; Abbott Diagnosis, Abbott Park, IL), 10 used prenatal vitamin supplements (1000 µg folic acid/d) such as Materna (Lederle Laboratories, Pearl River, NY) and Natalins (Mead Johnson, Evansville, IL), and 17 women reported using both. At the postpartum sampling, none of the 40 women reported using folic acid.

Blood sampling and analysis

Serial venous blood samples were collected at GW 9, 16, 20, 24, 28, 32, and 36 and postpartum. Women were instructed not to consume breakfast before sampling, which took place at ≈0800. Samples were taken by venipuncture. EDTA plasma was prepared by centrifugation (2500 rpm, 10 min, -4 °C) within 1 h, and aliquots of plasma were stored at -70 °C until analyzed. The storage time was 4 –9 y. Plasma tHcy, plasma folate, and plasma vitamin B-12 were analyzed by competitive protein binding assays with the use of an immunochemistry analyzer (IMX; Abbott Laboratories, Chicago, IL) in the Analytic Diagnostic Center, Curaçao, Netherlands Antilles. Plasma choline, plasma betaine, and plasma DMG were analyzed at the Institute of Pharmacology, University of Bergen, Bergen, Norway, by using a method based on normal-phase chromatography tandem mass spectrometry (27). Creatinine and hematocrit were measured immediately

after sampling with the use of a Vitros 9550 (Johnson and Johnson, New Brunswick, NJ) and a Celldyne 3200 (Abbott Diagnostics), respectively. Samples from individual subjects were analyzed in a single run to minimize the effect of analytic variance.

Statistical analysis

Logarithmic transformation was performed to approximate normal distribution for plasma tHcy, folate, vitamin B-12, choline, betaine, and DMG. Geometric means were used when appropriate. Changes in variables throughout gestation were analyzed with repeated-measures analysis of variance. Post hoc analyses were performed with the Student's *t* test for paired samples. Data at each sampling were compared with those of the previous sampling; postpartum data were compared with those of GW 36 and GW 9. The results were corrected for multiple testing by using the Bonferroni method to minimize type I errors. Correlations were investigated by using Spearman's rank-test. Longitudinal values of 5th, 50th, and 95th percentiles for plasma choline and betaine throughout pregnancy were estimated with a linear mixed model for repeated measurements (19). Multiple regression analysis was used to study predictors of plasma tHcy at each sampling point during the course of gestation and postpartum. Most statistical analyses were performed by using SPSS version 10 (SPSS Inc, Chicago, IL). The linear mixed model for repeated measurements was performed by using SAS version 8.2 (SAS Institute Inc, Cary, NC).

RESULTS

Characteristics of the study population

The mean (±SD) age of the study population was 29 ± 5.4 y, 30% were nullipara, and the women delivered at a mean (±SD) gestational age of 39.6 ± 1.0 wk. The mean (±SD) birth weight of their babies was 3260 ± 297 g. The percentage of women who reported using folic acid increased with advancing gestation; only 20.9% of the women reported using folic acid at GW 9, whereas 69.8% and 62.8% of them reported using folic acid at GW 32 and GW 36, respectively.

Changes in blood indexes during pregnancy

The geometric means and 95% reference intervals for plasma tHcy, folate, vitamin B-12, choline, betaine, and DMG at GW 9, 16, 20, 24, 28, 32, and 36 and postpartum are presented in **Table 1**. All of these variables changed significantly (by repeated-measures analysis of variance) during gestation: $P < 0.0001$ for plasma tHcy, vitamin B-12, choline, and betaine; $P = 0.01$ for plasma folate; and $P = 0.02$ for plasma DMG.

A post hoc analysis showed that plasma tHcy decreased in early pregnancy (from GW 9 to GW 16; $P < 0.0001$). Although plasma tHcy tended to decrease until GW 28, these decrements were not significant. Plasma folate increased from GW 9 to GW 16 ($P = 0.01$). Plasma vitamin B-12 decreased from GW 9 to GW 16, from GW 16 to GW 20 ($P < 0.0001$ for both), and from GW 28 to GW 32 ($P = 0.009$). Plasma choline increased from

GW 9 to GW 16 ($P = 0.03$), from GW 16 to GW 20 ($P = 0.02$), from GW 20 to GW 24 ($P < 0.0001$), and from GW 32 to GW 36 ($P = 0.002$). Plasma betaine decreased from GW 9 to GW 16 and from GW 16 to GW 20 ($P < 0.0001$ for both). Plasma DMG decreased from GW 9 to GW 16 ($P < 0.0001$).

The 5th, 50th, and 95th percentiles of longitudinal values of plasma choline and plasma betaine throughout pregnancy, estimated by using a linear fixed model for repeated measures, are shown in **Figures 2** and **3**, respectively.

Postpartum blood indexes

Three months after delivery, plasma tHcy, vitamin B-12, betaine, and DMG were higher, whereas plasma folate and choline were lower than the concentrations at GW 36 ($P < 0.0001$ for all except folate; $P = 0.004$). Postpartum concentrations of plasma betaine and DMG were also higher than the concentrations at GW 9 ($P < 0.0001$ for both). On an individual basis, 35 of 40 women had a lower plasma choline concentration postpartum and 40 of 40 had a higher plasma betaine concentration postpartum than at late pregnancy (GW 36).

Univariate correlations

Spearman coefficients for the correlations of plasma tHcy with plasma folate, vitamin B-12, and betaine are listed in **Table 2**. Plasma tHcy was significantly and inversely related to plasma folate throughout gestation and postpartum and with plasma vitamin B-12 between GW 16 and GW 28. Plasma tHcy became inversely related to plasma betaine at GW 16, and this relation was maintained throughout gestation (except at GW 32) and postpartum. Plasma tHcy was unrelated to plasma choline, DMG, or creatinine (data not shown)

Plasma betaine was positively related to plasma choline at each sampling point during pregnancy and postpartum. The weakest correlation was found at GW 28 ($r = 0.35$, $P = 0.02$), whereas the strongest correlation was found at GW 20 ($r = 0.56$, $P < 0.0001$). The relation between plasma betaine and plasma DMG during pregnancy was significant at GW 9 ($r = 0.38$, $P = 0.006$), GW 16 ($r = 0.48$, $P = <0.0001$), GW 28 ($r = 0.41$, $P = 0.004$), GW 36 ($r = 0.33$, $P = 0.02$), and postpartum ($r = 0.45$, $P = 0.004$). Plasma betaine was related to plasma folate at GW 16 ($r = 0.40$, $P = 0.004$) and GW 20 ($r = 0.31$, $P = 0.03$).

Hematocrit was not significantly related to plasma tHcy or betaine at any time during pregnancy, except at GW 36. At this time point, an inverse relation with hematocrit and plasma tHcy was observed ($r = -0.36$, $P = 0.01$).

Multiple regression analysis

Predictors of plasma tHcy in all 50 women were determined by multiple regression. The model included maternal age, folate, vitamin B-12, choline, betaine, DMG, creatinine, and hematocrit as independent variables. Plasma folate, vitamin B-12, betaine, and hematocrit were significantly associated with plasma tHcy. The results for plasma folate, vitamin B-12, and betaine are listed in **Table 3**. Plasma folate was the strongest predictor

Table 1. Plasma concentrations of total homocysteine (tHcy), folate, vitamin B-12, choline, betaine, and dimethylglycine (DMG) throughout gestation and postpartum (PP)[1]

	GW 9	GW 16	GW 20	GW 24	GW 28	GW 32	GW 36	PP
tHcy (μmol/L)								
Geometric $\bar{x}$	9.42	7.28[2]	7.33	7.11	6.89	7.17	7.60	10.15[2]
95% RI	5.50, 16.12	4.28, 12.40	4.25, 12.64	4.03, 12.55	3.93, 12.06	4.38, 11.73	4.46, 12.96	5.51, 18.70
Folate (nmol/L)								
Geometric $\bar{x}$	16.38	19.61[3]	18.46	17.31	19.98	20.04	20.46	14.93[4]
95% RI	7.92, 33.85	9.05, 42.52	8.44, 40.39	6.52, 45.91	8.13, 49.13	7.14, 56.25	7.35, 56.91	6.43, 34.67
Vitamin B-12								
(pmol/L)	293.2	221.2[2]	195.4[2]	185.5	179.3	164.4[4]	164.1	276.7[2]
Geometric $\bar{x}$	122.7, 700.7	102.5, 477.4	89.4, 427.1	81.9, 420.0	80.9, 397.3	73.8, 366.3	72.7, 370.2	118.8, 644.4
95% RI								
Choline (μmol/L)	6.62	7.32[3]	7.94[3]	8.87[2]	9.36	9.78	10.77[4]	7.92[2]
Geometric $\bar{x}$	4.51, 9.70	4.89, 10.97	5.62, 11.20	6.38, 12.33	6.61, 13.26	7.12, 13.41	7.45, 15.58	5.44, 11.52
95% RI								
Betaine (μmol/L)	16.27	11.47[2]	10.29[2]	10.29	10.73	10.76	10.93	27.45[2,5]
Geometric $\bar{x}$	8.59, 30.78	6.77, 19.45	6.56, 16.15	6.96, 15.20	7.41, 15.53	7.18, 16.13	7.13, 16.75	12.63, 59.70
95% RI								
DMG (μmol/L)	1.96	1.54[2]	1.57	1.61	1.74	1.71	1.76	2.66[2,5]
Geometric $\bar{x}$	0.89, 4.28	0.74, 3.19	0.73, 3.39	0.67, 3.92	0.59, 5.15	0.85, 3.44	0.84, 3.68	1.43, 4.96
95% RI								

[1] Blood samples were collected from 50 women with uncomplicated pregnancies at their initial visit [gestational week (GW) 9] and subsequently at GW 16, 20, 24, 28, 32, and 36. PP blood samples were obtained from 40 women approximately 14.5 wk after delivery. The value were logarithmically transformed to approximate normality. RI, reference interval. All variables were analyzed with repeated-measures ANOVA. All variables changed during gestation: $P < 0.001$ for tHcy, vitamin B-12, choline, and betaine; $P = 0.01$ for folate; and $P = 0.02$ for DMG. Data at each time point were compared with data for the preceding time point by Student's t test for paired samples. The results were corrected for multiple testing by using the Bonferroni method to minimize type 1 errors.

[2–4] Significantly different from previous time point (Student's t test): [2]$P < 0.001$, [3]$P < 0.05$, [4]$P < 0.01$.

[5] Significantly different from GW 9, $P < 0.001$ (Student's t test).

of tHcy at GW 9 ($P = 0.01$) and postpartum ($P < 0.0001$), whereas plasma betaine was the strongest predictor at GW 20 ($P = 0.02$), GW 24 ($P = 0.04$), GW 28 ($P = 0.003$), and GW 36 ($P < 0.0001$). Plasma vitamin B-12 was related to tHcy only at GW 16 ($P = 0.04$; Table 3). Hematocrit was related to tHcy only at GW 36 ($P = 0.03$). A repeat of the multiple regression analyses after the exclusion of 7 women who reported not taking folic acid supplements gave essentially the same results (data not shown).

DISCUSSION

In the present longitudinal study, we measured metabolites and vitamins involved in one-carbon metabolism at 7 time points during the course of uncomplicated pregnancies and once postpartum in 50 women.

Plasma tHcy, betaine, DMG, and vitamin B-12 decreased during pregnancy, whereas folate increased. The decrease in plasma tHcy reached a nadir in the early third trimester (GW 28), which is in general agreement with previous reports (15–23). The observed increase in plasma folate concentrations during pregnancy may reflect the reported folic acid use of the women. The steady reduction in plasma vitamin B-12 was in agreement with the findings of previous studies (28, 29). The novel and most important findings are a steady increase in plasma choline throughout gestation and an inverse relation between plasma betaine and tHcy with advancing gestation. There seems to be a concurrent attenuation of the folate-tHcy relation, especially in midtrimester.

Table 2. Univariate correlations (r) of plasma total homocysteine (tHcy) with plasma folate, vitamin B-12, and betaine throughout gestation and postpartum (PP)[1]

	tHcy and folate	tHcy and vitamin B-12	tHcy and betaine
GW 9	-0.351[2]	-0.088	-0.077
GW 16	-0.352[2]	-0.454[3]	-0.440[3]
GW 20	-0.470[3]	-0.430[3]	-0.322[2]
GW 24	-0.306[2]	-0.393[3]	-0.456[3]
GW 28	-0.289[2]	-0.353[2]	-0.379[3]
GW 32	-0.311[2]	-0.272	-0.258
GW 36	-0.377[3]	-0.202	-0.472[3]
PP	-0.534[4]	-0.169	-0.378[2]

[1] Blood samples were collected from 50 women with uncomplicated pregnancies at their initial visit [gestational week (GW) 9] and subsequently at GW 16, 20, 24, 28, 32, and 36. PP blood samples were obtained from 40 women approximately 14.5 wk after delivery. Univariate analysis was performed with Spearman's rank-sum test.

[2] $P < 0.05$.

[3] $P < 0.01$.

[4] $P < 0.001$.

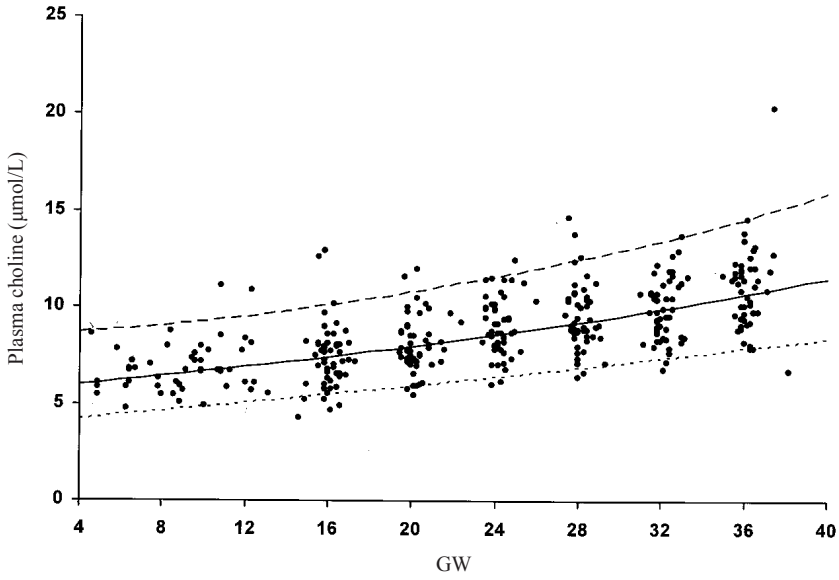

Figure 2. Plasma choline throughout normal human pregnancy. Blood samples were taken from 50 women at the initial visit [gestational week (GW) 9] and subsequently at GW 16, 20, 24, 28, 32, and 36. Data are presented as individual values (●) and the 5th (lower, - - -), 50th (——) and 95th (upper, - - -) percentiles, as estimated with a linear mixed model for repeated measurements (19).

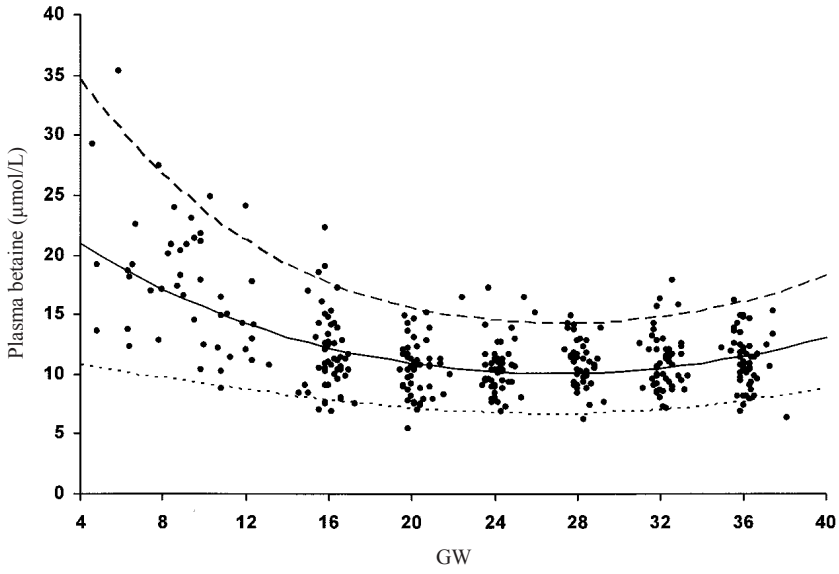

Figure 3. Plasma betaine throughout normal human pregnancy. Blood samples were taken from 50 women at the initial visit [gestational week (GW) 9] and subsequently at GW 16, 20, 24, 28, 32, and 36. Data are presented as individual values (●) and the 5th (lower, - - -), 50th (——) and 95th (upper, - - -) percentiles, as estimated with a linear mixed model for repeated measurements (19).

Animal experiments have shown that tHcy in mice with low 5,10-methylenetetrahydrofolate reductase (MTHFR) activity was more responsive to betaine than was tHcy in wild-type animals (14). However, we did not determine the *MTHFR* 677C→T genotype in this study, but a previous study of 178 women from the same population showed a homozygous *MTHFR* 677C→ T genotype frequency of only 1.7% (FV Velzing-Aarts et al, unpublished observations, 2000). Therefore, it is unlikely that the tHcy-folate and tHcy-betaine relations observed by us were significantly influenced by the *MTHFR* 677C→T polymorphism.

Two cross-sectional studies have addressed free choline concentrations in pregnancy (9, 10). Lower plasma choline concentrations were found in women at the time they gave birth than in nonpregnant women in an early study (9), and a more recent study showed higher serum free choline concentrations in the second and third trimesters of pregnant women than in nonpregnant women (10). These latter findings essentially agree with our finding of a progressive increase in choline throughout gestation (Figure 2). In the same studies, choline concentrations in the amniotic fluid (10) and in newborns (9, 10) were almost 2-fold those of maternal concentrations. Conceivably, the observed increase in maternal plasma choline ensures choline availability for (active) placental transfer (30) to meet fetal choline requirements (3).

Table 3. Predictors of plasma total homocysteine (tHcy) throughout gestation and postpartum (PP) by multiple regression anaysis[1].

	β			B			
	Folate	Vitamin B-12	Betaine	Folate	Vitamin B-12	Betaine	R^2
GW 9	-0.405[2]	-0.011	0.068	-0.297	-0.007	0.059	0.186
GW 16	-0.209	-0.296[2]	-0.326	-0.144	-0.205	-0.332	0.396
GW 20	-0.253	-0.233	-0.402[2]	-0.176	-0.163	-0.486	0.394
GW 24	-0.232	-0.222	-0.337[2]	-0.134	-0.153	-0.486	0.421
GW 28	-0.067	-0.174	-0.507[3]	-0.043	-0.122	-0.788	0.406
GW 32	-0.163	-0.173	-0.259	-0.076	-0.111	-0.327	0.222
GW 36	-0.276[2]	0.065	-0.643[4]	-0.139	0.045	-0.774	0.596
PP	-0.677[4]	0.061	-0.117	-0.511	0.045	-0.097	0.608

[1] Blood samples were collected from 50 women with uncomplicated pregnancies at their initial visit [gestational week (GW) 9] and subsequently at GW 16, 20, 24, 28, 32, and 36. PP blood samples were obtained from 40 women approximately 14.5 wk after delivery. J3, standardized coefficient; B, unstandardized coefficient. Multiple regression analysis was performed with plasma tHcy as a dependent variable and maternal age, plasma folate, vitamin B-12, betaine, choline, dimethylglycine, creatinine, and hematocrit as independent variables. Of the other independent variables, only hematocrit was related to plasma tHcy (at GW 36: β = -0.256, $P = 0.03$; data not shown).

[2] $P < 0.05$.

[3] $P < 0.01$.

[4] $P < 0.0001$.

In animals, maternal hepatic choline is mobilized to supply the placenta and fetus (6). Compared with nonpregnant rats, pregnant rats have a 24% higher hepatic activity of phosphatidylethanolamine-*N*-methyltransferase, which is the enzyme responsible for de novo choline synthesis (6). This increased de novo choline synthesis cannot compensate for the Predictors of plasma total homocysteine (tHcy) throughout gestation and postpartum (PP) by multiple regression analysis[1] mobilization of choline stores, as indicated by the depletion of maternal hepatic choline stores at the end of pregnancy (6). Thus, both mobilization of maternal choline stores and enhanced de novo choline synthesis may cause maternal plasma choline concentrations to rise. However, the net effect may be the diminution of maternal choline reserves. This emphasizes the importance of a higher dietary intake of choline during pregnancy.

A notable finding in this study was the inverse relation between plasma betaine and tHcy with advancing gestation. In nonpregnant subjects, plasma betaine is strongly related to the increase in tHcy after methionine loading, particularly in subjects who did not take B vitamin supplements (31). Plasma betaine is only weakly related to fasting tHcy in healthy subjects and in patients with cardiovascular disease (14). The relation between fasting tHcy and betaine becomes more pronounced in subjects with low folate concentrations (13), but still does not reach the strength that we observed during pregnancy. Thus, the reported irregular folic acid use in our study population is expected to attenuate the association between tHcy and folate (31) and cannot explain the enforcement of the tHcy-betaine relation. The tHcy-betaine relation observed in the present study emphasizes the importance of betaine in one-carbon metabolism during pregnancy. The exact role of betaine is not known, but conserving methionine via a folate-independent route may be beneficial during pregnancy.

The hormonal environment of pregnancy, characterized by increased concentrations of estrogens (32) and cortisol (33), may affect BHMT-catalyzed homocysteine remethylation. The recently cloned human BHMT gene contains consensus sites for steroid hormone receptors, including estrogens and glucocorticoids (26). Cortisol is reported to increase BHMT gene expression (26), whereas cortisol-treated animals exert a 300% increase in liver BHMT activity (25). Estrogens are well-established tHcy-lowering agents in humans and may contribute to pregnancy-associated decreases in tHcy (15, 18, 20, 24).

Both cortisol (34) and estrogens (32, 35) increase continuously throughout gestation, which parallels our finding of a stronger betaine-tHcy relation with advancing gestation. Plasma betaine concentrations are nearly 50% lower during pregnancy than at postpartum (Table 1) or in nonpregnant women (31). Hemodilution cannot explain this reduction, because plasma betaine concentrations were unrelated to hematocrit. Increased consumption through the BHMT pathway, distribution to intracellular compartments, placental transfer, and reduced synthesis from choline may account for the reduction. The latter may have a choline-sparing effect but may also reflect a suboptimal choline status.

In conclusion, the second half of pregnancy in humans is characterized by a progressive increase in plasma choline and a strong inverse relation between plasma betaine and tHcy. The steady increase in plasma choline throughout gestation may ensure

choline availability for placental transfer, with subsequent use by the growing fetus. The inverse relation between plasma betaine and tHcy emphasizes the role of betaine during normal pregnancy. This relation also points to the possibility that a low betaine status may predispose to pregnancy complications associated with high tHcy (36). Our results emphasize the importance of choline and betaine status during normal human pregnancy and encourage further studies, including intervention trials.

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FVV-A designed the study, participated in the organization of data collection, and wrote the manuscript. PIH and PMU performed the data analysis and contributed to the writing of the manuscript. MRF performed the statistical analysis and contributed to the writing of the manuscript. FPvdD participated in the organization of data collection. FAM contributed to the writing of the manuscript. No conflicts of interest were declared.

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Abnormal relationships between plasma homocysteine, folate and vitamin B_{12} in preeclampsia

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ABSTRACT

Introduction. Healthy pregnant women have lower plasma homocysteine than healthy non-pregnant counterparts. Preeclamptic women have higher homocysteine than healthy pregnant controls.

Objective en Study Design. Investigate relationships between plasma homocysteine, folate and vitamin B_{12} in 43 preeclamptic women and 43 pregnancy duration- and parity-matched pregnant controls.

Results. Preeclamptic women had higher plasma homocysteine ($p<0.0001$), higher vitamin B_{12} ($p=0.025$) and similar folate. There were inverse relations between homocysteine and folate (both preeclampsia and controls: $p<0.0001$), and homocysteine and vitamin B_{12} (preeclampsia $p=0.008$; controls $p=0.020$). Unlike controls, preeclamptic women exhibited steep homocysteine increases below folate and vitamin B_{12} levels of about 10 nmol/l and 200 pmol/l, respectively.

Conclusion. Homocysteine-folate and homocysteine-vitamin B_{12} relationships in preeclampsia are similar to those of healthy non-pregnant women. These abnormal relationships suggest high dependency of their homocysteine metabolism on the folate- and vitamin B_{12}-dependent remethylation pathway.

INTRODUCTION

The amino acid homocysteine is exclusively formed as an intermediate in methionine metabolism.[1] Homocysteine is metabolically cleared by its remethylation to methionine, or its degradation to cysteine. Remethylation of homocysteine occurs via methionine synthase with vitamin B_{12} as cofactor and 5-methyl tetrahydrofolate (5-methyl THF) as methyl-donating substrate, or via betaine-homocysteine *S*-methyltransferase (BHMT) with betaine as methyl-donating substrate. Degradation of homocysteine to cysteine is vitamin B_6 dependent. Homocysteine is excreted from the cells, when its formation exceeds metabolic clearance.[2] Elevated plasma homocysteine is recognized as an independent risk factor for atherosclerosis.[1] The underlying causes of its detrimental effects are as yet poorly understood, but endothelial injury and dysfunction are likely to be involved.

Healthy pregnant women have lower plasma homocysteine compared with non-pregnant controls.[3-6] Rajkovic *et al*.[7] were the first to report higher plasma homocysteine in women with preeclampsia compared with healthy pregnant counterparts. Others[8-10] subsequently confirmed this observation. Rajkovic *et al*.[11] reported higher postpartum plasma homocysteine levels in 33 eclamptic and 138 preeclamptic African women, Powers *et al*.[8] showed a relation between plasma homocysteine and cellular fibronectin, suggesting that homocysteine plays a role in promoting endothelial dysfunction in preeclampsia,

Renal function, genetic factors, vitamin B_6-, vitamin B_{12}- and folate status are the main plasma homocysteine determinants in non-pregnant subjects. Plasma folate proved the major determinant of plasma homocysteine in the general population.[12] Brouwer *et al*.[13] reported a significant increase of plasma homocysteine in 103 non-pregnant subjects when plasma folate reaches levels below 10 nmol/l.

The lower plasma homocysteine levels in pregnancy may result from adaptation to efficient homocysteine metabolism as a physiologic response to the altered needs of pregnancy.[3-6] In contrast to non-pregnant subjects, low maternal plasma folate and vitamin B_{12} do not give rise to elevated homocysteine levels in pregnant subjects.[14,15] The disparity is partly explained by fetal uptake of maternal homocysteine and altered hormonal status in pregnancy.[14]

We elaborated on the relationship between plasma homocysteine, folate and vitamin B_{12} in normal and preeclamptic pregnancies. For this, we determined plasma homocysteine, folate and vitamin B_{12} levels in 43 preeclamptic pregnancies and 43 pregnancy duration and parity matched controls. We were particularly interested to see whether preeclamptic women and their controls exhibited different relationships between homocysteine and folate and between homocysteine and vitamin B_{12}. All participants lived in the island of The Netherlands Antilles (mainly Curaçao), which is inhabited by a population of predominantly West African origin with ample Caucasian mixture.

MATERIAL AND METHODS

Study group

The study was performed in the islands of The Netherlands Antilles (mainly Curaçao). The study population (Table 1) was composed of 43 preeclamptic women and 43 controls, who were matched for pregnancy duration (±1 week) and parity (nullipara *versus* multipara). The women were predominantly from Afro-Caribbean descent. None of them had diabetes, pre-existent hypertension, or pre-existing renal disorders**.** Preeclampsia was clinically diagnosed upon admittance to the obstetric ward of the St. Elisabeth Hospital, Curaçao (The Netherlands Antilles). Preeclampsia was defined as the combination of hypertension (≥90 mm Hg diastolic) and proteinuria (≥0.3 g/l) according to Davey and MacGillivray.[16] Forty-three apparently healthy non-proteinuric normotensive outclinic pregnant women served as controls. The study conformed to local ethical standards and the Helsinki declaration of 1975, as revised in 1989.

Study design

Blood samples were collected from preeclamptic women and matched controls during pregnancy (Table 1). None of the women was at labor at the time of blood sampling. Samples were taken by venepuncture, EDTA-plasma was prepared by centrifugation and aliquots of plasma were stored at -70°C until further analysis. Plasma homocysteine, folate and vitamin B_{12} were analyzed by competitive protein binding assays using an immunochemistry analyzer (IMX, Abbott Laboratories, USA) in the Public Health Laboratory (Curaçao).

Statistical analyses

Between-group differences of subject characteristics were analyzed with the Student's t-test for paired samples. Between group-differences of plasma homocysteine, plasma folate and plasma vitamin B_{12} levels were analyzed using the Wilcoxon matched-pairs signed-ranks test. Correlations were investigated with the Spearman rank-test[17] and $p<0.05$ was considered significant.

RESULTS

Preeclampsia *versus* controls

Table 1 shows the study group characteristics together with plasma concentrations of homocysteine, folate and vitamin B_{12}. Compared with their matched controls, preeclamptic women were older ($p=0.003$), had children with lower birth weight ($p<0.0001$) and had lower gestational age at delivery ($p<0.0001$). Plasma homocysteine and plasma vitamin B_{12} levels were higher in preeclampsia, compared to controls ($p<0.0001$ and $p=0.025$, respectively). There was no between-group difference in plasma folate.

Table 1. Subject characteristics, homocysteine, folate and vitamin B_{12} for preeclamptic and control women.

	Controls	Preeclampsia
Number	43	43
Maternal age (years)	25.7±7.2	27.5±7.3[1]
Parity (nulli/multi)	20/23	20/23
Birth weight (g)	3350±342	1923±840[2]
Gestational age at delivery (weeks)	39.8±1.0	34.5±4.0[2]
Pregnancy duration at blood sampling	33.8±4.2	33.9±4.0
Plasma homocysteine (μmol/l)	6.6 (3.4-21.1)	11.6 (4.9-40.5)[4]
Plasma folate (nmol/l)	14.0 (2.7-36.5)	11.1 (2.7-36.5)
Plasma vitamin B_{12} (pmol/l)	156 (44-393)	189 (81-945)[3]

Data represent mean±SD or median (range). Statistically different from controls by matched pair Student T-test at [1]$p<0.01$, [2]$p<0.001$, and by Wilcoxon matched pairs analysis at [3]$p<0.05$ and [4]$p<0.001$.

Correlations plasma homocysteine, folate and vitamin B_{12}

Maternal age was a possible cofounder (see Table 1), but it proved unrelated to plasma homocysteine, folate and vitamin B_{12} in the entire study population, preeclamptic women or controls. Matching of preeclamptic women and controls on the basis of pregnancy duration precluded any confounding influence of birth weight and gestational age at delivery.

There were inverse relations between plasma homocysteine and plasma folate (Figure 1; upper panel) in the entire study group ($r= -0.50$, $p<0.0001$ for n=86), preeclamptic women ($r= -0.59$, $p<0.0001$; for n=43) and controls ($r= -0.60$, $p<0.0001$; n=43). Plasma vitamin B_{12} correlated inversely with plasma homocysteine (Figure 1; lower panel) in preeclamptic women ($r= -0.41$, $p=0.008$; for n=41) and controls ($r= -0.35$, $p=0.020$; for n=43), but not in the entire study group. There was a positive relation between plasma folate and plasma vitamin B_{12} levels for the entire study population ($r= 0.30$, $p=0.005$; for n=84) and women with preeclampsia ($r= 0.46$, $p=0.002$; for n=41), but not for the controls (data not shown).

DISCUSSION

We determined the plasma homocysteine, folate and vitamin B_{12} levels of 43 preeclamptic women and compared the outcome with that of 43 controls matched for pregnancy duration and parity. In accordance with many other investigators,[7-10] we found higher plasma homocysteine in preeclampsia. There were no between-group differences in plasma folate, but in contrast to Laivuori *et al.*[9] we found higher plasma vitamin B_{12} in preeclampsia. The most remarkable observation was, however, that preeclamptic

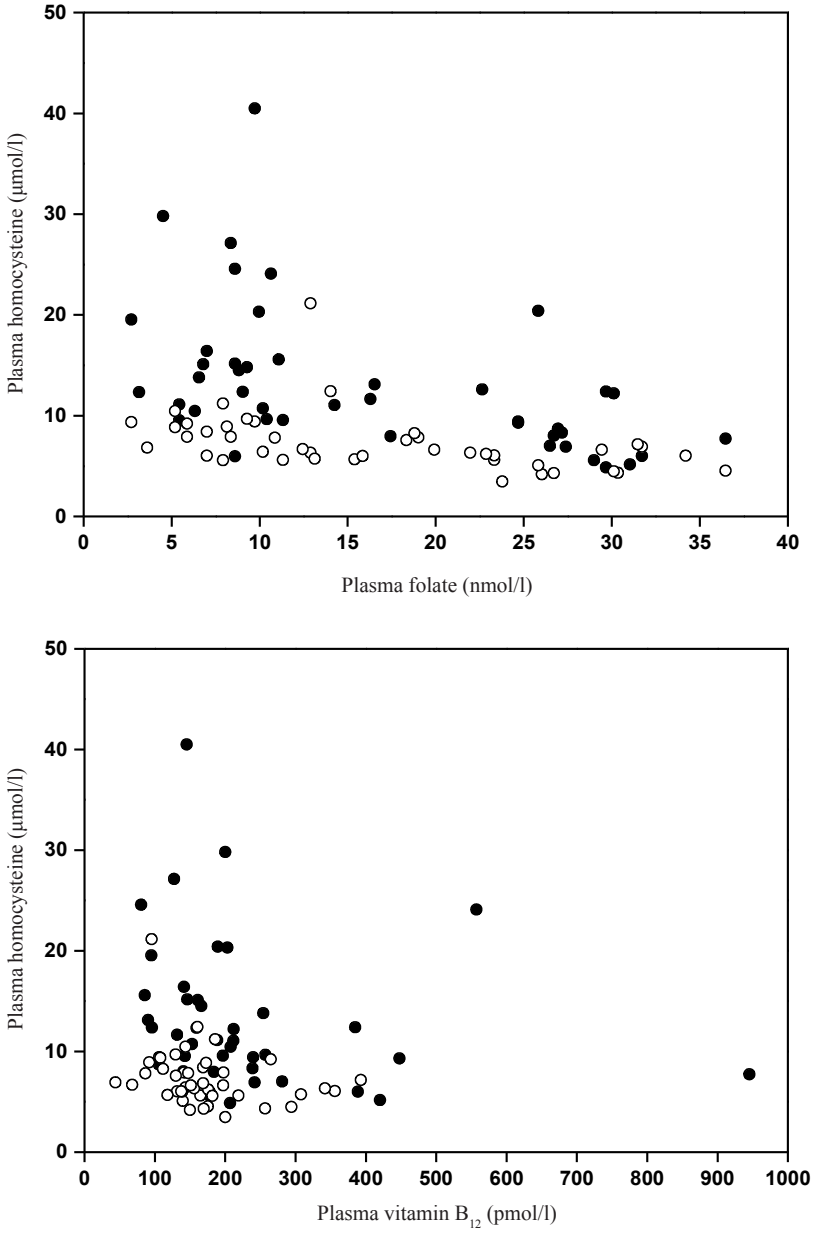

Figure 1. Plasma homocysteine-folate (upper) and plasma homocysteine-vitamin B_{12} (below) for preeclamptic women and controls.
Controls were matched for pregnancy duration and parity. Spearman rank-test for homocysteine-folate relation: r = -0.59, p<0.0001 (43 preeclamptic women) and r = -0.60, p<0.0001 (43 controls): for homocysteine-vitamin B_{12} relation: r = -0.41, p=0.008 (41 preeclamptic women) and r = -0.35, p=0.020 (43 controls).
● patients with pre-eclampsia; ○ controls.

women had altered relationships between plasma homocysteine and folate (Figure 1; upper panel), and between plasma homocysteine and vitamin B_{12} (Figure 1; lower panel). Plasma homocysteine and plasma folate were inversely related both in preeclamptic women and their controls. A steep rise of plasma homocysteine in preeclampsia was observed at lower folate levels, but a much less prominent relationship was found for normotensive controls. The plasma homocysteine and vitamin B_{12} correlations showed similar disparities.

Pregnant women have relatively low plasma homocysteine. Malinow *et al.*[14] and Metz *et al.*[15] noted that, in contrast to non-pregnant subjects, low plasma folate and vitamin B_{12} levels do not result in high homocysteine during normal pregnancy. The observed, rather flat, relationships in healthy pregnant women of homocysteine and folate (Figure 1; upper panel) and homocysteine and vitamin B_{12} (Figure 1; lower panel) confirm this notion. It suggests that homocysteine metabolism during normal pregnancy becomes less dependent on the folate- and vitamin B_{12}-dependent remethylation pathway. Theoretically, employment of less folate and vitamin B_{12} for homocysteine remethylation, results in higher folate and vitamin B_{12} availability for other processes, such as synthesis of purines and thymine, and transfer of folate and vitamin B_{12} to the fetus. Folate redistribution in favor of purine and thymine synthesis at the expense of 5-methyl THF synthesis, the methyl-donating substrate of homocysteine remethylation, is considered to secure DNA-synthesis in folate-deficient states.[18] Such a 'sparing effect' may also be essential during conditions of enhanced growth and could be part of the physiologic adaptation to the altered needs of pregnancy. As suggested by others,[3-9,14,19] this adaptation may be orchestrated by the hormonal environment of pregnancy or by fetal uptake of maternally derived homocysteine, which can be considered an additional clearance pathway of plasma homocysteine during pregnancy.

Preeclamptic women exhibit higher homocysteine levels, notably at lower folate and vitamin B_{12} levels compared to controls. A steep rise of plasma homocysteine in preeclampsia seems to take place when the plasma folate reaches levels below about 10 nmol/l (Figure 1; upper panel) or when plasma vitamin B_{12} levels reaches below 200 pmol/l (Figure 1; lower panel). The homocysteine-folate relationship observed in preeclampsia is consistent with the previously noted relationship in non-pregnant controls as reported by Brouwer *et al.*[13] Preeclamptic women seem in this respect similar to apparently healthy non-pregnant subjects. It therefore seems that, analogous to non-pregnant subjects, homocysteine metabolism in preeclamptic pregnancies is largely dependent on folate- and vitamin B_{12}-mediated remethylation. Our data give support to the suggestion of Powers *et al.* that the high homocysteine of preeclampsia relates to a deficit in mechanisms that decrease homocysteine during normal pregnancy. These mechanisms may relate to altered hormonal environment[8,20] and reduced placental perfusion,[21] which may reduce fetal uptake of maternally-derived homocysteine.

We conclude that preeclamptic women have higher homocysteine and vitamin B_{12}, compared to pregnancy duration and parity matched controls. The homocysteine-folate and homocysteine-vitamin B_{12} relationships in preeclampsia are similar to those in healthy

non-pregnant women, suggesting high dependence of their homocysteine metabolism on the folate- and vitamin B_{12}-dependent remethylation pathway.

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Value of the soluble transferrin receptor during uncomplicated pregnancy

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ABSTRACT

Background. Establishment of iron deficiency in pregnancy is troublesome, since pregnancy *per se* alters most iron status parameters. Soluble transferrin receptor (sTfR) is a recently recognized parameter that also depends on erythroid mass. We investigated the added value of sTfR for iron status assessment in pregnancy.

Methods. Blood was obtained from 82 apparently healthy pregnant women at gestational weeks (GW) 10, 16, 20, 24, 28, 32, and 36, and at 14.5 weeks postpartum. Plasma sTfR, serum ferritin and C-reactive protein (CRP) were measured by immunochemical methods. Women with CRP >0.8 mg/l were excluded. Women with ferritin ≤12 μg/l at GW 28, 32 and 36 were classified as an iron depleted group (n=19); those with ferritin >12 μg/l as an iron adequate group (n=22).

Results. sTfR increased during pregnancy and decreased postpartum in the total population. Comparison of sTfR levels of women in the depleted group with those in the adequate group revealed that sTfR was higher in the depleted group at GW 32 and 36. sTfR increased significantly in the depleted group from GW 28 to 36, but remained constant in the adequate group.

Conclusion. sTfR may detect iron deficiency beyond depleted stores in the third trimester of pregnancy.

INTRODUCTION

Detecting iron deficiency in pregnancy is troublesome since hemodynamic adaptations and altered iron needs change most traditional indices, independent of iron status.[1-4] Iron deficiency develops over a prolonged period of negative iron balance, in sequential stages from depleted iron stores to insufficient tissue iron supply and finally the development of iron-deficient erythropoiesis. The various indices of iron status detect iron deficiency at different stages. The amount of stainable bone marrow iron is considered to be the 'gold standard'.[4] Serum ferritin is a strong and practical surrogate marker, because it correlates positively with stainable bone marrow iron.[5] Serum ferritin reflects iron stores. Levels below consensus cut-off values are used to detect depleted iron stores. The concentration of truncated fragments of the transferrin receptor in the circulation (sTfR) becomes elevated beyond the depletion of iron stores and in the presence of insufficient tissue iron supply.[4,6-8] The transferrin receptor mediates cellular iron uptake and its surface expression becomes up-regulated in iron-deprived tissues. sTfR levels are proportionate to the number of cellular surface receptors and thereby reflect cellular iron needs.[9] TfR are widely expressed on placental and erythroid tissue, and sTfR increases upon augmented erythropoiesis.[10]

Kohgo *et al.*[11] were the first to report sTfR levels in 90 pregnant women. The rapid increase of sTfR in the second half of pregnancy was explained by TfR derivation from placental syncytiotrophoblasts. Beguin *et al.*[6] used sTfR as a marker for erythropoiesis in pregnancy. Levels decreased in the first two trimesters and increased later in pregnancy. Akesson *et al.*[12] explained the 74% sTfR increase from GW 11 to GW 36 in 29 women with ferritin <20 μg/l by increasing erythropoiesis on which a developing iron deficiency is superimposed. Choi *et al.*[13] demonstrated in iron-sufficient women a positive correlation between sTfR and the reticulocyte maturity index; which is a sensitive predictor of erythropoiesis. Both parameters proved 2-3 fold higher in the third, compared to the first, trimester. They concluded that sTfR increments in pregnancy were influenced by increasing erythropoiesis rather than deteriorating iron status.[13] A limitation of sTfR studies reported so far is that most were cross-sectional[6,11,13] or employed blood sampling with large intervals (i.e. GW 11 to 36) in pregnancy.[12]

We studied sTfR as a parameter for iron status during uncomplicated pregnancy. For this, we analyzed samples of 82 women who had uncomplicated pregnancies. Women were sampled at 7 occasions throughout pregnancy, starting from GW 10 to GW 36, and 14.5 weeks postpartum. In addition, women with normal C-reactive protein (CRP) were classified into those with adequate iron stores and those with depleted iron stores using a serum ferritin cut-off value of 12 μg/l. Classification was restricted to GW 28, 32 and 36, since serum ferritin reaches steady state values from GW 28. Using this classification we investigated the course of sTfR from GW 28-36 to determine the added value of sTfR for iron status assessment in this period.

MATERIALS AND METHODS

Study design

The study population was recruited from a larger study on the establishment of clinical chemical reference values in pregnancy in the island of Curaçao (Netherlands Antilles). Curaçao is predominantly inhabited by a population of West African (mostly Ghana) descent with ample Caucasian admixture. The dietary habits are essentially Western. Pregnant women attending antenatal outpatient clinics were asked for participation at their first visit. Upon agreement, gestational age was assessed by ultrasound before GW 20. Venous blood samples were obtained at intake (further referred to as GW 10) and subsequently at GW 16, 20, 24, 28, 32, 36 and 14.5 weeks postpartum. The women were asked about the use of drugs, vitamins and iron at each blood sampling occasion. Various clinical chemical parameters were measured, including serum ferritin and CRP. Of the 194 women who entered the study, 108 had uncomplicated pregnancies and delivered apparently healthy newborns at term. Eighty-two of these were randomly selected for sTfR measurements. Serum ferritin was determined by IMx (Abbott), CRP by an immunochemical method (Beckmann Array) and plasma sTfR with an immunoturbidimetric assay (Orion Diagnostics). Samples for sTfR assays were stored at –70 °C until analysis. All sTfR samples were analyzed in 6 runs on a single day. All participants gave their written consent. The study conformed to local ethical standards and the Helsinki declaration of 1975, as revised in 1989.

Classification

The women were classified according to their serum ferritin levels on three consecutive occasions. Classification was performed into those with adequate and depleted iron stores using serum ferritin cut-off values of 12 µg/l. The cut-off value is widely used to define depleted iron stores throughout pregnancy.[3,6,7,11-13,15] Classification was restricted to GW 28-36. In that part of gestation, serum ferritin reaches steady state values, supporting the use of a single cut-off value for all three sampling occasions. Since ferritin levels may also increase during inflammatory states, we excluded 21 women exhibiting one or more CRP values above the locally used reference value of <0.8 mg/l. We prefer to use this CRP cut-off value to guarantee the exclusion of every subject with an acute-phase response, although it reduces sample size.

The remaining 61 women were classified into those having depleted iron stores (i.e. ferritin ≤12 µg/l; n=19) at GW 28, GW 32 ad GW 36 and those having adequate stores (i.e. ferritin >12 µg/l; n=22) at all three occasions. The consistent classification of women in the adequate group on three consecutive occasions ensures the maintenance of adequate iron stores during 3 months of gestation (from GW 28 to 36). In absence of deteriorating iron status, influence of increased erythropoiesis and/or placental TfR expression on sTfR levels can be studied in this group. The consistent classification of women in the depleted group ensures the persistence of depleted iron stores during 3 months of gestation. These women are at high risk of deteriorating iron status, with concurrent elevations of sTfR.

The consistent classification of women in either the depleted or adequate group on three consecutive occasions, minimizes the confounding effects of irregular iron use on iron status within these groups. Women who did not fulfill these criteria were classified into an intermediate group (n=20). The inconsistency of their iron status is likely to be related to the irregular intake of iron supplements and prompted us to exclude them from evaluation as a well-defined group.

Quality control

Two commercially available quality control samples with low (mean 1.45 mg/l; target range 1.16-1.74, as stated by the manufacturer; Orion Diagnostics) and high (5.71 mg/l; target range 4.57-6.85) were used for establishment of the within-run and between-run precisions. The within-run precision (6 analyses) amounted to: 0.91% (at a mean of 1.49 mg/l) and 0.58% (at a mean of 5.79 mg/l). The between-run precisions of 6 runs (1 quality control sample per run) were: 1.97% (at a mean of 1.52 mg/l) and 0.84% (at a mean of 5.98 mg/l).

Statistical analyses

Ferritin and sTfR distributions proved skewed and were approximated towards normal distribution after logarithmic transformation. sTfR and ferritin changes throughout gestation for the total population, as well as for the adequate and depleted groups separately (GW 28-postpartum), were analyzed with Repeated Measures ANOVA. Post-hoc analyses were performed with the Student's T-test for paired samples, corrected for multiple testing to minimize type I errors.

Between-group differences of clinical characteristics were analyzed with the Student's T-test for independent samples (means) or chi square test (frequencies). Differences in sTfR and ferritin levels in the adequate group compared to the depleted group were analyzed with the Student's T-test for independent samples. $p<0.05$ was considered significant.

We calculated 95% reference intervals in the adequate group for the purpose of this study, although it is recommended to base them on data from at least 40 subjects.[16] The sTfR upper reference limit of the adequate group was used as cut-off levels for the evaluation of sTfR levels in the depleted group. Percentage of women with above normal sTfR values was calculated.

RESULTS

Clinical characteristics

The women were 28.3±5.9 years old at entry and 41.5% were nulliparous. The mean (sd) birth weight amounted to 3,238 (306) g and mean gestational age at delivery was 39.8 (1.1) weeks. Women with adequate iron stores at GW 28, 32 and 36 were frequently more nulliparous (77.3%) than women with depleted stores (42.1%, $p<0.05$).

sTfR and ferritin in the total population

Repeated Measures ANOVA showed significant changes over time for both sTfR and ferritin ($p<0.0001$). sTfR levels increased during pregnancy and decreased postpartum (Table 1). Postpartum levels of sTfR proved higher compared to levels at GW 10, 16 and 20. Ferritin decreased until GW 28, to reach steady state levels up to GW 36 at least. Postpartum ferritin levels were higher compared to GW 36, but below levels of GW 10 and 16 (Table 1).

Table 1. Soluble TfR and serum ferritin levels in 82 women during uncomplicated pregnancy and at 14.5 weeks postpartum.

GA[a] (wks)	sTfR[c] (mg/l)	Ferritin (µg/l)
10.0± 3.3	1.31 (0.79-2.20)	42.4 (9.1-197.4)
16.1± 0.5	1.36 (0.77-2.37)	39.0 (10.4-145.7) [1]
20.3± 0.6	1.50 (0.86-2.60) [1]	24.7 (6.1-100.3) [1]
24.2± 0.6	1.70 (0.97-2.99) [1]	17.3 (5.4- 55.3) [1]
28.2± 0.4	1.86 (1.00-3.49) [1]	14.6 (4.8- 44.9) [1]
32.3± 0.5	1.96 (1.02-3.76)	14.5 (4.6- 46.4)
36.2± 0.5	2.17 (1.07-4.41) [1]	14.6 (4.4- 48.3)
14.5± 1.9 [b]	1.76 (1.34-3.17) [1,2]	23.1 (4.9-109.3) [1,3]

[a] GA, gestational age at blood sampling; [b] postpartum blood sampling; [c] sTfR, plasma soluble transferrin receptor.

GA data represent means±SD. sTfR and ferritin data represent geometric means (95% reference interval). Within-group differences of sTfR and ferritin were analyzed by ANOVA for Repeated Measures, followed by Student's T-tests for paired samples with correction for type I errors. Repeated Measures ANOVA showed significant changes over time for both sTfR and ferritin ($p<0.0001$). Post-hoc Student's T-test for paired samples [1]Significantly different from previous level; [2]Postpartum sTfR levels were significantly different from levels at 10, 16 and 20 wks ; [3]Postpartum ferritin levels were significantly different from levels at 10 wks and 16 wks.

sTfR and ferritin in the depleted and adequate group

sTfR in the adequate group did not change over time ($p=0.509$), in contrast to the depleted group ($p<0.001$) (Figure 1A). Post-hoc analyses revealed that sTfR in the depleted group increased from GW 32 to 36 ($p<0.001$) and decreased from GW 36 to 14.5 weeks postpartum ($p=0.003$). Between-group differences showed higher sTfR in the depleted group at GW 32 and 36 (both $p<0.001$). At 14.5 weeks postpartum the sTfR difference between both groups disappeared (Figure 1B).

Ferritin in the adequate group did not change over time. There was a significant change in the depleted group ($p=0.049$). There was, however, no change over time after excluding postpartum levels from the Repeated Measures ANOVA ($p=0.599$). Classification resulted in higher serum ferritin levels (geometric mean) in the adequate group compared with the depleted group; 23.5 *versus* 8.3 µg/l, 24.1 *versus* 7.9 µg/l and 26.5 *versus* 8.0 µg/l for

GW 28, 32 and 36, respectively (all p<0.001). Postpartum ferritin levels remained higher in women maintaining adequate iron stores during pregnancy compared to women who developed depleted stores (27.1 *versus* 13.0 µg/l; p=0.004).

sTfR values in the adequate group

The geometric mean (95% reference interval) of sTfR values of the adequate group amounted to 1.64 mg/l (0.90-2.98; n=21) at GW 28, 1.64 mg/l (0.93-2.91; n=22) at GW 32, and 1.70 mg/l (1.05-2.76; n=21) at GW 36 and 1.60 mg/l (0.18-3.15; n=20) postpartum. Percentage women with depleted iron stores that exceeded the cut-off values amounted 15.8% (GW 28), 15.8% (GW 32), 55.6% (GW 36) and 0.0% (14.5 weeks postpartum).

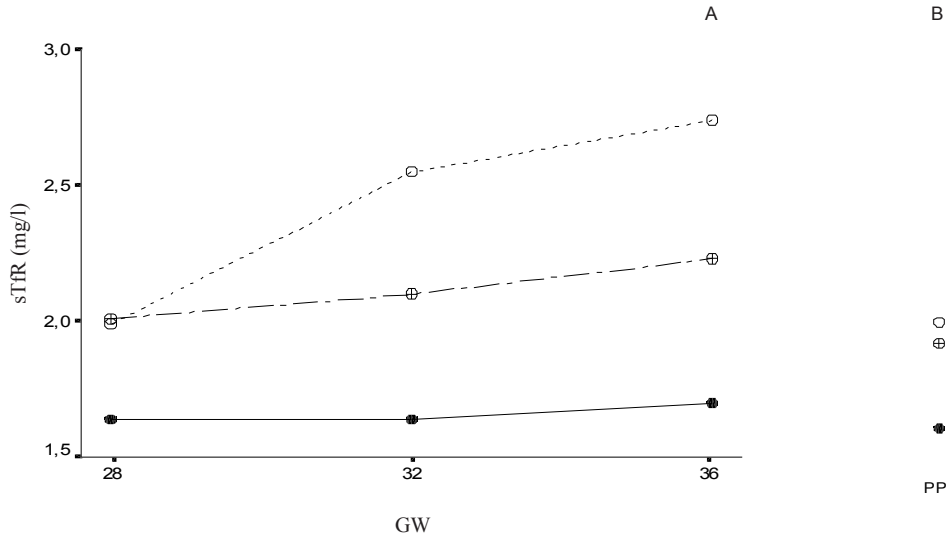

Figure 1. Courses of sTfR (A) and postpartum levels (B) in healthy pregnant women classified according to iron stores.
Data in geometric means. GW, gestational week at blood sampling. Women were classified on the basis of ferritin cut-off value of 12 µg/l for GW 28, 32 and 36, into 22 women with adequate iron stores (●), 19 women with depleted iron stores (○) and 20 women in the intermediate group (⊗). All had normal C-reactive protein.

DISCUSSION

We studied the course of sTfR during uncomplicated pregnancy. sTfR courses of women with persistently depleted iron stores at GW 28-36 were compared to counterparts maintaining adequate iron stores. Depletion of iron stores was assessed by using the widely recognized ferritin cut-off value of 12 µg/l. Ferritin is known to reach steady state values in that period (Table 1).

We found that sTfR increases during pregnancy and decreases postpartum, which is consistent with data of others.[6,7,11-13] Recently, Townsend and Drakesmith[8] proposed a model addressing the effect of sTfR on crypt cells and reticuloendothelial cells. By binding of the HFE protein on the membranes of these cells, sTfR may participate in programming the enterocyte to increase dietary iron absorption and the reticuloendothelial cell to increase iron export.[8] In this manner, sTfR may be involved in the maternal adaptation to higher iron needs in pregnancy.

Substantial sTfR differences were found between women with persistent depleted iron stores compared to women maintaining adequate iron stores. sTfR levels in the adequate group proved almost constant from GW 28 to 36. Apparently, increasing erythropoiesis and/or placental TfR expression do not affect sTfR levels in this period of gestation. In contrast, we observed a steep increase of sTfR in the depleted group (Figure 1A), resulting in higher levels at GW 32 and GW 36 as compared to the adequate group. Moreover, we observed an increasing percentage of women in the depleted group with above normal sTfR levels with increasing duration of pregnancy. Deteriorating iron status seems therefore to be the principal factor that causes the sTfR increase in this group. These findings suggest that sTfR in this period of pregnancy could detect those who develop iron deficiency beyond the depletion of stores. The upper limit of sTfR reference values of an adequate group in the third trimester can be used as a cut-off to further define iron status in depleted subjects. Since the presented reference ranges were based on only 22 subjects, these cut-offs should be subject of further investigation in larger study groups.

Two cross-sectional studies[7,13] with women who had ferritin ≥12 µg/l reported that sTfR increases in the third trimester. Carriaga *et al.*[7] reported a 12% increase from early to late third trimester and Choi *et al*.[13] noted a 20.9% increase from second to third trimester. These figures contrast with the constancy of sTfR levels in our women who had consistent ferritin levels ≥12 µg/l from GW 28-36. The discrepancy is likely to derive from our exclusion of women who in this study were classified in the 'intermediate' group. This sizeable group comprised approximately 33% of our study population and showed an appreciable 10.9% sTfR increase in the third trimester (Figure 1A). During prolonged observation these women exhibit ferritin levels that are occasionally above and below 12 µg/l. Possible explanations for the observed ferritin fluctuations around the employed cut-off value are irregular use of iron supplements or genuine ferritin biological variation.[17,18]

In conclusion, our findings suggest that sTfR measurements after GW 28 of pregnancy are able to detect iron deficiency beyond depletion of stores.

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Summary, the present state of the art, and epilogue

ABBREVATIONS

AA: Arachidonic acid (20:4ω6)
ACKR: Atypical chemokine receptor
ALA: α-Linoleic acid (18:3ω3)
BHMT: Betaine-homocysteine *S*-methyltransferase
CRP: C-reactive protein
CT: Cytotrophoblasts, cytotrophoblast cells
DAMPs: Damage-associated molecular patterns
DARC: Duffy antigen receptor for chemokines
EPA. Eicosapentaenoic acid (20:5ω3)
EV: Extra-cellular vesicle
DHA: Docosahexaenoic acid (22:6ω3)
GW: Gestational week
Hcy: Homocysteine
HFE: Human hemochromatosis
IL-8: Interleukin-8
LA: Linoleic acid (18:2ω6)
LDL: Low-density lipoprotein
LCP: Long-chain polyunsaturated fatty acids
MTHFR: Methylenetetrahydrofolate reductase
PC: Phosphatidylcholine
PE: Phosphatidylethanolamine
PEMT: Phosphatidylethanolamine *N*-methyltransferase
PGI2/3: Prostacyclin I2/3
PP: Postpartum
sTfR: soluble transferrin receptor
SNP: Single nucleotide polymorphism
SIRS: Systemic inflammatory response syndrome
ST: Syncytiotrophoblast
THF: Tetrahydrofolate
TNF-α: Tumor Necrosis Factor-a
TFR: Transferrin receptor
UPR: Unfolded protein response
Th2: Thelper2 cells
TxA2/3: Thromboxane A2/3
vWF: von Willebrand Factor
UA: Umbilical arteries
UV: Umbilical vein
VLDL: Very low-density lipoprotein

SUMMARY

Chapter 1: General introduction

In Chapter 1.1 we discuss the physiology of a normal healthy pregnancy and the pathophysiology of preeclampsia. Maternal and gestational tissues are subject to a tightly regulated and coordinated program. Maternal tissues, including decidual tissues, white adipose tissue and the cervix each undergo developmental trajectories of their own. The placenta and fetal membranes develop, mature, and age in a temporally and spatially coordinated manner. The functional layer of the placenta, the multinucleated syncytiotrophoblast (ST), acts as a true barrier. The ST is a syncytium with many nuclei that results from multiple fusions of mononucleated cytotrophoblast cells. Due to its syncytial nature, the ST is resistant to apoptosis. Apoptosis would spread through the entire functional layer and thereby render the condition incompatible with pregnancy continuation. The ST ends its lifespan (even when stressed) by placental delivery at the final stage of human parturition. The underlying mononucleated cytotrophoblast cells (CT) act as stem cells. CT proliferate continuously, differentiate and fuse with the ST to supply new organelles, like nuclei and mitochondria. The terminally differentiated ST is unable to dilute its cellular waste (such as defective organelles, modified proteins) by division and relies on effective clearing, degradative machineries. We argue that when, towards the end of pregnancy, this capacity falls short, cellular waste becomes disposed in the maternal circulation. Remarkably, the ST encounters its most intensive functional period towards the end of its lifespan, which coincides with the time that adaptive stress responses and degradative capacity start losing efficacy. This induces a well-controlled, physiologically-induced stress, notably of oxidative nature, that results in an aged placenta towards terms. The physiological ST stress is 'communicated' to the mother and is employed for functional purposes. The release of so-called extra-cellular vesicles (EVs) is an inventive way for inter-cellular communication that allows transfer of numerous components (e.g. lipids, proteins, mRNA species) as opposed to single molecules. The ST is a vivid releaser of EVs. Exosome and micro-vesicle release is shared with other cell types, but the release of larger-sized macro-vesicles is unique to the placenta. Macro-vesicles have been implicated in maternal physiological adaptations, e.g. cardiovascular adaptations, from the first trimester on. Exosomes are immuno-suppressive and contribute for instance to the Th2 bias of normal pregnancy. Via the release of pro-inflammatory micro-vesicles and "damage-associated molecular patterns" (DAMPs; released by stressed cells and also named "danger signals"), the aging and stressed ST may trigger the mother to mobilize her resources (heighten maternal inflammatory response) and prepare her for the upcoming parturition (activation of maternal immune cells in the decidua, myometrium and cervix). In addition, micro-vesicles carry pro-coagulant factors, with the intention to minimize blood loss during delivery. At a certain stage, maternal substrate supply cannot longer meet the increasing fetal oxygen/metabolic needs. The fetus will start to experience stress, even in normal uncomplicated pregnancies. The onset of human parturition is a synchronized process, integrating various inputs from the fetus, including

fetal stress, growth and maturation. Finalization of the gestational trajectory is considered successful when delivery is perfectly timed, resulting in the birth of a fully developed newborn.

In preeclamptic pregnancies, the normal aging and stress trajectory of the ST is disturbed. This may be induced by stresses of different nature, severity and timing. Increased oxidative stress as a consequence of poor placentation is often present early in gestation and responsible for most cases of severe, "early-onset preeclampsia" with intra-uterine growth restriction. Metabolically-triggered inflammation within the ST, induced by an insulin-resistant obese state, often occurs in later pregnancy and associates with "late-onset preeclampsia". The adaptive ST stress responses aim to minimize the consequences of experienced stresses. Decisive responses are autophagy (centrally positioned because it eliminates damaged structures) and the "unfolded protein response" (UPR). Both responses are found to be challenged and can become compromised, in particular in placentas of severely preeclamptic pregnancies. Mild ST stress accelerates the normal aging process, causing a relatively older ST for the actual stage of pregnancy. This leads to amplification of normal late gestational events (excessive maternal inflammatory response and angiogenic/hemostatic imbalance) with the onset of proteinuria and hypertension ("term preeclampsia"). It coincides with increments in the release of pro-inflammatory micro-vesicles and DAMPs and the disposal of waste into the maternal circulation, as seen in late normal pregnancy. The maternal manifestations are rather mild and uniform, and sometimes even hard to differentiate from late pregnancy changes. Profound stress may result in severe disease and may onset secondary stresses. For instance, excessive oxidative stress can induce severe endoplasmic reticulum stress. Micro-vesicle and DAMP release is intensified and display changes in cargo and signature, respectively. A compromised degradative capacity results in more waste disposal into the mother, which may be of different signature, such as toxic amyloid aggregates or damaged mitochondrial proteins. The maternal manifestations are more severe and less uniform.

The severity, nature and timing of the stress, and the coping efficiencies of the ST and the mother ultimately determine the maternal manifestations of the syndrome of preeclampsia. The true danger of preeclampsia lies in the fact that the loss of ST homeostasis (which normally leads to a regulated cell death scenario) is not necessarily synchronized to parturition onset and that the harmful ST remains *in situ*.

Chapter 2: Genetics

The way the mother copes with the stresses of a preeclamptic placenta is the sum of many factors of either intrinsic, extrinsic (lifestyle) or racial nature. Any (adverse) factor, even of modest nature, may have impact. In Chapter 2 we investigated three single nucleotide polymorphisms (SNPs) for their association with preeclampsia. Our study population comprised the inhabitants of the island of Curaçao. This is a population of predominantly West African descent with ample Caucasian admixture. The first investigated SNP is the Duffy negative phenotype (also referred to as the Duffy-null trait) (Chapter 2.1). The Duffy negative phenotype is rather common in Curaçao (estimated prevalence of 37%).

The second SNP is the thermo-labile MTHFR (methylenetetrahydrofolate reductase) variant, commonly present in Caucasian populations with less prevalence in African populations or their descendants (Chapter 2.2) . The final SNP investigated is the C282Y mutation in the *HFE* (human hemochromatosis) gene. The C282Y-allele has a very low prevalence amongst West Africans, but heterozygosity for the *HFE* C282Y mutation is common in Caucasian populations (Chapter 2.3).

Chapter 2.1. The Duffy blood group antigens Fy^a and Fy^b are the expression products of the FY*A and FY*B alleles. Sub-Saharan Africans carry a FY*B allele with a single T to C substitution at nucleotide -67, which disrupts a binding site of the erythroid transcription factor. The SNP gives rise to the Duffy negative phenotype. Individuals with the Duffy negative phenotype do not express Duffy blood group antigens on erythroid cells. The Duffy antigen proved identical to a non-signaling, high-affinity, promiscuous (e.g. interleukin-8) chemokine-receptor expressed on erythrocytes. This receptor was designated the Duffy antigen receptor of chemokines (DARC). In a retrospective study design we phenotyped 72 women with a history of preeclampsia and 55 counterparts with uncomplicated pregnancies, all of West-African descent living in the island of Curaçao. Women with a history of preeclampsia had a higher Duffy negative phenotype frequency compared with women with a history of uncomplicated pregnancies, i.e. 52.8% versus 27.3%, respectively; odds ratio 2.98; 95% CI 1.40-6.32, p=0.004. Erythroid DARC may act as a sink for circulating chemokines, like interleukin-8 (IL-8), especially at higher blood levels. IL-8 bound to erythroid DARC is unable to activate neutrophils. Lacking erythroid IL-8 binding capacity increases the accessibility of IL-8 to other receptors on e.g. immune cells, which may be of importance in the pathophysiology of preeclampsia.

In Chapter 2.2 we studied the thermo-labile MTHFR-variant. MTHFR is a central enzyme in one-carbon metabolism, i.e. a fundamental biochemical cycle that provides methyl groups for important methylation reactions. Proper functioning of enzymes in one-carbon metabolism is dependent on genetic and environmental (e.g. dietary) factors. The thermo-labile MTHFR variant is common in Caucasian populations, but has low prevalence in African populations. MTHFR reduces 5, 10-methylene tetrahydrofolate (THF) to 5-methyl THF, which is one of the important methyl-donors in one-carbon metabolism. By donating its methyl-group, 5-methyl THF methylates homocysteine to methionine, and is thereby a determinant of the plasma homocysteine level. A common C to T substitution at nucleotide -677 (named thermo-labile or MTHFR C677T variant) reduces MTHFR activity, especially at low folate status. Subjects homozygous for the MTHFR C677T variant (TT genotype) display an increased plasma homocysteine response at low folate status. High plasma homocysteine has been reported in preeclampsia and is related to endothelial damage. Therefore, we genotyped, in a retrospective study design, 89 women with a history of preeclampsia and 89 race- and parity-matched controls with uncomplicated pregnancies. We also genotyped 49 children born to preeclamptic women and 49 children born to healthy control women. All were Caribbean women of West

African descent with ample Caucasian admixture. We found no significant case-control differences in MTHFR C677T genotype and T-allele frequencies between mothers, their children and the mother/child combinations. A coincidental finding was that women with a history of mild preeclampsia had higher MTHFR C677T genotype and T-allele frequencies than women with a history of severe preeclampsia. Similar maternal C677T MTHFR TT genotype frequencies in preeclampsia and controls is in agreement with findings in white Americans, Dutch Caucasians and Zimbabwean women, but contrasts with reported higher frequencies of the C677T MTHFR TT genotype in Japanese, Jewish and European women with preeclampsia.

Different underlying etiologies and pathophysiological cascades leading to the single syndrome of preeclampsia may help explain the apparent discrepancies in the observed associations between MTHFR C677T and preeclampsia. Some evidence for this may come from the observed higher frequency of the MTHFR C677T variant in women with a history of mild versus severe preeclampsia, where different etiologies/pathophysiological cascades are suspected.

In Chapter 2.3 we investigated the C282Y mutation in the *HFE* gene, which encodes the HFE protein. This protein is involved in the uptake of iron from the diet. Homozygosity for the C282Y mutation explains the majority of cases with hereditary hemochromatosis (HH). Heterozygous C282Y individuals do not usually develop clinical manifestations of HH, but they have higher body iron stores. Moderate excessive body iron may be involved in iron-mediated lipid oxidation, e.g. low-density lipoprotein (LDL)-oxidation, suggested to promote atherogenesis. A case-control study conducted in Curaçao demonstrated a higher frequency of C282Y heterozygosity in patients with coronary heart disease as compared to controls, 9.6% versus 1.2%, respectively. As preeclampsia shares risk factors with atherosclerosis, we investigated, in a retrospective study design, whether preeclampsia associates with C282Y heterozygosity. For this, we genotyped 74 women with a history of preeclampsia and 84 controls. C282Y heterozygosity amounted to 4.1% (3/74) in women with previous preeclampsia and 2.4% (2/84) in controls (p=0.66). The prevalence was in general agreement with an admixture of West African women (Ghana populations do not carry the C282Y allele) and Caucasians, notably Dutch (7.2% C282Y heterozygosity). We concluded that our low-prevalent study population shows no evidence for an association between preeclampsia and C282Y heterozygosity. This is agreement with the finding of similar C282Y genotype frequencies in preeclamptic and control women in a Dutch population with heterozygosity prevalence of ~11% (Senden, 2004).

Chapter 3: Immunology

It has become evident that the maternal inflammatory response of a normal pregnancy becomes exaggerated during a preeclamptic pregnancy. We were among the first to report on higher levels of IL-8 in preeclamptic women in a small sample-sized, matched case-control study.

As neutrophils are activated in preeclampsia, we measured serum levels of the neutrophil chemo-attractant and activator IL-8 in 13 preeclamptic women and 13 normotensive, non-proteinuric pregnant controls. We concomitantly measured serum tumor necrosis factor-α (TNF-α) levels, because of its involvement in IL-8 production. Most women were phenotyped for the Duffy blood antigen, which is involved in the binding of circulating IL-8 (see Chapter 2.1). To confirm that preeclampsia is an endothelial cell disorder, we also determined plasma von Willebrand factor (vWF) as a general marker of endothelial dysfunction. Our study group was exclusively composed of African-Caribbean women, without evident signs of infection or labor, and matched for parity (null versus multipara) and time of blood sampling (± one week). We found that preeclamptic women had higher IL-8 ($p=0.0033$), TNF-α ($p=0.0067$) and vWF ($p=0.019$), as compared to control women. IL-8 levels correlated positively with TNF-α and vWF (entire study group). IL-8 levels varied widely among the preeclamptic women, but all of them had higher IL-8 levels compared to their matched controls. Almost all (9/10) women in the preeclamptic group were phenotyped as Duffy negative, as compared to only 3/11 women in the control group ($p=0.0037$). Higher serum IL-8 levels in preeclamptic women may be the result of increased production (secondary to increased TNF-α levels) and/or reduced clearance (related to the high frequency of the Duffy negative phenotype). The positive correlation between serum IL-8 and vWF (a marker of endothelial dysfunction) suggest that high serum IL-8 levels in pregnancy are unfavorable. The high frequency of the Duffy negative phenotype in the preeclamptic group prompted us to conduct the larger retrospective study described in Chapter 2.1.

Chapter 4: Nutrition

In Chapter 4 we studied the influence in pregnancy and preeclampsia of long-chain polyunsaturated fatty acids (Chapter 4.1), nutrients involved in one-carbon metabolism (Chapter 4.2) and iron (Chapter 4.3).

Chapter 4.1 Long-chain polyunsaturated fatty acids (LCP). LCP are fatty acids with ≥20 carbon atoms and ≥3 double bonds. LCP are an important class of fatty acids, since they are involved in a variety of biological processes, e.g. as precursors of eicosanoids and determinants of the physical-chemical properties of membranes. LCP derive from the diet, or are synthesized from the "parent" essential fatty acids, linoleic acid (LA; 18:2ω6) and α-linoleic acid (ALA; 18:3ω3) yielding LCPω6 (arachidonic acid, AA;) and LCPω3 (eicosapentaenoic acid, EPA, docosahexaenoic acid, DHA), respectively. The synthesis rate of notably EPA and DHA in humans is believed to be rather low. Consequently, the majority of LCPω3 must derive from the diet, with EPA (20:5ω3) and DHA (22:6ω3) originating especially from marine based foods, meat and eggs, and AA (20:4ω6) from meat, poultry, and eggs.

Adequate LCP status is notably important during pregnancy as AA, 22:4ω6 and DHA are amongst the quantitatively most important fatty acids in brain. There is little evidence that fetal LCP synthesis is sufficient to cover the high intrauterine needs. Consequently,

the fetus relies heavily on maternal LCP supply, rendering the LCP in this period to be conditionally essential.

In Chapter 4.1.1 we investigated the LCP composition in preeclamptic and normotensive pregnancies. In Chapter 4.1.2 we studied in an open label trial design the effect of low-dose LCPω3 supplementation of healthy pregnant women on the umbilical LCP status at birth.

In Chapter 4.1.1 we studied the LCP composition of maternal and umbilical platelets and of umbilical veins (UV) and umbilical arteries (UA). The study group was composed of 27 preeclamptic and 24 normotensive women. Preeclampsia is characterized by enhanced platelet aggregation and vasoconstriction, which may at least in part be caused by abnormal eicosanoid production with elevated platelet-derived thromboxane A2 (TxA2) relative to endothelial-derived prostacyclin I2 (PGI2). AA and EPA are precursors of eicosanoids of the 2-series and 3-series, respectively. Endothelial (AA-derived) PGI2 and endothelial (EPA-derived) PGI3 are equipotent vasodilators and platelet inhibitors. In contrast, AA-derived TxA2 in platelets is a more potent vasoconstrictor and platelet aggregator than EPA-derived TxA3. Therefore, we were interested in the LCP status, and especially the AA and EPA contents of platelets, of preeclamptic and control women. The women in our study population lived in the island of Curaçao and were mostly of mixed African-Western descent. Their dietary habits were essentially Western, characterized by a high intake of ω6 fatty acids (notably from LA-rich vegetable oils) and a low intake of ω3 fatty acids (from ALA-rich vegetable oils and fish). The ensuing ω6 predominance may favor platelet aggregation and vasoconstriction. In addition, fetal LCP status seems marginal even under normal conditions, because of the lower LCP status of UA, as compared to UV. The seemingly uneven LCP distribution may become accentuated in preeclampsia. We therefore determined the LCP composition of both UA and UV in preeclamptic and control pregnancies. Several key findings are discussed here. First, platelets of preeclamptic women contained lower amounts of EPA and a higher AA/EPA ratio, compared to normotensive women. Although differences were rather small, this finding might be in line with a "TxA2 over TxA3 dominance" and the state of platelet aggregation and vasoconstriction seen in preeclampsia. Second, compared to controls, UV and especially UA of preeclamptic pregnancies contained lower percentages fatty acids of both the ω3- and the ω6-series, including their LCP members. Lower LCPω3 and LCPω6 in preeclamptic UA may reflect impaired fetal LCP accrual, causing insufficient LCP availability for incorporation into fetal tissues located most distal from the placental supply (e.g. UA). The lower fetal ω3 and ω6 status of mothers with adequate ω6 fatty acid intake argues in favor of insufficient trans-placental transport, and may thereby be related to preeclampsia pathogenesis. Third, we found a higher 20:3ω9/AA ratio in preeclamptic UA. Mead acid (20:3ω9) is a marker of essential fatty acid deficiency. In the literature, the 20:3ω9/AA ratio has been found to relate inversely to UA prostacyclin production. A higher 20:3ω9/AA ratio may consequently be in line with the lower prostacyclin production by preeclamptic UA. In summary, the platelets of women with preeclampsia in Curaçao exhibit somewhat higher AA/EPA ratio, while the umbilical vessels of their fetuses harbor low relative amounts of both LCPω3 and LCPω6.

In Chapter 4.1.2 we studied the effect of three low-dose fish oil supplements, administered during uncomplicated pregnancies, on fetal LCP status at birth. Fetal LCP status was assessed by measurements of the LCP compositions of UA and UV. The fetus relies strongly on maternal LCP supply. It is nowadays widely accepted that the current maternal LCP, notably LCPω3 status, is insufficient to prevent maternal LCPω3 depletion during pregnancy, suggesting possible adverse effects in both mother and fetus. LCPω3 supplementation studies, notably those employing high doses, have been conducted to augment maternal and neonatal LCPω3 status and improve clinical outcomes such as preeclampsia and birth weight. It has been shown that high dose LCP (2,200-2,600 mg LCPω3 daily) improves neonatal LCPω3 status with a concomitant reduction of LCPω6. Reduction of fetal LCPω6 status is considered undesirable, since a reduction of its main member AA is related to retarded growth. Therefore, we investigated, in an open label trial design, the effect of three low-dose LCPω3 supplements, either as fish oil capsules ("fish-1" group; 336 mg/d LCPω3, n=15, "fish-3" group; 1,008 mg/d LCPω3, n=20) or as a milk-based supplement ("Mum" group; 528 mg/d LCPω3, n=24). Fifty-seven untreated women served as controls. Supplements were administered from the second trimester to delivery. The mean supplement duration was 19 weeks. The most important outcome of this study was that daily supplementation with 528 and 1,008 mg LCPω3 from second trimester on significantly augments neonatal LCPω3 status, without affecting the LCPω6 status to an appreciable extent. More specific, compared to untreated controls, the Mum (528 mg/d) group had higher EPA, 22:5ω3, DHA, LCPω3 and DHA/22:5ω6 ratio in both UV and UA. The fish-3 (1,008 mg/d) group had higher 20:3ω6, LCPω3 and DHA/22:5ω6 ratio in UV and higher 22:5ω3, DHA, LCPω3 and DHA/22:5ω6 ratio in UA. A dose of 336 mg/d did not show an effect when compared with untreated controls. The incorporation efficiencies of LCPω3 in the Mum and fish-3 groups were similar to those observed following supplementation with 2,200 mg/d LCPω3 in pregnant Danish women. Incorporation of LCPω3 in umbilical vessel walls of the pregnancies in Curaçao seemed dose-dependent, but may reach a steady state from 528 mg/d. Although statistically insignificant, the lower-dosed milk-based supplement performed better than the higher-dosed fish-oil supplement. This may be caused by the additional daily ingestion of 178 mg 18:3ω3 from the milk-based supplement. Its intake favorably increased the dietary 18:3ω3/18:2ω6 ratio and may thereby have augmented the conversion of 18:3ω3 to EPA and DHA. Improved conversion may, on its turn, have been driven by the gestational hormonal milieu. In the UV of the Fish-3 group we found an unexpected increase of 20:3ω6, which is the precursor of the seemingly favorable eicosanoids of the 1-series. It is in this context of note that high LCPω3 status of Inuits coincides with higher 20:3ω6 status. UV 20:3ω6 was also found to be the best indicator of intrauterine fetal growth. We have previously reported on low 20:3ω6 in preeclamptic UV. In summary, a 500-1,000 mg LCPω3/d supplement, taken either as fish oil capsules or as a milk-based supplement effectively increased fetal LCPω3 status, without affecting LCPω6 status.

Chapter 4.2 One-carbon metabolism. In Chapter 4.2, we studied the relationships of plasma total homocysteine levels (tHcy) with plasma folate and plasma betaine throughout normal pregnancy (4.2.1) and between plasma tHcy and plasma folate during preeclamptic pregnancy (Chapter 4.2.2). Studying these relationships provides insight into the relative contributions of folate and betaine as methyl-donors to intracellular homocysteine remethylation, and thereby, to important maternal methylation reactions as part of the methionine cycle.

Folate-dependent homocysteine remethylation takes place in almost all tissues, catalyzed by methionine synthase, with MTHFR-derived 5-methyl THF as methyl-donor and vitamin B_{12} as cofactor. By contrast, an alternative homocysteine remethylation route uses betaine as methyl-donor, is catalyzed by betaine-homocysteine *S*-methyltransferase (BHMT), and notably takes place in the liver and kidney. To be complete, intracellular homocysteine can also become metabolized to cysteine in the vitamin B_6-dependent transsulfuration pathway located in the liver and kidneys. In non-pregnant individuals, plasma folate is the main determinant of plasma tHcy. We sought to find whether the high nutritional demands in human pregnancy leads to adaptations of the homocysteine-methionine metabolic cycle.

Chapter 4.2.1. Many studies reported on lower plasma concentrations of tHcy in pregnant women compared to non-pregnant women, with lowest concentrations found in the second trimester. This finding suggests the presence of an "effective homocysteine-lowering mechanism" in normal pregnancy. BHMT is known to be hormonally (e.g. estrogens) regulated in rats. A possible role for the BHMT pathway was even more suspected by the finding that low plasma folate did not lead to higher tHcy in pregnant women. No study had until then addressed the possible roles of betaine and its precursor choline as alternative methyl-donors to folate. We investigated the courses of plasma choline, betaine, folate, and vitamin B_{12} during normal human pregnancy and their relations to plasma tHcy. Blood samples were obtained monthly. The initial samples were taken at 9 gestational weeks (GW) and the last at 3 months postpartum (PP). The study population comprised 50 women of West African descent with uneventful pregnancies. Most of the subjects took a folic acid supplement on an irregular basis. We found that plasma choline (geometric means; 95% CI) increased continuously during pregnancy, from 6.6 (4.5, 9.7) µmol/L at GW 9 to 10.8 (7.4, 15.6) µmol/L at GW 36, which is in general agreement with the finding of others. Plasma betaine decreased in first half of pregnancy, from 16.3 (8.6, 30.8) µmol/L at GW 9 to 10.3 (6.6, 16.2) µmol/L at GW 20, and to remain constant thereafter. We confirmed a reduction of plasma tHcy with lowest concentrations in the second trimester. Three months after delivery, plasma tHcy and betaine were higher, whereas plasma folate and choline were lower, as compared to the last sampling during pregnancy. From GW 16 onwards, we observed an inverse relation between plasma tHcy and betaine (except for GW 32). Multiple regression analyses showed that plasma folate was the strongest predictor of tHcy at GW 9 and after delivery, but that plasma betaine was the strongest predictor of plasma tHcy at GW 20, 24, 28 and 36. The increase of plasma choline levels ensures choline availability for

placental transfer. Both mobilization of maternal hepatic choline stores and enhanced *de novo* choline synthesis, may explain the observed rise of maternal plasma choline. The appearance of an inverse relationship between plasma tHcy and betaine was a notable and novel finding. This gestational relationship was stronger than the relationship in non-pregnant individuals, even at low folate status. It stresses the importance of betaine in one-carbon metabolism during pregnancy, and aligns with higher (hormonally driven) BHMT activity. The almost halving of plasma betaine during pregnancy may be explained by enhanced consumption via the BHMT pathway, placental transfer, or reduced synthesis from choline, the latter may reflect a sub-optimal choline status. Our results emphasizes the importance of choline and betaine status during normal human pregnancy.

Chapter 4.2.2. In contrast to normal pregnant women, preeclamptic women have higher plasma homocysteine levels, which proved correlated to fibronectin, a marker of endothelial disorders. In a case-control study design, we determined third trimester plasma homocysteine, plasma folate and plasma vitamin B_{12} in 43 women with proven preeclampsia and in 43 controls that were matched for parity and pregnancy duration (sampled in GW 33). All participants lived in the island of Curaçao. We confirmed higher plasma homocysteine levels in preeclamptic women ($p<0.0001$), as reported by others. In addition, plasma vitamin B_{12} levels were slightly higher ($p=0.025$) in preeclamptic women, while plasma folate was similar. We found inverse relationships between plasma homocysteine and plasma folate in both preeclamptic ($r= -0.59$, $p<0.0001$) and control women ($r= -0.60$, $p<0.0001$). Less strong inverse relationships were found between plasma homocysteine and vitamin B_{12} in both preeclamptic ($r= -0.41$, $p=0.008$) and control women ($r= -0.35$, $p=0.020$). In healthy pregnant women, low plasma folate and vitamin B_{12} did not result in high homocysteine as noted by others. Unlike controls, preeclamptic women exhibited steep plasma homocysteine increases at low levels of plasma folate (below ~10 nmol/l) and at low vitamin B_{12} (below~ 200 pmol/l). During normal pregnancy, folate and vitamin B_{12} seem 'spared' for other processes, such as placental transfer. The steep increase of plasma homocysteine at low folate levels in preeclamptic women is similar to that of healthy non-pregnant subjects, as reported by our group. Our findings in preeclamptic women suggest high dependency of homocysteine metabolism on the folate- and vitamin B_{12}-dependent remethylatio.

Chapter 4.3 Iron. In Chapter 4.3 we investigated the value of the "soluble transferrin receptor" to identify pregnant women at risk of becoming iron deficient. Detection of iron deficiency in pregnancy is troublesome, because of hemodynamic adaptations and altered iron needs change most traditional indices, independent of iron status. The amount of stainable bone marrow iron is the 'gold standard', with serum ferritin as a strong and practical surrogate marker, because of its positive correlation with stainable bone marrow iron. The transferrin receptor (TfR) mediates cellular iron uptake and its surface expression becomes up-regulated in iron-deprived tissue. Its truncated fragment in the circulation (soluble TfR; sTfR) is proportional to the number of cellular surface receptors and reflects cellular iron needs.

We investigated the added value of the soluble transferrin receptor (sTfR) for assessing tissue iron deficiency in pregnancy. For this, blood was obtained from 82 apparently healthy pregnant women who were sampled from GW 10, 16, 20, 24, 28, 32, and 36 and at 14.5 weeks PP. The study population was recruited from a larger sample used for the establishment of clinical chemical reference values in pregnancy in the island of Curaçao. The women reported irregular iron use. Plasma sTfR and serum ferritin were measured and women with C-reactive protein (CRP) >0.8 mg/l were excluded. Nineteen women with ferritin levels ≤12 µg/l at GW 28, 32, and 36 were classified as iron depleted. Twenty-two women with ferritin levels >12 µg /l at all three occasions were classified as iron replete. The influences of increased erythropoiesis during pregnancy and/or placental TfR expression on sTfR levels could be studied in this latter group. In addition, the upper reference limit of sTfR values in this group was used for the establishment of sTfR cut off values. In the total study population, ferritin decreased until GW 28 to a reach steady state up to GW 36 and to increase PP. sTfR increased during pregnancy and decreased PP, consistent with previous reports. sTfR levels increased significantly in the iron-depleted women from GW 28 to GW 36, but remained constant in the adequate group. The percentage women in the depleted group with sTfR values above our established cut off values increased from 15.8% at GW 28/32 to 55.6% at GW 36. The relevance of the findings in these well-defined (strength of the study), small-sized study population (limitation of the study) are two-fold. First, the constancy of sTfR levels in the replete group indicates that increasing erythropoiesis and/or placental TfR expression have no effect on sTfR values at that period of gestation. Second, the observed steep increase in sTfR values in the depleted group together with the increase in the percentage women with sTfR value above cut off, indicate that more women develop tissue iron deficiency beyond depletion of iron stores. We conclude that sTfR may detect iron deficiency beyond depleted stores in the third trimester of pregnancy.

In later studies by others, the levels of sTfR alone, or as a ratio to log serum ferritin (ferritin index), were found to correlate with the severity of iron deficiency anemia in pregnancy. sTfR and the ferritin index are more useful than traditional parameters like MCV (mean corpuscular volume), MCHC (mean corpuscular hemoglobin concentration), or serum ferritin (Sharma, 2016). However, the value of the sTfR assay may lie primarily in the assessment of complicated anemic cases in pregnancy (Weyers, 2016).

THE PRESENT STATE OF THE ART

Many new data on the investigated subjects in this thesis became available after the publication of our studies or after their description in the unpublished manuscripts. The following short review describes the current state of the art on relevant information regarding “IL-8 and DARC”, “LCPω3 in pregnancy and preeclampsia” and “One-carbon metabolism”.

Interleukin-8 (IL-8) and the Duffy antigen receptor of chemokines (DARC)

Numerous later reports confirmed our findings of high maternal TNF-α and high IL-8 levels in preeclamptic pregnancies (**Chapter 3**). In a recent systematic review, CRP, TNF-α, IL-6 and IL-8 were considered to be prominent pro-inflammatory markers in preeclampsia from the second trimester onwards (Black, 2018).

Micro-vesicles and/or DAMPs, all released from a stressed preeclamptic placenta, have been studied as novel candidates (**Chapter 1**) to explain the higher circulating IL-8 levels in preeclampsia. Mitochondrial DAMPs can stimulate circulating neutrophils to produce and secrete IL-8. By acting both on maternal immune cells and endothelial cells, micro-vesicles-associated DAMPs can provoke pro-inflammatory cytokine production and increase endothelial cell permeability (Collett, 2018). Our suggestion (**Chapter 1**) that preeclampsia resembles the clinical systemic inflammatory response syndrome (SIRS), provoked by massive release of DAMPs, as in e.g. trauma patients, fits this concept. In this context, chemokine binding to erythroid DARC (~2,000 binding sites per erythrocyte) is believed to buffer excess chemokines during inflammatory conditions (Hansell, 2011). Lacking erythroid chemokine binding capacity may augment activation of circulating neutrophils, and their infiltration into e.g. the systemic arterial vasculature, causing vascular dysfunction.

To date, however, the exact clinical significance of erythrocyte DARC, now named "atypical chemokine receptor-1" (ACKR-1) remains to be fully delineated. The buffering capacity of erythrocyte DARC/ACKR-1 during inflammatory conditions is nowadays well accepted and confirmed *in vivo* (Mayr, 2008). Erythrocyte DARC/ACKR-1 only minimally degrades its cognate chemokines (Pruenster, 2009), unlike other atypical chemokine receptors. Erythroid DARC/ACKR1 may even release its chemokines under basal conditions (Hansell, 2011). Collectively, the primary function of erythrocyte DARC/ACKR-1 is currently thought to regulate chemokine availability, acting as a buffer in inflammatory states and as a depot/reservoir during basal states. Of note, DARC/ACKR-1 expression is not restricted to erythroid cells, but also takes place on the endothelium lining of post-capillary and collecting venules (Thiriot, 2017), even in Duffy negative individuals (specific erythroid loss). Endothelial DARC/AKCR-1 presents its cognate chemokines on the apical endothelial surface (Pruenster, 2009), and proved essential for chemokine-driven leukocyte trafficking (Horuk, 2015, Novitzky-Basso, 2012, Thiriot, 2017).

Given its buffering capacity during inflammatory conditions, the role of the Duffy negative phenotype was investigated in inflammatory disorders, notably sickle cell disease (Schnog, 2000, Afenyi-Annan, 2008, Mecabo, 2010, Nebor, 2010, Drasar, 2013, Araujo, 2015, Farawela, 2016). The majority of studies, however, did not find a significant impact of the Duffy negative phenotype on e.g. the severity of disease symptoms. The only solid link of the Duffy negative phenotype is that with ethnic benign neutropenia, a condition believed to have no clinical impact (Thobakgale, 2014). The buffering function of erythrocyte DARC/ACKR-1 is believed to be rather complex (Novitsky-Basso, 2012) and context-dependent. Erythrocyte DARC/ACKR-1 chemokine binding

is saturable, and its numerous (>20 CXC- and CC chemokines) cognate chemokines may bind with different affinities (Mei, 2010). At extensive inflammatory conditions, erythrocyte DARC/ACKR-1 binding capacity may simply be overwhelmed, in which case the presence or absence of its buffering capacity may no longer be of (clinical) significance. Therefore, the encountered association by us between the Duffy negative phenotype and preeclampsia suggests that inflammatory conditions in preeclampsia may fall within the functional range of erythroid DARC/ACKR-1, at which its presence or absence does indeed matter.

The Duffy negative phenotype confers resistance against *Plasmodium vivax* infection. These malarial parasites enter host erythrocytes via erythroid DARC/ACKR-1. Its knock-out by mutation is thought to be the evolutionary drive for its dissemination and fixation in sub-Saharan populations. Recently, Duchene et al. discovered another putative benefit of the Duffy null phenotype (Duchene, 2017). Neutrophils formed in DARC/ACKR-1 deficient bone marrow of chimeric mice displayed phenotypical changes, e.g. up-regulation of molecules for antimicrobial defense (Duchene, 2017). When endothelial DARC/ACKR-1 expression is preserved in these mice (mimicking the human Duffy null phenotype), these neutrophils traffic easily into various tissues, with the "selection advantage" of increased innate immune tissue surveillance (Duchene, 2017, Palmblad, 2018). In case of a similar scenario in humans, it may explain the close link between the Duffy negative phenotype and ethnic benign neutropenia (Duchene, 2017, Palmblad, 2018). A downside of having increased numbers of tissue neutrophils, however, may be an excessive response to various pro-inflammatory stimuli (Palmblad, 2018).

In conclusion, the genuine clinical impact of missing erythroid DARC/ACKR-1 in the sense of being protective or detrimental, is still unclear. Our finding in **Chapter 2.1** of an association between the Duffy negative phenotype and preeclampsia still needs to be confirmed or refuted by others.

Long-chain polyunsaturated fatty acids ω3 (LCPω3) in pregnancy and preeclampsia

Our finding in 1999 of low LCPω3 and LCPω6 contents in preeclamptic umbilical vessel walls (**Chapter 4.1.1**) became confirmed by a 2009 study of ourselves in Africa, Tanzania (Huiskes, 2009). This study with Tanzanian women with high DHA, EPA and AA status showed similar preeclampsia versus control differences in UA and UV, although differences were less pronounced as compared to the Curaçao women with lower dietary DHA and EPA intakes. Our findings are in general agreement with later studies examining different umbilical cord compartments, including umbilical plasma (Mehendale, 2008, Wadhwani, 2014) and erythrocytes (Mehendale, 2008, Mackay, 2012, Wadhwani, 2016). Lower LCPω3 status, in particular DHA, in preeclamptic umbilical vessels has been reported by most (Mackay, 2012, Wadhwani, 2014, 2016), but not all (Mehendale, 2008) authors. Lower LCPω6 in preeclamptic umbilical vessels as found by us was also noted in umbilical erythrocytes (20:3ω6, 22:4ω6, 22:5ω6) (Mackay, 2012) and -plasma (AA) (Wadhwani, 2014).

Mechanistically, it seems possible that the LCPω6 status becomes down-regulated at low LCPω3 status to maintain balance between these two important LCP families. The rationale might be to preserve the beneficial effects of LCPω3 on blood clotting, vessel diameter and inflammation (Calder, 2018). Such a synergetic relationship of AA with LCPω3 at low DHA+EPA status, and an antagonistic relationship at high DHA+EPA status, has indeed been demonstrated by us (Luxwolda, 2011).

Studies measuring both maternal and umbilical LCP levels at delivery revealed that in preeclamptic pregnancies, umbilical DHA and AA levels were higher compared to their respective maternal levels (Wadhwani, 2014, 2016). A healthy human placenta preferentially takes up and transfers LCP, such as AA and DHA, to the fetal circulation. This results in higher relative amounts in umbilical plasma lipids and erythrocytes compared with maternal levels. This underlying phenomenon is designated "bio-magnification" (Crawford, 1976). The preservation of bio-magnification in preeclamptic pregnancies is indicative for the efficiency by which LCP transport is prioritized in favor of the fetus. Prioritization may even be at the expense of the placenta's own DHA and AA demands in preeclampsia. Indeed, DHA levels were found lower in preeclamptic placentas (Wang, 2005, Kulkarni, 2011a, Wadhwani, 2014). Impaired placental uptake, and increased oxidative stress in preeclamptic placentas "attacking" LCPs have been put forward as other explanations (Wang, 2005, Kulkarni, 2011a).

LCPω3 levels, in particular DHA, are lower in plasma and erythrocytes of preeclamptic women compared to control women investigated across trimesters (Wadhwani, 2016, 2014) or at a single occasion in the third trimester (Mackay, 2012, Mehendale, 2008, Kulkarni, 2011b). The physiological adaptations of the mother to augment LCPω3 availability for placental transfer, either by increasing LCP synthesis, mobilization of LCP stores, or both, seems to fail in preeclampsia. The deviant hormonal milieu (e.g. low estrogen levels) in preeclampsia may preclude augmented LCP synthesis capacity. There is good evidence to show that augmented LCP synthesis is driven by estrogens (Burdge, 2005). Similarly, an important DHA mobilization route for the mother is to mobilize DHA from her liver, via the synthesis of DHA-enriched phosphatidylcholine (PC) with use of the PEMT pathway (phosphatidylethanolamine N-methyltransferase; discussed in more detail in "One-carbon metabolism"). This pathway is also believed to be regulated by estrogens (Resseguie, 2007). The recently discovered placental expression of the Mfsd2a receptor (Toufaily, 2013), thereby shared with brain, may be involved in the uptake of DHA-enriched PC, with concurrent choline uptake. Downregulation of this receptor has been reported in preeclamptic placentas (Toufaily, 2013). In pregnancies complicated by gestational diabetes mellitus (GDM), placental Mfsd2a protein was found reduced and related with lower DHA in cord serum lipids (Prieto-Sánchez, 2017).

In our study in Tanzania, we noticed higher contents of potentially *de novo* synthesized fatty acids in preeclamptic umbilical vessels, in addition to lower LCPω3 and LCPω6 (Huiskes, 2009). Similar findings were reported by our group in umbilical vessels of diabetic pregnancies (Type 1 diabetes and gestational diabetes) (Dijck-Brouwer, 2005). This led us to propose that the shared state of excessive loss of sensitivity to insulin

in preeclamptic and diabetic pregnancies may contribute to similar derangements of umbilical LCP contents (Huiskes, 2009). Failure of preeclamptic women to adapt their LCP metabolism may thus, in part, be ascribed to the metabolic state. Mechanistically, the exaggerated insulin resistance in preeclamptic and diabetic pregnancies may drive hepatic *de novo* lipogenesis, from polar precursors, notably glucose. The ensuing *de novo* synthesized fatty acids may 'dilute' the LCP content of very low-density lipoprotein (VLDL)-TG, which is an important source of placental (lipoprotein lipase-mediated) fatty acid uptake, to become ultimately reflected in preeclamptic umbilical vessels (Huiskes, 2009). Increased *de novo* lipogenesis is an important feature of a fatty liver (i.e. non-alcoholic fatty liver disease: NAFLD) as known in the non-pregnant state and to be driven by insulin resistance (Engin, 2017). Non-pregnant obese patients with NAFLD exhibit reduced Δ5- and especially Δ6-desaturase activities and reduced LCP synthesis (Araya, 2010). It is hypothesized that a similar scenario of fat accumulation occurs in the preeclamptic liver, with decreased hepatic LCP synthesis (Mackay, 2012).

A longitudinal study revealed that reductions of DHA in erythrocytes of preeclamptic women are already demonstrable at GW 16-20. These lower levels proved related to lower umbilical vessel levels at a later stage (Wadhwani, 2016). Apparently, failure of the mother to adapt her LCP metabolism is already present in early pregnancy. Nervonic acid (24:1ω9) is a fatty acid involved in the synthesis of myelin and white matter development. The concomitant decrease of 24:1ω9 in both maternal and umbilical erythrocytes raised concern on the neurodevelopment of the preeclamptic offspring (Wadhwani, 2016). A systematic review demonstrated that preeclamptic offspring have altered cognitive, behavioral and mood outcomes as compared to offspring from normal pregnancies (Figueiró-Filho, 2017). MRI neuroimaging was in line with e.g. behavioral deviations in preeclamptic offspring (Mak, 2018). Other meta-analyses revealed a ~30% increased risk for autism-spectrum disorders (Dachew, 2018, Maher, 2018, Xu, 2018) and attention deficit hyperactivity disorder (ADHD) (Maher, 2018) in preeclamptic offspring; similar to offspring from other hypertensive disorders in pregnancy (Maher, 2018, Xu, 2018).

It is commonly accepted that the current maternal LCPω3 status of many women is insufficient to prevent maternal LCPω3 depletion during pregnancy, suggesting possible adverse effects for both mother and fetus. This fueled interest for antenatal LCPω3 supplementation to prevent maternal depletion (with e.g. consequences for her mental health) and to secure fetal LCPω3 status. Indeed, several observational studies reported positive associations between maternal intake of fish and neurodevelopmental and behavioral outcomes in their infants studied at various ages (between 6 months and 9 years), as reviewed by Starling et al. (Starling, 2015). We studied, in an open trial design, the effect of three low-dose LCPω3 supplements on the fetal LCPω3 status during uncomplicated pregnancies (**Chapter 4.1.2**). We found biochemical improvements of the fetal LCPω3 status at LCPω3 doses of 528 mg/d (DHA ~185 mg/d) and 1,008 mg/d (DHA ~369 mg/d). These supplemental DHA doses are lower than, and similar to, respectively, the current DHA recommendation of 300 mg/d for pregnant women (Koletzko, 2014). In a recent dose-finding study conducted by our group, a 750 mg/d LCPω3 (DHA+EPA)

supplement proved necessary to reach the optimal maternal LCPω3 status (Stoutjesdijk, 2018), as compiled from Tanzanian women living near Lake Victoria with life-long high intakes of LCPω3 from fish (Kuipers, 2011).

Nevertheless, a recent Cochrane-based systematic review reported very few differences in the infant's cognitive and behavioral outcomes following antenatal LCPω3 supplementation versus placebo or no ω3 in randomized controlled trials (Middleton, 2018). There was insufficient evidence for beneficial effects on maternal mental health (postnatal depression) (Middleton, 2018). Antenatal LCPω3 reduced the risk of preterm and very preterm birth, and "possibly" reduced preeclampsia risk (Middleton, 2018). Apparently, LCPω3 act on pregnancy disorders with inflammatory elements by improving the LCPω3/CPUFAω6 ratio. Further studies are needed to unravel the exact underlying mechanisms (Middleton, 2018), e.g. the role of the recently discovered specialized pro-resolving mediators generated by EPA and DHA (Serhan, 2004). The authors (Middleton, 2018) concluded that maternal LCPω3 supplementation is a simple, inexpensive way to reduce the risk of preterm delivery. However, to better assess maternal and child outcomes, efforts must be made to identify the best type and LCPω3 dose and also those women who will benefit most (Middleton, 2018). Baseline LCPω3 status has been subject to variation in randomized controlled trials (Middleton, 2018). Responses are more pronounced at lower LCPω3 status (Stoutjesdijk, 2018) and women with poor habitual LCPω3 intakes (a determinant of LCPω3 stores) will benefit most of preventive LCPω3 supplements. In women who fail to adapt their LCP metabolism (irrespective of LCPω3 stores), like preeclamptic women, favorable effects are to be expected for the mother, the placenta and the offspring.

One-carbon metabolism

We did not find an association between preeclampsia and maternal and fetal MTHFR C677T in our study population composed of African-Caribbean women with ample Caucasian admixture (**Chapter 2.2**). Numerous studies have reported on the association between preeclampsia and maternal MTHFR C677T. They produced inconclusive results. Variations in folate status of the study populations may be a plausible explanation since the presence of sufficient folate stabilizes the variant MTHFR C677T enzyme. In addition, preeclampsia is a heterogeneous disorder, with distinct pathophysiological cascades and a wide variety of inter-individual genetic predispositions. A very recent meta-analysis did not find an association between MTHFR C677T and preeclampsia (Zhang, 2019). This outcome contrasts with a previous meta-analysis that included 54 studies with 7,398 cases and 11,222 controls (Wu, 2015). This analysis allowed stratification for ethnicity to find that the MTHFR C677T allele is modestly associated with preeclampsia, especially in Caucasian and Asian populations (Wu, 2015).

In contrast to the maternal genotype, only a few studies addressed the fetal/placental MTHFR C677T genotype. These studies reported an association (Chedraui, 2015) and no association (del Gobbo, 2018, Mislanova, 2011) with preeclampsia; the latter in support of our unpublished data from 2001 (**Chapter 2.2**).

Studying the relationships of plasma tHcy with plasma betaine and plasma folate, we proposed that the relative contribution of betaine in homocysteine remethylation increases during pregnancy (**Chapter 4.2.1**). *In vivo* support of our findings came from a study on the dynamics of deuterium-labeled choline, administered to third trimester pregnant women, as compared with non-pregnant women (Yan, 2013). Tracing of the isotopic enrichments of choline's metabolites revealed that in pregnant women, as hypothesized by us, more betaine is used as a methyl-donor, but in addition that less of the ingested choline becomes oxidized to betaine (Yan, 2013). This explains the lower plasma betaine levels in pregnant women (Yan, 2013) and is in agreement with the gestational decrease of plasma betaine documented by us. Dietary choline is highly needed for PC synthesis via the so-called "CDP pathway", required for VLDL biosynthesis and its subsequent export from the liver; a process profoundly augmented in pregnancy. Non-pregnant women with various SNPs in folate metabolism, including MTHFR C677T, showed changes in choline dynamics similar to those observed in pregnancy, i.e. using more betaine for methionine synthesis and more choline partitioning to the CDP pathway (Ganz, 2016). In pregnant women, choline dynamics did not differ amongst genotypes (Ganz, 2016). Apparently, one-carbon metabolism in pregnant women is reprogrammed to better deal with reduced folate availability for its use as methyl-donor in homocysteine remethylation.

Increased use of betaine for homocysteine remethylation contributes to the lower homocysteine levels in pregnancy, in addition to e.g. placental uptake of homocysteine. Reduction of the homocysteine/methionine ratio via remethylation serves several important maternal methylation reactions (i.e. methionine cycle). One of them is the sequential methylation of phosphatidylethanolamine (PE) to PC. This reaction, catalyzed by PEMT, is the preferred pathway to produce DHA-enriched PC (in contrast to the CDP-pathway). After PC incorporation into the phospholipid fraction of VLDL (and possibly HDL), DHA-enriched PC becomes exported from the liver, rendering hepatic DHA available to other tissues, brain included. In pregnant rats, PEMT is up-regulated rendering DHA-enriched PC a major contributor to increased maternal plasma DHA levels (Chalil, 2018). PEMT (Ressequie, 2007) and probably BHMT are both up-regulated by estrogens, and pregnant women use more betaine in the PEMT pathway *in vivo* (Yan, 2013). Such a dual up-regulation mobilizes maternal hepatic DHA from the liver to serve the high fetal (brain) DHA demands. Selective transfer of PEMT-derived PC relative to CPD-derived PC, into the umbilical cord strongly supports this concept (Yan, 2013).

Preeclamptic women have higher circulating homocysteine, as reported by many authors, including ourselves (**Chapter 4.2.2**). A recent meta-analysis confirmed the association of elevated plasma homocysteine with preeclampsia (Gaiday, 2018). Our findings of higher plasma homocysteine levels in preeclampsia and especially the steep increase of plasma homocysteine at lower plasma folate/vitamin B_{12} levels suggests that preeclamptic women have lost the importance of betaine to act as a methyl-donor, as opposed to healthy pregnant counterparts. The aberrant hormonal status of preeclampsia is a likely explanation for this loss. An ensuing consequence may be that in preeclamptic pregnancies, less maternal hepatic DHA becomes available for placental uptake and

subsequent deposition in the fetus, notably when also the status of folate and vitamin B_{12} are low.

In conclusion, it has become evident that there is an intimate relationship between DHA and one-carbon metabolism with important roles for choline and its oxidized metabolite betaine. Mobilization of maternal DHA from the liver via the PEMT pathway is a mechanism to augment DHA availability and thereby to cover the high fetal needs, especially in third trimester (see LCP). This process is likely to be comprised in preeclampsia. The reliance on folate/vitamin B_{12} status during preeclampsia and in the non-pregnant state, and the shift to reliance on choline/betaine in healthy pregnancies, emphasize the importance of nutritional interactions and its variation in different (hormonal) circumstances.

EPILOGUE

Obesity, lifestyle in general and especially insulin sensitivity, are among the few modifiable risk factors for the prevention of preeclampsia, notably preeclampsia of the "late-onset type". The current obesity epidemic in Western societies predicts that the incidence of his type of preeclampsia will increase profoundly over the next decades. Modification of obesity-related risk for preeclampsia should preferably begin (long) before conception. Promoting a healthy lifestyle is important not only for obese women, but for every woman with a pregnancy wish, but actually for all of us.

It is of utmost importance that pregnant women do not adopt a lifestyle in which "eating for two" or "taking lots of rest" is recommended. They should rather remain engaged in normal daily activities, and participate in the working process, as long as compatible with their physical condition. However, in our current, high-tech Western society, daily engagements in physical activities are declining, both during working hours, and in leisure time, in which the latter has become replaced in part, by other activities like keeping contact via the social media and internet shopping. Daily physical activities have substantial impact on energy expenditure, but maintenance or improvement of insulin sensitivity and metabolic flexibility might be the most important. The existence of "healthy obesity" suggests that "fitness" might be more important than "fatness". Even subtle imperfections in our daily energy balance will have impact over time, with the surplus deposited in white adipose tissue stores. This compartment has a remarkable, seemingly unlimited, capacity to expand, which to some extent is necessary in the first half of pregnancy; i.e. during the expansion phase for the building of fat stores necessary for later pregnancy. During this period, physical activity is of utmost importance, notably for the obese. Walking is cheap, and can in most cases become easily implanted in the daily routine.

A balanced diet is the second pillar of a healthy lifestyle, both before and during pregnancy. Meeting nutrient recommendations like those for the LCPω3 and the nutrients involved in the one-carbon metabolism (folate, vitamin B_{12}, vitamin B_6, betaine, choline)

are important for maintaining the anti-inflammatory/pro-inflammatory balance and the numerous important methylation reactions, respectively. Preeclampsia compromises the normal gestational adaptations taking place in e.g. LCP and one-carbon metabolism, which strengthens the special needs for these nutrients, both for the benefit of the mother and her baby. Since preeclampsia onset in the obese is of inflammatory nature, an optimal LCPω3/ω6 balance may be of great importance. A high quality diet with fish, fresh fruits, vegetables and fiber together with avoidance of rapid carbohydrates, strongly processed foods and "junk foods" might have advantage over supplements, because of many nutrient interaction. Care should be taken not to exceed PCB, dioxin, mercury and vitamin A upper limits, while the current recommendations for supplemental folic acid and vitamin D are to be obeyed. The need of critical nutrients in many Western societies, like vitamin B_{12}, iodine and selenium might require coverage by supplements, or the, as yet poorly investigated, consumption of shellfish and seaweed.

The currently most effective preventive strategy with drugs to reduce preeclampsia risk in high-risk populations is treatment with low dose aspirin (aspirin for evidenced-based preeclampsia prevention trial), preferably starting before 16 weeks of gestation (ACOG Committee, 2018). It is intriguing that aspirin is at present regarded to be a so-called "caloric restriction mimetic"; i.e. a pharmacological agent that induces autophagy similar to starvation. Autophagy induced by calorie restriction generates energy by catabolization of endogenous sources, at which damaged and near-damaged organelles/proteins are cleared from cells ("wiping cells clean"). Autophagy is regarded to be an "anti-aging response". Fibroblasts of centenarians look like they are "naturally caloric restricted". Optimizing the placenta's degradative capacity and her ability to handle stresses might be a preventive mechanism by which aspirin reduces preeclampsia risk. Nutritional abundance inhibits autophagy, and keeping energy balance following the simple advice to respect normal overnight fasts may protect against such an inhibitory effect.

There is epidemiological evidence that infants born to preeclamptic mothers are at increased risk of future cardiovascular and metabolic diseases. A genetic component in the efficiency of adaptive stress responses dictates similar efficiency in the placental syncytiotrophoblast (ST) and offspring. If the ST has poorly coped with experienced stresses, the preeclamptic offspring may also cope poorly with future stresses. The future risks associated with birth following a preeclamptic pregnancy are currently recognized. The infant nevertheless faces the temptations of the current affluent society and a healthy lifestyle should be the primary advice. Such recommendations also applies for their mothers, who are also at increased risk for the above mentioned diseases.

Preeclampsia bears remarkable similarity with age-related disorders like neurodegenerative diseases. The preeclamptic placenta may share the release of toxic amyloid proteins with neurodegenerative neurons, causing the "spreading" of these proteins in the mother and the neuronal environment, respectively. Fundamentally similar pathophysiological cascades may underpin these similarities, like a compromised lysosomal compartment. The sharing of insights, discoveries, and novel strategies may be to the benefit of both research fields.

Early detection of disease is a major focus in preeclampsia research. A promising and rather new research field is the release of vesicles (micro-vesicles and exosomes) into the extra-cellular compartment, currently investigated in e.g. oncology. It has been shown that the placental ST is a vivid releaser of exosomes. Micro-vesicle and exosome cargoes become selected and affected by the physical condition of the releasing cell. These particles may have functions for surveillance in diverse pathogenic states and ideally reflect early changes in these states. They may consequently be of future use as early biomarkers of preeclampsia. Exciting is the possibility to modify the cargo of these particles. If instructed by the right "address labels", such tags may deliver drugs or particular cargoes (e.g. RNA species) to designated target cells. Implementations of technological innovations are likely to become reality and thereby become an increasing part of the current clinical practice.

Research on the prevention and management of preeclampsia, and as a matter of fact on many other diseases, may be more than ever benefit from an open mind by adopting insights from different scientific fields. The development of pharmacological agents, and the implementation of high-tech innovative solutions are important, but exclusively when paralleled by the propagation of a healthy lifestyle.

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Samenvatting, de huidige stand van zaken, en epiloog

AFKORTINGEN

AA: Arachidonzuur (20:4ω6)
ACKR: Atypische chemokine receptor-1
ALA: α-Linoleenzuur (18:3ω3)
BHMT: Betaïne-homocysteïne *S*-methyltransferase
CRP: C-reactief proteïne
CT: Cytotrophoblasts, cytotrophoblast cellen
DAMPs: "Damage" geassocieerde moleculaire patronen
DARC: Duffy antigeen receptor voor chemokinen
EPA: Eicosapentaeenzuur (20:5ω3)
EV: Extra-cellulair vesikel
DHA: Docosahexaeenzuur (22:6ω3)
GW: Gestatie week
Hcy: Homocysteïne
HFE: Humane hemochromatose
IL-8: Interleukine-8
LA: Linolzuur (18:2ω6)
LDL: Low-density lipoproteïne
LCP: Lange keten meervoudig onverzadigde vetzuren
MTHFR: Methyleentetrahydrofolate reductase
PC: Fosfatidylcholine
PE: Fosfatidylethanolamine
PEMT: Fosfatidylethanolamine *N*-methyltransferase
PGI2/3: Prostacycline I2/3
PP: Postpartum
sTfR: Soluble transferrine receptor
SNP: Single nucleotide polymorfisme
SIRS: Systemisch inflammatoire response syndroom
ST: Syncytiotrophoblast
THF: Tetrahydrofolaat
TNF-α: Tumor Necrose Factor-a
TfR: Transferrine receptor
UPR: Unfolded proteïne response
Th2: Thelper2 cells
TxA2/3: Thromboxaan A2/3
vWF: von Willebrand Factor
UA: Navelstreng arteriën
UV: Navelstreng vene
VLDL: Very low-density lipoproteïne

SAMENVATTING

Hoofdstuk 1: Algemene introductie

In Hoofdstuk 1.1 worden de fysiologie van een normale zwangerschap en de pathofysiologie van pre-eclampsie besproken. Maternale en gestationele weefsels zijn onderworpen aan een streng gereguleerd en gecoördineerd programma. Maternale weefsels, zoals de decidua, wit vetweefsel en de cervix, doorlopen een eigen ontwikkelingstraject. De placenta en foetale membranen ontwikkelen, rijpen en verouderen op een in tijd en plaats gecoördineerde wijze. De functionele laag van de placenta, de meerkernige syncytiotrophoblast (ST), gedraagt zich als een echte barrière. De ST is een syncytium met veel kernen vanwege meerdere fusies van eenkernige cytotrophoblast cellen. Door de syncytiële aard is de ST resistent voor apoptose. Apoptose zou zich verspreiden door de gehele functionele laag, waardoor de omstandigheden incompatibel zouden worden met het continueren van de zwangerschap. De levensduur van de ST eindigt (ook onder stress) met de geboorte van de placenta in de laatste fase van het baringsproces. De onderliggende eenkernige cytotrophoblast (CT) cellen functioneren als stamcellen. De CT cellen prolifereren, differentiëren en fuseren continu met de ST om nieuwe organellen te leveren, zoals kernen en mitochondriën. De terminaal gedifferentieerde ST is niet in staat om zijn cellulaire afval (zoals defecte organellen, gemodificeerde eiwitten) te verdunnen door te delen, en is afhankelijk van efficiënte opruimmechanismen. We beargumenteren dat wanneer, aan het einde van de zwangerschap deze opruimcapaciteit tekort schiet, cellulair afval in de maternale circulatie terecht komt. Het is opmerkelijk dat de meest intensieve functionele periode van de ST intreedt aan het eind van zijn levensduur, wat samenvalt met de periode waarin de stress-responsen en opruimcapaciteit hun werkzaamheid beginnen te verliezen. Dit geeft aanleiding tot een goed gecontroleerde, fysiologisch-geïnduceerde stress, voornamelijk oxidatief van aard, die aan het eind van de zwangerschap resulteert in een verouderde placenta. Deze fysiologische stress wordt 'doorgegeven' naar de moeder en wordt benut voor functionele doeleinden. De uitscheiding van zogenaamde extra-cellulaire vesikels (EVs) is een inventieve manier voor communicatie tussen cellen die het transport van verschillende componenten (zoals lipiden, eiwitten, mRNA exemplaren) mogelijk maak; dit in tegenstelling tot enkelvoudige moleculen. De ST is een actieve uitscheider van deze EVs. Het uitscheiden van exosomen en micro-vesikels wordt gedeeld met andere celtypen, maar de uitscheiding van grotere macro-vesikels is uniek voor de placenta. Macro-vesikels zijn betrokken bij de fysiologische aanpassingen van de moeder, zoals cardiovasculaire aanpassingen, al vanaf het eerste trimester. Exosomen zijn immuno-suppressief en dragen bij aan de Th2 bias in de normale zwangerschap. Via de uitscheiding van pro-inflammatoire micro-vesikels en "damage geassocieerde moleculaire patronen" (DAMPs; vrijgemaakt door gestreste cellen en ook wel "gevaarsignalen" genoemd), zou de verouderde en gestreste ST de moeder kunnen aanzetten om haar reserves te mobiliseren (verhoogde maternale inflammatoire response) en haar voor te bereiden op de naderende bevalling (activatie van maternale immune cellen in de decidua, myometrium en cervix). Micro-vesikels vervoeren

bovendien pro-coagulatie factoren met als doel het bloedverlies tijdens de bevalling te minimaliseren. Op een gegeven moment kan de maternale substraatvoorziening niet meer voldoen aan de groeiende foetale zuurstof/metabole behoeftes. De foetus begint stress te ondervinden, zelfs in ongecompliceerde zwangerschappen. De aanvang van de bevalling is in de mens een gesynchroniseerd proces, waarbij verschillende signalen van de foetus worden geïntegreerd, zoals foetale stress, groei en maturatie. De voltooiing van het zwangerschapstraject kan als succesvol worden beschouwd als de bevalling perfect is getimed en resulteert in de geboorte van een volledig ontwikkelde baby.

In pre-eclamptische zwangerschappen is het normale verouderings- en stresstraject verstoord. Dit kan worden geïnduceerd door stress van verschillende aard, ernst en timing. Verhoogde oxidatieve stress veroorzaakt door een slechte placentaire ontwikkeling is vaak reeds vroeg in de zwangerschap aanwezig en verantwoordelijk voor de meeste gevallen van ernstige, "early-onset pre-eclampsie" met intra-uteriene groeiachterstand. Metabool getriggerde inflammatie in de ST, veroorzaakt door een insuline-resistente obese staat, vindt vaak later in de zwangerschap plaats en is geassocieerd met "late-onset pre-eclampsie". De adaptieve ST stress-responsen hebben als doel om de consequenties van doorgemaakte stressen te beperken. Bepalende stress-responsen zijn autofagie (centraal gepositioneerd omdat het beschadigde structuren elimineert) en de "unfolded proteïne response" (UPR). Beide responsen worden op de proef gesteld en kunnen worden gecompromitteerd, met name in placenta's van ernstig pre-eclamptische zwangerschappen. Milde ST stress versnelt het normale verouderingsproces, wat een relatief ouder ST veroorzaakt voor het werkelijke stadium van de zwangerschap. Dit leidt tot een versterking van de gebeurtenissen die normaliter in de late zwangerschap plaatsvinden (excessieve maternale inflammatoire response en een angiogenetische/hemostatische disbalans), met het ontstaan van proteïnurie en hypertensie ("term pre-eclampsie"). Dit gaat samen met verhogingen van de uitscheiding van pro-inflammatoire micro-vesikels en DAMPs, en het vrijkomen van cellulaire afval in de maternale circulatie, zoals gezien aan het eind van de normale zwangerschap. De maternale verschijnselen zijn dan vrij mild en uniform, en soms zelfs moeilijk te onderscheiden van de veranderingen die gezien worden in de late normale zwangerschap. Uitgesproken stress kan resulteren in ernstige ziekte en kan leiden tot het ontstaan van secundaire stress. Ernstige oxidatieve stress kan bijvoorbeeld leiden tot ernstige stress in het endoplasmatisch reticulum. De uitscheiding van micro-vesikels en DAMPs is toegenomen en laten respectievelijk veranderingen zien in lading en signatuur. Een gecompromitteerde afbraakcapaciteit leidt tot meer afvoer van afval naar de moeder, wat ook van een andere signatuur kan zijn, zoals toxische amyloïd aggregaten of beschadigde mitochondriële eiwitten. De maternale verschijnselen zijn ernstiger en minder uniform.

De ernst, de aard en de timing van de stress, en de efficiëntie waarmee de ST en de moeder hiermee omgaan, bepalen uiteindelijk de maternale verschijnselen van het pre-eclampsie syndroom. Het echte gevaar in pre-eclampsie ligt in het feit dat het verlies van ST homeostase (wat normaliter zou leiden tot een scenario van gereguleerde celdood) niet noodzakelijkerwijs gesynchroniseerd is met het begin van de bevalling, en dat een schadelijk ST *in situ* blijft.

Hoofdstuk 2: Genetica

De manier waarop de moeder omgaat met de stress die uitgaat van een pre-eclamptische placenta is de som van vele factoren van intrinsieke, extrinsieke (lifestyle) of raciale aard. Elke (ongunstige) factor, zelfs van bescheiden aard, kan impact hebben. In Hoofdstuk 2 hebben we 3 "single nucleotide polymorfismen" (SNPs) onderzocht voor hun associatie met pre-eclampsie. Onze studiepopulatie bestond uit de inwoners van het eiland Curaçao. Dit is een populatie van voornamelijk West-Afrikaanse afkomst met veel Kaukasische vermenging. De eerste onderzochte SNP is het Duffy negatieve fenotype (ook wel de "Duffy-null trait" genoemd) (Hoofdstuk 2.1). Het Duffy negatieve fenotype komt tamelijk vaak voor op Curaçao (geschatte prevalentie 37%). De tweede SNP is de thermolabiele MTHFR (methyleentetrahydrofolate reductase) variant, die veel voorkomt in Kaukasische populaties, met een lagere prevalentie in Afrikaanse populaties en hun afstammelingen (Hoofdstuk 2.2). De laatst onderzochte SNP is de C282Y mutatie in het *HFE* (humane hemochromatose) gen. Het C282Y-allel heeft een lage prevalentie in West-Afrikanen, maar heterozygotie voor de HFE C282Y mutatie komt veel voor in Kaukasische populaties (Hoofdstuk 2.3).

Hoofdstuk 2.1. De Duffy bloedgroep antigenen Fy^a en Fy^b zijn de expressieproducten van de FY*A en FY*B allelen. Sub-Sahara Afrikanen zijn dragers van een FY*B allel met een enkele T naar C substitutie in nucleotide -67, die een bindingsplaats van de erythroïde transcriptie factor ontwricht. De SNP veroorzaakt het Duffy negatieve fenotype. Individuen met het Duffy negatieve fenotype vertonen geen expressie van Duffy bloedgroepantigenen op erythroïde cellen. Het Duffy antigeen bleek identiek aan een niet-signalerende receptor, hoog-affiniene, promiscue (zoals interleukine-8) chemokien-receptor die op erythrocyten tot expressie wordt gebracht. Deze receptor werd aangeduid als de Duffy antigeen receptor voor chemokinen (DARC). In een retrospectieve studie-opzet fenotypeerden we 72 vrouwen met een voorgeschiedenis van pre-eclampsie en 55 tegenhangers met ongecompliceerde zwangerschappen, allemaal van West-Afrikaanse origine en woonachtig op het eiland Curaçao. Vrouwen met pre-eclampsie in de voorgeschiedenis hadden een hogere frequentie van het Duffy negatieve fenotype, vergeleken met vrouwen met ongecompliceerde zwangerschappen, respectievelijk 52,8% tegen 27,3%; odds ratio 2,98; 95% CI 1,40-6,32, p=0,004. Erythroïd DARC zou kunnen fungeren als afvoersysteem voor circulerende chemokinen, zoals interleukine-8 (IL-8), in het bijzonder bij hogere bloedspiegels. IL-8 dat gebonden is aan erythroïd DARC is niet in staat neutrofielen te activeren. Het missen van een erythroïd IL-8 bindingcapaciteit verhoogt de toegankelijkheid van IL-8 voor andere receptoren, bijvoorbeeld op immuun cellen, wat belangrijk zou kunnen zijn in de pathofysiologie van pre-eclampsie.

In Hoofdstuk 2.2 bestudeerden we de thermolabiele MTHFR-variant. MTHFR is een centraal enzym in het 1-koolstofmetabolisme, i.e. een fundamentele biochemische cyclus die methyl- groepen aanlevert voor belangrijke methylerings-reacties. Het goed functioneren van de enzymen in het 1-koolstofmetabolisme is afhankelijk van

genetische en omgevingsfactoren (zoals voedingsfactoren). De thermolabiele MTHFR-variant komt veel voor in Kaukasische populaties, maar heeft een lage prevalentie in Afrikaanse populaties. MTHFR reduceert 5, 10-methyleentetrahydrofolaat (THF) naar 5-methyl THF, wat een belangrijke methyl-donor is in het 1-koolstofmetabolisme. Door het doneren van zijn methyl-groep methyleert 5-methyl THF homocysteïne naar methionine, en is daarmee een determinant van de plasma homocysteïne concentratie. Een veel voorkomende C naar T substitutie in nucleotide -677 (genaamd de thermolabiele C677T MTHFR-variant) vermindert de MTHFR activiteit, in het bijzonder bij een lage folaatstatus. Homozygote individuen voor de C677T MTHFR-variant (het TT genotype) vertonen een verhoogde plasma homocysteïne response bij een lage folaatstatus. Een hoog plasma homocysteïne is gevonden in pre-eclampsie en is gerelateerd aan endotheel schade. Daarom genotypeerden we in een retrospectieve studie-opzet, 89 vrouwen met een voorgeschiedenis van pre-eclampsie en 89 ras- en pariteit- gematchte controles met ongecompliceerde zwangerschappen. Ook genotypeerden we 49 kinderen van de pre-eclamptische moeders en 49 kinderen van de gezonde moeders. Allen waren Caribische vrouwen van West-Afrikaanse origine met ruime Kaukasische vermenging. We vonden geen significante case-control verschillen in de MTHFR C677T genotype en T-allel frequenties tussen moeders, hun kinderen en de moeder/kind combinaties. Een toevalsbevinding was dat vrouwen met een voorgeschiedenis van milde pre-eclampsie hogere frequenties hadden van het MTHFR C677T genotype en van het T-allel, dan vrouwen met een voorgeschiedenis van ernstige pre-eclampsie. Gelijke maternale C677T MTHFR TT genotype frequenties in pre-eclamptische en controle vrouwen is in overeenstemming met bevindingen in blanke Amerikaanse, Nederlands Kaukasische en Zimbabwaanse vrouwen, maar is in tegenspraak met de hogere frequenties van het C677T MTHFR TT genotype in Japanse, Joodse en Europese vrouwen met pre-eclampsie.

Verschillende onderliggende ethologiën en pathofysiologische cascaden die leiden naar een enkel syndroom van pre-eclampsie, zouden kunnen helpen om de schijnbare discrepanties te verklaren in de gevonden associaties tussen MTHFR C677T en pre-eclampsie. Enig bewijs hiervoor zou kunnen worden gevonden in de waargenomen hogere frequentie van de C677T MTHFR-variant in vrouwen met een milde pre-eclampsie versus een ernstige pre-eclampsie in hun voorgeschiedenis, alwaar verschillende ethologiën/ pathofysiologische processen worden vermoed.

In Hoofdstuk 2.3 onderzochten we de C282Y mutatie in het *HFE* gen, dat codeert voor het HFE eiwit. Dit eiwit is betrokken bij de opname van ijzer uit de voeding. Homozygotie voor de C282Y mutatie verklaart de meerderheid van de gevallen met hereditaire hemochromatose (HH). C282Y heterozygoten ontwikkelen gewoonlijk geen klinische manifestaties van HH, maar ze hebben een hogere ijzervoorraad in hun lichaam. Een mild verhoogd lichaamsijzer zou betrokken kunnen zijn bij de oxidatie van lipiden door ijzer, bijvoorbeeld “low-density lipoproteïne” (LDL)-oxidatie, waarvan gesuggereerd is dat het atherosclerose bevordert. Een case-control studie uitgevoerd op Curaçao toonde aan dat patiënten met coronaire hartziekten een hogere C282Y heterozygotie frequentie

hebben, indien vergeleken met controle personen, respectievelijk 9,6% versus 1,2%. Omdat pre-eclampsie en atherosclerose risicofactoren delen, onderzochten we in een retrospectieve studie-opzet of pre-eclampsie geassocieerd is met C282Y heterozygotie. Hiervoor genotypeerden we 74 vrouwen met pre-eclampsie in de voorgeschiedenis en 84 controles. Heterozygotie voor C282Y bedroeg 4,1% (3/74) bij vrouwen met doorgemaakte pre-eclampsie en 2,4% (2/84) bij de controle vrouwen (p=0,66). De prevalentie was in grote lijnen in overeenkomst met de vermenging van West-Afrikaanse vrouwen (Ghanese populaties zijn geen dragers van het C282Y-allel) en Kaukasiërs, voornamelijk Nederlanders (7,2% C282Y heterozygotie). We concludeerden dat onze laag-prevalente studiepopulatie geen bewijs vertoont voor een associatie tussen pre-eclampsie en C282Y heterozygotie. Dit komt overeen met het vinden van gelijke C282Y genotype frequenties in pre-eclamptische en controle vrouwen in een Nederlandse populatie met een heterozygotie prevalentie van ~11% (Senden, 2004).

Hoofdstuk 3: Immunologie

Het is duidelijk geworden dat de maternale inflammatoire response in de normale zwangerschap wordt versterkt tijdens een pre-eclamptische zwangerschap. Wij waren één van de eersten die melding maakten van hogere IL-8 concentraties in pre-eclamptische vrouwen in een gematchte case-control studie van beperkte omvang.

Aangezien neutrofielen geactiveerd zijn in pre-eclampsie bepaalden we de serumconcentratie van de neutrofiele chemo-attractor en activator IL-8 in 13 pre-eclamptische vrouwen en in 13 zwangere controles met een normale bloeddruk en zonder proteïnurie. We bepaalden tegelijkertijd de serum tumor necrose factor-α (TNF-α) concentratie, vanwege zijn betrokkenheid in de productie van IL-8. De meeste vrouwen werden gefenotypeerd voor het Duffy bloedgroep antigeen, wat betrokken is bij de binding van circulerend IL-8 (zie Hoofdstuk 2.1). Om te bevestigen dat pre-eclampsie een endotheliale aandoening is bepaalden we eveneens de plasma von Willebrand factor (vWF) als een algemene marker voor endotheliale dysfunctie. Onze studiegroep bestond uitsluitend uit Afrikaans-Caraïbische vrouwen die geen evidente tekenen vertoonden van infecties of aan het bevallen waren, en die gematcht werden voor pariteit (nullipara versus multipara) en het moment van bloedafname (± een week). We vonden een hoger IL-8 (p=0,0033), TNF-α (p=0,0067) en vWF (p=0,019) bij pre-eclamptische vrouwen in vergelijk met de controle vrouwen. IL-8 spiegels waren positief gecorreleerd met TNF-α en vWF (in de totale studiegroep). IL-8 spiegels varieerden sterk bij de pre-eclamptische vrouwen, maar ze vertoonden allen hogere IL-8 spiegels dan hun gematchte controles. Bijna alle vrouwen (9/10) in de pre-eclampsie groep werden gefenotypeerd als Duffy negatief, tegenover slechts 3/11 in de controle groep (p=0,0037). Hoger serum IL-8 spiegels bij pre-eclamptische vrouwen is mogelijk het resultaat van een verhoogde productie (secundair aan verhoogde TNF-α spiegels) en/of gereduceerde klaring (gerelateerd aan de hoge frequentie van het Duffy negatieve fenotype). De positieve correlatie tussen serum IL-8 en vWF (een marker voor endotheliale dysfunctie) suggereert dat hoge serum IL-8 spiegels in de zwangerschap ongunstig zijn. De hoge frequentie van het Duffy negatieve

fenotype in de pre-eclampsie groep stimuleerde ons om de grotere retrospectieve studie uit te voeren die beschreven is in Hoofdstuk 2.1.

Hoofdstuk 4: Voeding
In Hoofdstuk 4 bestudeerden we de invloed tijdens zwangerschap en pre-eclampsie van lange keten meervoudig onverzadigde vetzuren (Hoofdstuk 4.1), nutriënten betrokken bij het 1-koolstofmetabolisme (Hoofdstuk 4.2) en ijzer (Hoofdstuk 4.3) .

Hoofdstuk 4.1 Lange keten meervoudig onverzadigde vetzuren (LCP). LCP zijn vetzuren met ≥20 koolstof atomen en ≥3 dubbele bindingen. LCP zijn een belangrijke klasse van vetzuren omdat ze betrokken zijn bij diverse biologische processen, bijvoorbeeld als precursors van eicosanoïden en als determinanten van de fysisch-chemische eigenschappen van membranen. LCP zijn afkomstig uit de voeding of worden gesynthetiseerd uit de essentiële "stam" vetzuren linolzuur (LA; 18:2ω6) en α-linoleenzuur (ALA;18:3ω3) wat de vorming oplevert van respectievelijk LCPω6 (arachidonzuur, AA) en LCPω3 (eicosapentaeenzuur, EPA en docosahexaeenzuur, DHA). In de mens wordt verondersteld dat de synthesesnelheden van met name EPA en DHA tamelijk gering zijn. Derhalve dient het merendeel van de LCPω3 uit de voeding te komen, waarbij EPA (20:5ω3) en DHA (22:6ω3) voornamelijk verkregen worden uit voedingsproducten uit de zee, vlees en eieren, en AA (20:4ω6) uit vlees, gevogelte en eieren.

Een adequate LCP status is met name belangrijk tijdens de zwangerschap omdat AA, 22:4ω6 en DHA tot de kwantitatief belangrijkste vetzuren in de hersenen behoren. Er is weinig bewijs dat de foetale LCP synthese voldoende is om in de hoge intra-uteriene LCP behoefte te voorzien. De foetus is derhalve in hoge mate afhankelijk van de maternale LCP voorziening, hetgeen de LCP in deze periode kwalificeert als conditioneel-essentieel. In Hoofdstuk 4.1.1 onderzochten we de LCP samenstelling in pre-eclamptische en normotensieve zwangerschappen. In Hoofdstuk 4.1.2 onderzochten we, in een open label studie-opzet, het effect van de suppletie van een lage dosis LCPω3 bij gezonde zwangere vrouwen, op de navelstreng LCP status bij de geboorte.

In Hoofdstuk 4.1.1 onderzochten we de LCP samenstelling van bloedplaatjes van de moeder en uit de navelstreng, en van de navelstrengvenen (UV) en de navelstrengarteriën (UA). De studiegroep bestond uit 27 pre-eclamptische en 24 normotensieve vrouwen. Pre-eclampsie wordt gekenmerkt door een verhoogde bloedplaatjes aggregatie en vasoconstrictie, wat ten minste deels veroorzaakt kan worden door een abnormale productie van eicosanoïden met een verhoogd gehalte van het uit bloedplaatjes-afkomstig thromboxaan A2 (TxA2), ten opzichte van endotheliaal-geproduceerd prostacycline I2 (PGI2). AA en EPA zijn voorlopers van eicosanoïden van respectievelijk de 2-serie en de 3-serie. Endotheliaal PGI2 (afkomstig van AA) en endotheliaal PGI3 (afkomstig van EPA) zijn equipotente vaatverwijders en remmers van bloedplaatjes aggregatie. Daarentegen is TxA2 uit bloedplaatjes en afkomstig van AA een sterkere vaatvernauwer en bloedplaatjes aggregator dan TxA3, afkomstig van EPA. We waren derhalve geïnteresseerd in de LCP status, en met name de AA en EPA gehaltes in bloedplaatjes van

pre-eclamptische en controle vrouwen. De vrouwen in onze studiepopulatie woonden op het eiland Curaçao, en waren vooral van een gemixte Afrikaans-Westerse afkomst. Hun voedingsgewoonte waren in wezen Westers, gekenmerkt door een hoge inname van ω6 vetzuren (met name uit LA-rijke plantaardige oliën) en een lage inname van ω3 vetzuren (uit ALA-rijke plantaardige oliën en vis). Het daaruit voortvloeiende ω6 overwicht kan bloedplaatjes aggregatie en vasoconstrictie bevorderen. Daarnaast lijkt, zelfs onder normale omstandigheden, de foetale LCP status marginaal, gezien de lagere LCP status van de UA indien vergeleken met de UV. Deze ogenschijnlijk ongelijkmatige LCP verdeling zou kunnen worden geaccentueerd tijdens pre-eclampsie. Daarom bepaalden we de LCP samenstelling van zowel de UA en de UV bij pre-eclamptische en controle zwangerschappen. Een aantal van de belangrijkste bevindingen worden hier besproken. Ten eerste, bloedplaatjes van pre-eclamptische vrouwen bevatten een lagere hoeveelheid EPA en hadden een hogere AA/EPA ratio vergeleken met normotensieve vrouwen. Hoewel de verschillen tamelijk klein waren, kan deze bevinding in overeenstemming zijn met een "overwicht van TxA2 ten opzichte van TxA3" en de toestand van bloedplaatjes aggregatie en vaatvernauwing zoals die wordt gezien bij pre-eclampsie. Ten tweede, vergeleken met controles bevatten de UV en UA van pre-eclamptische zwangerschappen lagere percentages vetzuren van zowel de ω3- als van de ω6-series, inclusief hun LCP leden. Lagere LCPω3 en LCPω6 in pre-eclamptische UA kan wijzen op een verlaagde foetale LCP aanwas, waardoor onvoldoende LCP beschikbaar komt voor incorporatie in foetale weefsels die het meest distaal van de placentaire toevoer liggen (zoals UA). De lagere LCPω3 en LCPω6 status bij moeders met adequate ω6 vetzuur inname wijst in de richting van onvoldoende trans-placentair transport, en zou dus gerelateerd kunnen zijn aan de pathogenese van pre-eclampsie. Ten derde, we vonden een hogere 20:3ω9/AA ratio in pre-eclamptische UA. "Meadzuur" (20:3ω9) is een marker voor een essentiële vetzuurdeficiëntie. In de literatuur is een omgekeerde relatie gevonden tussen de 20:3ω9/AA ratio en de prostacycline productie in de UA. Een hogere 20:3ω9/AA ratio kan daarom passen bij de lagere prostacycline productie in pre-eclamptische UA. Samengevat, bloedplaatjes van pre-eclamptische vrouwen op Curaçao vertoonden een iets hogere AA/EPA ratio, terwijl de navelstrengen van hun foetussen lagere relatieve hoeveelheden van zowel LCPω3 als LCPω6 bevatten.

In Hoofdstuk 4.1.2 bestudeerden we het effect van drie lage-doses visolie supplementen, gegeven tijdens ongecompliceerde zwangerschappen, op de foetale LCP status bij de geboorte. De foetale LCP status werd vastgesteld door de LCP samenstelling van de UA en de UV te bepalen. De foetus is in hoge mate afhankelijke van de maternale LCP aanvoer. Tegenwoordig wordt algemeen aanvaard dat de huidige maternale LCP status, en vooral die van LCPω3, onvoldoende is om maternale LCPω3 depletie tijdens de zwangerschap te voorkomen, hetgeen mogelijk nadelige effecten suggereert voor zowel de moeder als de foetus. LCPω3 suppletie studies, vooral de studies die hoge doses gebruikten, zijn uitgevoerd om de maternale en neonatale LCPω3 status te verhogen en de klinische uitkomst te verbeteren, zoals pre-eclampsie en het geboortegewicht. Het is aangetoond dat hoge doses LCPω3 (2.200-2.600 mg per dag) de neonatale LCPω3 status

verbeteren met een gelijktijdige reductie van LCPω6. Een reductie van de foetale LCPω6 status wordt gezien als ongewenst, omdat een reductie van diens voornaamste vetzuur AA gerelateerd is aan een geretardeerde groei. We onderzochten daarom, in a open label studie-opzet, het effect van drie lage-doses LCPω3 supplementen ofwel in de vorm van visolie capsules ("vis-1" groep; 336 mg/d LCPω3, n=15, "vis-3" groep; 1.008 mg/d LCPω3, n=20) of op basis van een melk supplement ("Mum" groep; 528 mg/d LCPω3, n=24). Zevenenvijftig onbehandelde vrouwen dienden als controles. Supplementen werden gegeven vanaf het tweede trimester tot aan de bevalling. De gemiddelde suppletieduur bedroeg 19 weken. De belangrijkste uitkomst van deze studie was dat een dagelijkse suppletie met 528 en 1.008 mg LCPω3 vanaf het tweede trimester de neonatale LCPω3 status significant verhoogt zonder merkbare invloed op de LCPω6 status. Meer specifiek, vergeleken met onbehandelde controles, had de Mum-groep (528 mg/d) een hogere EPA, 22:5ω3, DHA, LCPω3, en DHA/22:5ω6 ratio in zowel de UV als de UA. De vis-3-groep (1.008 mg/d) had een hogere 20:3ω6, LCPω3 en DHA/22:5ω6 ratio in UV en een hogere 22:5ω3, DHA, LCPω3 en DHA/22:5ω6 ratio in de UA. Een dosis van 336 mg/d liet geen effect zien indien vergeleken met onbehandelde controles. De incorporatie efficiënties van LCPω3 in de Mum- en vis-3-groepen kwamen overeen met hetgeen gezien werd na suppletie met 2.200 mg/d LCPω3 bij zwangere Deense vrouwen. Incorporatie van LCPω3 in de navelstrengwand bij de Curaçaose zwangerschappen leek dosis-afhankelijk, maar bereikte waarschijnlijk een steady-state vanaf 528 mg/d. Alhoewel statistisch niet significant deed het lager gedoseerde melk-gebaseerde supplement het beter dan het hoger gedoseerde visolie supplement. Dit wordt mogelijk veroorzaakt door een additionele dagelijkse inname van 178 mg 18:3ω3 via het op melk-gebaseerde supplement. Deze inname gaf een gunstige toename van de 18:3ω3/18:2ω6 ratio, en kan daarmee de omzetting van 18:3ω3 naar EPA en DHA hebben verhoogd. Een verbeterde omzetting kan, op haar beurt, zijn gedreven door het hormonale zwangerschapsmilieu. In de UV van de visolie-3-groep vonden we een onverwachte verhoging van 20:3ω6, hetgeen de precursor is van de schijnbaar gunstige eicosanoïden van de 1-serie. Het is hierbij van belang te vermelden dat de hoge LCPω3 status van de Inuit samengaat met een hogere 20:3ω6 status. Het is ook gevonden dat 20:3ω6 in de UV de beste indicator is van intra-uteriene foetale groei. Eerder rapporteerden we een lage 20:3ω6 in pre-eclamptische UV. Samengevat, een 500-1.000 mg/d LCPω3 supplement, ingenomen als visolie capsules dan wel als een op melk-gebaseerd supplement, was effectief voor de verhoging van de foetale LCPω3 status, zonder de LCPω6 status te beïnvloeden.

Hoofdstuk 4.2 1-Koolstofmetabolisme. In Hoofdstuk 4.2 bestudeerden we de relaties tussen het plasma totale homocysteïne (tHcy) gehalte met dat van het plasma folaat en het plasma betaïne tijdens normale zwangerschappen (Hoofdstuk 4.2.1) en tussen plasma tHcy en plasma folaat tijdens pre-eclamptische zwangerschappen (Hoofdstuk 4.2.2). Het bestuderen van deze relaties geeft inzicht in de relatieve bijdrages van folaat en betaïne als methyl-donoren aan de intracellulaire homocysteïne remethylering, en daarmee aan belangrijke maternale methylerings-reacties als onderdeel van de methionine cyclus.

Folaat-afhankelijke homocysteïne remethylering vindt plaats in bijna alle weefsels, gekatalyseerd door methionine synthase, met MTHFR-afkomstig 5-methyl THF als methyl-donor en vitamine B12 als cofactor. Daarentegen gebruikt een alternatieve homocysteïne remethylerings route betaïne als methyl-donor, hetgeen gekatalyseerd wordt door betaïne-homocysteïne *S*-methyltransferase (BHMT) en met name plaatsvindt in de lever en nieren. Om volledig te zijn, kan intracellulair homocysteïne ook worden gemetaboliseerd naar cysteïne in de vitamine B_6-afhankelijke transsulfuratie route die plaatsvindt in lever en nieren. In niet-zwangere personen is het plasma folaat de belangrijkste determinant van plasma tHcy. We trachtten uit te vinden of de hoge nutritionele behoeften tijdens de humane zwangerschap tot aanpassingen leidt in de homocysteïne-methionine metabole cyclus.

Hoofdstuk 4.2.1. Vele studies rapporteerden lagere plasma concentraties van tHcy in zwangere vrouwen indien vergeleken met niet-zwangere vrouwen, waarbij de laagste concentraties werden gevonden in het tweede trimester. Deze bevinding suggereert de aanwezigheid van een "effectief homocysteïne-verlagend mechanisme" in de normale zwangerschap. Het is bekend dat BHMT in ratten hormonaal (bijvoorbeeld door oestrogenen) gereguleerd wordt. Een mogelijke rol voor de BHMT-route werd nog sterker vermoed door de bevinding dat een laag plasma folaat in zwangere vrouwen niet leidt tot een hogere tHcy. Tot dan had geen enkele studie de mogelijke rol van betaïne en zijn precursor choline als een alternatieve methyl-donor aan de orde gesteld. Wij onderzochten het verloop van plasma choline, betaïne, folaat en vitamine B_{12} tijdens de normale humane zwangerschap en hun relaties met plasma tHcy. Bloedmonsters werden maandelijks afgenomen. De eerste bloedafname vond plaats in "gestatieweek" (GW) 9, en de laatste 3 maanden postpartum (PP). De studiepopulatie bestond uit 50 vrouwen van West-Afrikaanse afkomst met ongecompliceerde zwangerschappen. De meeste deelneemsters gebruikten een foliumzuur-supplement op onregelmatige basis. We vonden een continue stijging van het plasma choline (geometrisch gemiddelde; 95% CI) gedurende de zwangerschap, van 6,6 (4,5, 9,7) µmol/L in GW 9 naar 10,8 (7,4, 15,6) µmol/L in GW 36, wat overeenkomt met de bevindingen van anderen. Plasma betaïne daalde in het eerste gedeelte van de zwangerschap, van 16,3 (8,6, 30,8) µmol/L in GW 9 naar 10,3 (6,6, 16,2) µmol/L in GW 20, om daarna constant te blijven. We bevestigheden een reductie in plasma tHcy met laagste concentraties in de tweede trimester. Drie maanden na de bevalling waren het plasma tHcy en betaïne hoger, terwijl het plasma folaat en choline lager waren ten opzichte van de laatste bemonstering tijdens de zwangerschap. Vanaf GW 16 zagen we een omgekeerde relatie tussen plasma tHcy en plasma betaïne (met uitzondering van GW 32). Multipele regressieanalyses lieten zien dat plasma folaat de beste voorspeller was van tHcy in GW 9 en na de bevalling, maar dat plasma betaïne de beste voorspeller was van tHcy in GW 20, 24, 28 en 36. De stijging van het plasma choline waarborgt de beschikbaarheid van choline voor placentair transport. Zowel mobilisatie van de maternale choline voorraad in de lever als toegenomen *de novo* choline synthese kunnen de waargenomen stijging van het maternale plasma choline verklaren. Het verschijnen van een omgekeerde relatie tussen plasma tHcy en betaïne

was een opmerkelijke en nieuwe bevinding. Deze relatie tijdens de zwangerschap was sterker dan de relatie bij niet-zwangere personen, zelfs bij een lage folaatstatus. Het benadrukt het belang van betaïne in het 1-koolstofmetabolisme tijdens de zwangerschap en is in overeenstemming met een hogere (hormonaal gestuurde) BHMT activiteit. Het bijna halveren van het plasma betaïne tijdens de zwangerschap wordt mogelijk verklaard door een verhoogd verbruik in de BHMT-route, door placentair transport of door een verminderde synthese uit choline, dat laatste kan mogelijk een suboptimale choline status weergeven. Onze resultaten benadrukken het belang van de choline en betaïne status tijdens de normale zwangerschap van de mens.

Hoofdstuk 4.2.2. In tegenstelling tot vrouwen met een normaal verlopende zwangerschap, hebben pre-eclamptische vrouwen een hoger plasma homocysteïne, dat gecorreleerd bleek met fibronectine, een marker voor endotheliale aandoeningen. We bepaalden in een case-control studie-design het plasma homocysteïne, plasma folaat en plasma vitamine B_{12} van 43 vrouwen met bewezen pre-eclampsie in hun derde trimester en in 43 controle zwangeren die waren gematcht voor pariteit en zwangerschapsduur (bloedafname GW 33). Alle deelnemers waren woonachtig op Curaçao. We bevestigden hoger plasma homocysteïne bij pre-eclamptische vrouwen ($p<0,0001$), zoals gerapporteerd door anderen. Daarnaast was het plasma vitamine B_{12} iets hoger ($p=0,025$) bij pre-eclamptische vrouwen, terwijl het plasma folaat gelijk was. We vonden omgekeerde relaties tussen plasma homocysteïne en plasma folaat bij zowel de pre-eclamptische ($r= -0,59$, $p<0,0001$) als bij de controle vrouwen ($r= -0,60$, $p<0,0001$). Minder sterke omgekeerde relaties werden gevonden tussen plasma homocysteïne en vitamine B_{12} bij zowel pre-eclamptische ($r= -0,41$, $p=0,008$) als controle vrouwen ($r= -0,35$, $p=0,020$). Bij gezonde zwangeren leidde een laag plasma folaat en vitamine B_{12} niet tot hoog plasma homocysteïne, zoals opgemerkt door anderen. In tegenstelling tot de controles vertoonden de pre-eclamptische vrouwen een sterke stijging in het plasma homocysteïne bij een laag plasma folaat (onder ~10 nmol/l) en een laag vitamine B_{12} (onder ~200 pmol/l). Tijdens de normale zwangerschap lijken folaat en vitamine B_{12} te worden 'gespaard' voor andere processen, zoals placentair transport. De sterke stijging van het plasma homocysteïne bij een laag folaat bij pre-eclamptische vrouwen is vergelijkbaar met de stijging bij gezonde niet-zwangere personen, zoals beschreven door onze research groep. Onze bevindingen in pre-eclamptische vrouwen suggereert een grote afhankelijkheid van het homocysteïne metabolisme van folaat- en vitamine B_{12}-afhankelijke remethylering.

Hoofdstuk 4.3 IJzer. In Hoofdstuk 4.3 onderzochten we de waarde van de "soluble transferrine receptor" om zwangere vrouwen te identificeren die het risico lopen om ijzerdeficiënt te worden. Het opsporen van een ijzerdeficiëntie in de zwangerschap is lastig, omdat de hemodynamische aanpassingen en de veranderende vraag naar ijzer de meeste traditionele indices veranderen, onafhankelijk van de ijzerstatus. De hoeveelheid "aangekleurd" ijzer in het beenmerg is de 'gouden standaard' met serum ferritine als een sterke en praktische surrogaatmarker, door zijn positieve correlatie met "aangekleurd" ijzer in het beenmerg. De transferrine receptor (TfR) is betrokken bij de cellulaire opname

van ijzer en zijn expressie op het celoppervlak wordt geupreguleerd in ijzer gedepriveerd weefsel. Een afgesplitst fragment van de receptor in de circulatie (soluble TfR; sTfR) is proportioneel aan het aantal receptoren op het celoppervlak en weerspiegelt de cellulaire vraag naar ijzer.

We onderzochten de toegevoegde waarde van de soluble transferrine receptor (sTfR) voor het vaststellen van een ijzergebrek in de zwangerschap. Hiervoor werd bloed verkregen van 82 ogenschijnlijk gezonde zwangere vrouwen vanaf GW 10, en daarna in GW 16, 20, 24, 28, 32 en 36, en 14,5 weken PP. De onderzoekspopulatie werd gerekruteerd uit een grotere populatie voor het vaststellen van klinisch chemische referentiewaarden voor zwangeren op het eiland Curaçao. De vrouwen rapporteerden een onregelmatig gebruik van ijzer. Plasma sTfR en serum ferritine werden bepaald en vrouwen met een C-reactief proteïne (CRP) >0,8 mg/l werden uitgesloten. Negentien vrouwen met serum ferritine ≤12 µg/l in GW 28, 32 en 36 werden geclassificeerd als ijzer "gedepleteerd". Tweeëntwintig vrouwen met serum ferritine >12 µg/l tijdens alle drie de momenten werden geclassificeerd als ijzer "verzadigd". De invloeden van een toegenomen erytropoëse tijdens zwangerschap en/of van placentaire TfR expressie op de sTfR gehaltes konden worden bestudeerd in deze laatste groep. Daarnaast werd de bovenste sTfR referentiewaarde in deze groep gebruikt als sTfR afkapgrens. In de totale populatie daalde het serum ferritine tot aan GW 28 om daarna een steady state te bereiken tot aan GW 36, waarna het PP steeg. sTfR steeg gedurende de zwangerschap en daalde PP in overeenstemming met eerdere studies. sTfR steeg significant in de ijzer "gedepleteerde" vrouwen van GW 28 naar GW 36, maar bleef constant in de adequate groep. Het percentage vrouwen in de "gedepleteerde" groep met sTfR waarden boven de door ons vastgestelde afkapgrens, steeg van 15,8% in GW 28 en 32 naar 55,6% in GW 36. De relevantie van de bevindingen in deze goed definieerde (sterkte van de studie), kleine studiepopulatie (limitatie van de studie) is tweevoudig. Ten eerste, de constante sTfR waarden in de "verzadigde" groep is een indicatie dat de toegenomen erytropoëse en/of placentaire sTfR expressie geen invloed hebben op de sTfR waarden in deze periode van de zwangerschap. Ten tweede, de waargenomen scherpe toename van de sTfR waarden in de "gedepleteerde" groep tezamen met het hogere percentage vrouwen met sTfR waarden boven de afkapgrens, geeft aan dat meer vrouwen een ijzerdeficiëntie in de weefsels ontwikkelen bovenop een uitgeputte ijzervoorraad. We concluderen dat de sTfR bepaling een ijzerdeficiëntie kan detecteren bovenop een uitgeputte ijzervoorraad in de derde trimester van de zwangerschap.

Latere studies door anderen vonden dat de sTfR concentraties alleen, of als een ratio met log serum ferritine (ferritine index), correleerden met de ernst van een ijzergebreksanemie in de zwangerschap. sTfR en de ferritine index zijn bruikbaarder dan de traditionele parameters zoals MCV (gemiddelde corpusculaire volume), MCHC (gemiddelde corpusculaire hemoglobineconcentratie) of het serum ferritine (Sharma, 2016). Echter, de waarde van de sTfR bepaling zal primair liggen in de beoordeling van gecompliceerde anemische gevallen in de zwangerschap (Weyers, 2016).

DE HUIDIGE STAND VAN ZAKEN

Veel nieuwe gegevens over de bestudeerde onderwerpen in dit proefschrift kwamen beschikbaar na de publicaties van onze studies of na hun beschrijving in de ongepubliceerde manuscripten. Het volgende korte review beschrijft de huidige stand van zaken betreffende relevante informatie over "IL-8 en DARC", "LCPω3 in de zwangerschap en pre-eclampsie" en "1-Koolstofmetabolisme".

Interleukine-8 (IL-8) en de Duffy antigeen receptor van chemokines (DARC)
Talrijke latere studies bevestigden onze bevindingen van een hoog maternaal TNF-α en hoge IL-8 spiegels in pre-eclamptische zwangerschappen (**Hoofdstuk 3**). Een recente systematische review beschouwde CRP, TNF-α, IL-6 en IL-8 als prominente pro-inflammatoire markers in pre-eclampsie vanaf het tweede trimester (Black, 2018).

Micro-vesikels en/of DAMPs, allemaal vrijgekomen uit een gestreste pre-eclamptische placenta, zijn bestudeerd als nieuwe kandidaten (**Hoofdstuk 1**) om de hoge circulerende IL-8 spiegels in pre-eclampsie te verklaren. Mitochondriële DAMPs kunnen circulerende neutrofielen stimuleren om IL-8 te produceren en uit te scheiden. Door hun werking op zowel maternale immuun-cellen en endotheelcellen kunnen micro-vesikel-geassocieerde DAMPs een pro-inflammatoire cytokine productie en een verhoogde endotheelcel permeabiliteit teweegbrengen (Collett, 2018). Onze suggestie (**Hoofdstuk 1**) dat pre-eclampsie gelijkenis vertoont met het klinische systemisch inflammatoire response syndroom (SIRS), die wordt uitgelokt door een massale DAMP release, zoals bijvoorbeeld bij trauma patiënten, past in dit concept. In dit verband wordt verondersteld dat tijdens inflammatoire processen de binding van chemokinen aan erythroïd DARC (met ~2.000 bindingplaatsen per erythrocyt) een bufferende werking heeft op het overschot aan chemokinen (Hansell, 2011). Het ontbreken van een erythroïde chemokine bindingscapaciteit zou de activatie van circulerende neutrofielen en hun infiltratie in bijvoorbeeld het systemisch arteriële vaatstelsel kunnen versterken, en daarmee vasculaire dysfunctie veroorzaken.

Echter, tot op heden moet de exacte klinische significantie van erythroïd DARC, tegenwoordig genaamd "atypische chemokine receptor-1 (ACKR-1), nog tot volledigheid worden uitgezocht. De buffercapaciteit van erythroïd DARC/ACKR-1 tijdens inflammatoire omstandigheden is tegenwoordig goed aanvaard en bevestigd *in vivo* (Mayr, 2008). In tegenstelling tot andere atypische chemokine receptoren breekt erythroïd DARC/ACKR-1 zijn verwante chemokines slechts in geringe mate af (Pruenster, 2009). Erythroïd DARC/ACKR-1 zou onder basale condities zijn chemokines zelfs weer afgeven (Hansell, 2011). Alles tezamen wordt nu gedacht dat de primaire functie van erythroïd DARC/ACKR-1 ligt in de regulatie van de beschikbaarheid van chemokines door als een buffer op te treden onder inflammatoire omstandigheden en als een depot/reservoir onder basale condities. Opmerkelijk is dat DARC/ACKR-1 expressie zich niet beperkt tot erythroïde cellen, maar ook plaatsvindt op de endotheelbekleding van post-capillaire en "verzamelende" venulen (Thiriot, 2017), zelfs in Duffy negatieve individuen (specifiek

verlies van erythroïde expressie). Endotheliaal DARC/ACKR-1 presenteert zijn verwante chemokines op het apicale endotheeloppervlak (Pruenster, 2009) en is essentieel gebleken voor het chemokine-gestuurde leukocytenverkeer (Horuk, 2015, Novitzky-Basso, 2012, Thiriot, 2017).

In het licht van zijn buffercapaciteit tijdens inflammatoire omstandigheden werd de rol van het Duffy negatieve fenotype verder uitgezocht tijdens inflammatoire aandoeningen, in het bijzonder bij de sikkelcelziekte (Schnog, 2000, Afenyi-Annan, 2008, Mecabo, 2010, Nebor, 2010, Drasar, 2013, Araujo, 2015, Farawela, 2016). De meeste studies vonden echter geen significante invloed van het Duffy negatieve fenotype op bijvoorbeeld de ernst van de ziektesymptomen. De enige duidelijke link van het Duffy negatieve fenotype is die met een etnische benigne neutropenie, een conditie die waarschijnlijk geen klinische betekenis heeft (Thobakgale, 2014). De bufferfunctie van erythroïd DARC/ACKR-1 wordt beschouwd als vrij complex (Novitsky-Basso, 2012) en context-afhankelijk. De erythrocyt DARC/ACKR-1 binding van chemokinen is verzadigbaar, en zijn vele (>20 CXC- en CC-chemokinen) verwante chemokinen kunnen binden met verschillende affiniteiten (Mei, 2010). Onder extensieve inflammatoire omstandigheden zou de erythroïd DARC/ACKR-1 bindingscapaciteit eenvoudigweg kunnen worden overweldigd, in welk geval de aanwezigheid of afwezigheid van een buffercapaciteit niet meer van (klinische) betekenis is. Daarom wijst de door ons gevonden associatie tussen het Duffy negatieve fenotype en pre-eclampsie erop dat de inflammatoire condities in pre-eclampsie binnen de functionele range van erythroïd DARC/ACKR-1 vallen, en dat diens aanwezigheid of afwezigheid er dan wel degelijk toe doet.

Het Duffy negatieve fenotype biedt weerstand tegen infectie met *Plasmodium vivax*. Deze malariaparasieten dringen gast-erythrocyten binnen via erythroïd DARC/ACKR-1. De knock-out hiervan door mutatie wordt beschouwd als de evolutionaire drijfveer voor de verspreiding en fixatie in sub-Sahara-populaties. Recent ontdekte Duchene et al. een ander vermeend voordeel van het Duffy-null fenotype (Duchene, 2017). Neutrofielen gevormd in DARC/ACKR-1-deficiënt beenmerg van chimerische muizen vertoonden fenotypische veranderingen, zoals up-regulatie van moleculen voor de antimicrobiële verdediging (Duchene, 2017). Indien endotheliale DARC/ACKR-1 expressie behouden blijft in deze muizen (het menselijke Duffy negatieve fenotype nabootsend), trekken deze neutrofielen gemakkelijk naar de verschillende weefsels, met als "selectief-voordeel" een toegenomen toezicht in het weefsel door het "aangeboren" immuun systeem (Duchene, 2017, Palmblad, 2018). Indien eenzelfde scenario plaatsvindt bij de mens zou dit een verklaring kunnen zijn voor het nauwe verband tussen het Duffy negatieve fenotype en etnische benigne neutropenie (Duchene, 2017, Palmblad, 2018). Een keerzijde van verhoogde aantallen neutrofielen in de weefsels is echter een buitensporige response op diverse pro-inflammatoire stimuli (Palmblad, 2018).

Concluderend, de werkelijke klinische impact van het missen van erythroïd DARC/ACKR-1 in de zin van beschermend of nadelig, is nog steeds onduidelijk. Onze bevinding van een associatie tussen het Duffy negatieve fenotype en pre-eclampsie in **Hoofdstuk 2.1** moet nog door anderen worden bevestigd of weerlegd.

Lange keten meervoudige onverzadigde vetzuren ω3 (LCPω3) in de zwangerschap en pre-eclampsie

Onze bevinding uit 1999 van lage LCPω3 en LCPω6 gehaltes in de wanden van pre-eclamptische navelstrengbloedvaten (**Hoofdstuk 4.1.1**) werd geconfirmeerd in een door onszelf in 2009 uitgevoerde studie in Afrika, Tanzania (Huiskes, 2009). Deze studie met Tanzaniaanse vrouwen met een hoge DHA, EPA en AA status toonde soortgelijke pre-eclampsie versus controle verschillen in UA en UV, hoewel de verschillen minder uitgesproken waren vergeleken met de Curaçaose vrouwen met lagere innames van DHA en EPA. Onze bevindingen zijn in algemene overeenkomst met latere studies welke verschillende navelstrengcompartimenten onderzochten, zoals navelstrengplasma (Mehendale, 2008, Wadhwani, 2014) en -erythrocyten (Mehendale, 2008, Mackay, 2012, Wadhwani, 2016). Een lagere LCPω3 status, met name DHA, in pre-eclamptische navelstrengen werd vermeld door de meeste (Mackay, 2012, Wadhwani, 2014, 2016), maar niet alle (Mehendale, 2008) auteurs. Een lager LCPω6 in pre-eclamptische navelstrengen, zoals door ons gevonden, werd ook gevonden in navelstrengerythrocyten (20:3ω6, 22:4ω6, 22:5ω6) (Mackay, 2012) en -plasma (AA) (Wadhwani, 2014).

Mechanistisch is het mogelijk dat de LCPω6 status wordt gedownreguleerd bij een lage LCPω3 status met als doel om de balans tussen deze twee belangrijke LCPUFA families te bewaren. De rationale zou kunnen zijn het behoud van de voordelige effecten van LCPω3 op bloedstolling, vaatdiameter en inflammatie (Calder, 2017). Zo'n synergistische relatie tussen AA en LCPω3 bij een lage DHA+EPA status, en een antagonistische relatie bij een hoge DHA+EPA status, is inderdaad door ons aangetoond (Luxwolda, 2011).

Studies die zowel de maternale als de navelstreng LCP gehaltes hebben gemeten tijdens de bevalling, onthulden dat bij pre-eclamptische zwangerschappen de DHA en AA gehaltes in de navelstreng hoger zijn dan de respectievelijke gehaltes in de moeder (Wadhwani, 2014, 2016). Een gezonde placenta van de mens neemt bij voorkeur LCP, zoals AA en DHA, op en vervoert deze naar de foetale circulatie. Dit leidt tot hogere relatieve hoeveelheden in lipiden in navelstrengplasma en -erythrocyten vergeleken met maternale gehaltes. Het onderliggende fenomeen wordt "bio-magnificatie" genoemd (Crawford, 1976). Het behoud van "bio-magnificatie" in pre-eclamptische zwangerschappen is indicatief voor de efficiëntie waarmee het LCP transport wordt geprioriteerd ten gunste van de foetus. Deze prioritering zou tijdens pre-eclampsie zelfs ten koste kunnen gaan van de placenta's eigen behoefte aan DHA en AA. Inderdaad werd gevonden dat het DHA gehalte in placenta's van pre-eclamptische zwangerschappen lager is (Wang, 2005, Kulkarni, 2011a, Wadhwani, 2014). Een verminderde opname door pre-eclamptische placenta's, en een verhoogde oxidatieve stress in pre-eclamptische placenta's waarbij LCP worden "aangevallen", zijn naar voren gebracht als andere verklaringen (Wang, 2005, Kulkarni, 2011a).

LCPω3 gehaltes, en met name DHA, zijn lager in plasma en erythrocyten van pre-eclamptische vrouwen vergeleken met controle vrouwen die werden onderzocht in alle trimesters (Wadhwani, 2016, 2014), dan wel eenmalig in het derde trimester (Mackay, 2012, Mehendale, 2008, Kulkarni, 2011b). De fysiologische aanpassingen van de moeder

om de beschikbaarheid van LCPω3 voor placentair transport te vergroten, hetzij via een verhoging van haar LCP synthese, de mobilisatie van haar LCP voorraden, of beiden, lijkt tijdens pre-eclampsie te mislukken. Het afwijkende hormonale milieu tijdens pre-eclampsie (zoals lage oestrogeen spiegels) kan een toename van de LCP synthese capaciteit belemmeren. Er zijn goede aanwijzingen dat een toename van de LCP synthese wordt gedreven door oestrogenen (Burdge, 2005). Evenzo is een belangrijke route voor de moeder om DHA te mobiliseren uit haar lever, via de synthese van DHA-verrijkt fosfatidylcholine (PC) door middel van de PEMT route (fosfatidylethanolamine N-methyltransferase; in meer detail besproken bij het "1-Koolstofmetabolisme"). Deze route wordt waarschijnlijk ook door oestrogenen gereguleerd (Resseguie, 2007). De recent ontdekte expressie van de Mfsd2a receptor in de placenta (Toufaily, 2013), daarmee gedeeld met de hersenen, zou betrokken kunnen zijn bij de opname van dit DHA-verrijkte PC, met een gelijktijdig choline opname. Downregulatie van deze receptor is gerapporteerd in pre-eclamptische placenta's (Toufaily, 2013). In zwangerschappen die gecompliceerd waren door zwangerschapsdiabetes werd in de placenta's een reductie van het Mfsd2a proteïne gevonden, die gerelateerd was aan een lagere DHA in navelstrengserumlipiden (Prieto-Sánchez, 2017).

In onze studie in Tanzania zagen we hogere gehaltes van potentieel *de novo* gesynthetiseerde vetzuren in pre-eclamptische navelstrengvaten, naast een lager LCPω3 en LCPω6 (Huiskes, 2009). Soortgelijke bevindingen werden door onze groep gerapporteerd voor de navelstrengvaten van diabetische zwangerschappen (Type 1 diabetes en zwangerschapsdiabetes) (Dijck-Brouwer, 2005). Dit deed ons voorstellen dat het gezamenlijk overmatig verlies aan insuline-sensitiviteit in pre-eclamptische en diabetische zwangerschappen zou kunnen bijdragen aan de vergelijkbare verstoringen in navelstreng LCP gehaltes (Huiskes, 2009). Het mislukken bij pre-eclamptische vrouwen om hun LCP metabolisme aan te passen zou dus, gedeeltelijk, kunnen worden toegeschreven aan de metabole situatie. Mechanistisch kan de overmatige insuline-resistentie bij pre-eclamptische en diabetische zwangerschappen de drijver zijn van *de novo* lipogenese in de lever uit polaire voorlopers, voornamelijk glucose. De hieruit voorkomende *de novo* gesynthetiseerde vetzuren "verdunnen" mogelijk de LCP gehaltes in "very low-density lipoproteïne" (VLDL)-TG, hetgeen een belangrijke bron is voor placentaire (lipoproteïne lipase gemedieerde) vetzuuropname, om uiteindelijk te worden weerspiegelt in pre-eclamptische navelstrengvaten (Huiskes, 2009). Toegenomen *de novo* lipogenesis is een belangrijk kenmerk van leververvetting (i.e. non-alcoholische leververvetting; NAFLD), zoals bekend in de niet-zwangere situatie en te worden gedreven door insuline-resistentie (Engin, 2017). Niet-zwangere obese patiënten met NAFLD hebben een gereduceerde Δ5- en vooral Δ6- desaturase-activiteit en een gereduceerde LCP synthese (Araya, 2010). Een hypothese is dat een soortgelijk scenario van vetaccumulatie plaatsvindt in de pre-eclamptische lever, met afgenomen hepatische LCP synthese (Mackay, 2012).

Uit een longitudinale studie bleek dat de daling van het DHA in erythrocyten van pre-eclamptische vrouwen al kon worden aangetoond op GW16-20. Deze lagere gehaltes waren gecorreleerd met lagere gehaltes in navelstrengvaten in een later stadium

(Wadhwani, 2016). Blijkbaar is de moeder al vroeg in de zwangerschap niet in staat haar LCP metabolisme aan te passen. Nervonzuur (24:1ω9) is een vetzuur dat betrokken is bij de myelinesynthese en de ontwikkeling van de witte stof. De gelijktijdige daling van 24:1ω9 in erythrocyten van zowel de moeder als de navelstreng veroorzaakte bezorgdheid over de neurologische ontwikkeling van pre-eclamptische nakomelingen (Wadhwani, 2016). Een systematische review toonde aan dat pre-eclamptische nakomelingen andere uitkomstmaten hebben van cognitie, gedrag en stemming, indien vergeleken met nakomelingen uit normale zwangerschappen (Figueiró-Filho, 2017). Neurologische MRI beelden waren in overeenstemming met, bijvoorbeeld, gedragsafwijkingen bij pre-eclamptische nakomelingen (Mak, 2018). Andere meta-analyses lieten een ~30% verhoogd risico zien voor autisme-spectrum stoornissen (Dachew, 2018, Maher, 2018, Xu, 2018) en aandachtstekort-hyperactiviteitstoornis (ADHD) (Maher, 2018) bij pre-eclamptische nakomelingen; vergelijkbaar met nakomelingen uit andere hypertensieve stoornissen in de zwangerschap (Maher, 2018, Xu, 2018).

Het is algemeen aanvaard dat de huidige maternale LCPω3 status van vele vrouwen onvoldoende is om maternale LCPω3 depletie tijdens de zwangerschap te voorkomen, hetgeen mogelijke nadelige gevolgen suggereert voor zowel moeder als kind. Dit voedde de belangstelling voor antenatale LCPω3 suppletie ter voorkoming van maternale depletie (met bijvoorbeeld consequenties voor haar mentale gesteldheid) en om de foetale LCPω3 status veilig te stellen. Inderdaad rapporteerden verschillende observationele studies positieve associaties tussen de maternale visinname en uitkomsten voor neurologische ontwikkeling en gedrag van hun kinderen die bestudeerd werden op verschillende leeftijden (tussen 6 maanden en 9 jaar), en zoals gereviewed door Starling et al. (Starling, 2015). Wij onderzochten, in een open label studie-opzet, het effect van drie lage-doses LCPω3 supplementen op de foetale LCPω3 status tijdens ongecompliceerde zwangerschappen (**Hoofdstuk 4.1.2**). We vonden biochemische verbeteringen van de foetale LCPω3 status bij LCPω3 doseringen van 528 mg/d (DHA ~185 mg/d) en 1.008 mg/d (DHA ~369 mg/d). Deze DHA supplement-doseringen zijn respectievelijk lager dan, en gelijk aan, de huidige DHA aanbevelingen van 300 mg/d voor zwangere vrouwen (Koletzko, 2014). In een recente dosisbepalingsstudie uitgevoerd door onze groep, bleek een supplement van 750 mg/d LCPω3 (DHA+EPA) noodzakelijk voor het bereiken van de optimale maternale LCPω3 status (Stoutjesdijk, 2018), zoals ontleend aan de gegevens van Tanzaniaanse vrouwen die bij Lake Victoria wonen met een levenslange hoge inname van LCPω3 uit vis (Kuipers, 2011).

Niettemin rapporteerde een recente Cochrane-gebaseerde systematische review weinig verschillen in cognitieve- en gedrags-uitkomsten na antenatale LCPω3 suppletie versus placebo of geen ω3 in gerandomiseerde gecontroleerde trials (Middleton, 2018). Er was onvoldoende bewijs voor gunstige effecten op de maternale mentale gezondheid (postnatale depressie) (Middleton, 2018). Antenatale LCPω3 verlaagde het risico op pre-terme en zeer pre-terme geboorten en "mogelijk" het risico op pre-eclampsie (Middleton, 2018). Blijkbaar werken LCPω3 op zwangerschapsstoornissen met inflammatoire elementen door de LCPω3/LCPω6 ratio te verbeteren. Verdere studies zijn nodig om

de exacte onderliggende mechanismen te ontrafelen (Middleton, 2018), zoals de rol van de recent ontdekte gespecialiseerde "pro-oplossende" mediatoren die gegenereerd worden uit EPA en DHA (Serhan, 2014). De auteurs (Middleton, 2018) concludeerden dat maternale LCPω3 suppletie een simpele en goedkope manier is om het risico op een pre-terme geboorte te verlagen. Om echter de maternale uitkomsten en die van de kinderen beter te kunnen beoordelen dienen inspanningen te worden geleverd om het beste type LCPω3 en dosis te identificeren, als ook die vrouwen die er de meeste baat van zullen hebben (Middleton, 2018). De basale LCPω3 status was onderworpen aan variaties in gerandomiseerde gecontroleerde trials. De responsen zijn meer uitgesproken bij een lagere LCPω3 status (Stoutjesdijk, 2018), en vrouwen met een lage habituele LCPω3 inname (een determinant van de LCPω3 voorraad) zullen de meeste baat hebben van preventieve LCPω3 supplementen. Bij vrouwen die hun LCP metabolisme niet kunnen aanpassen (ongeacht de LCPω3 voorraad), zoals pre-eclamptische vrouwen, zijn gunstige effecten te verwachten voor de moeder, de placenta en de nakomelingen.

1-Koolstofmetabolisme

We vonden geen associatie tussen pre-eclampsie en maternaal en foetaal MTHFR C677T in onze studiepopulatie bestaande uit Afrikaans-Caraïbische vrouwen met ruime Kaukasische vermenging (**Hoofdstuk 2.2**). Talrijke studies hebben gerapporteerd over de associatie tussen pre-eclampsie en maternaal MTHFR C677T. Ze laten geen eenduidige resultaten zien. Variaties in de folaatstatus van de studiepopulaties kunnen een plausibele verklaring vormen omdat de aanwezigheid van voldoende folaat het variant MTHFR C677T enzym stabiliseert. Bovendien is pre-eclampsie een heterogene aandoening met onderscheidende pathofysiologische cascades en een wijde variëteit aan interindividuele genetische predisposities. Een zeer recente meta-analyse vond geen associatie tussen MTHFR C677T en pre-eclampsie (Zhang, 2019). Deze uitkomst contrasteert met die van een vorige meta-analyse die 54 studies insloot met 7.398 casussen en 11.222 controles (Wu, 2015). Deze analyse liet stratificatie toe voor etniciteit, waarbij een bescheiden associatie werd gevonden tussen het MTHFR C677T allel en pre-eclampsie, in vooral Kaukasische en Aziatische populaties (Wu, 2015).

In tegenstelling tot het maternale genotype, stelden slechts een paar studies het placentaire/foetale MTHFR C677T genotype aan de orde. Deze studies rapporteerden een associatie (Chedraui, 2015) en geen associatie (del Gobbo, 2018, Mislanova, 2011) met pre-eclampsie; waarbij laatstgenoemde in overeenstemming is met onze ongepubliceerde gegevens uit 2001 (**Hoofdstuk 2.2**).

Bij het bestuderen van de relaties tussen plasma tHcy met plasma betaïne en plasma folaat stelden we voor dat de relatieve contributie van betaïne in homocysteïne remethylering toeneemt tijdens de zwangerschap (**Hoofdstuk 4.2.1**). *In vivo* steun voor onze bevindingen kwam van een studie over de dynamiek van deuterium-gelabelde choline, toegediend aan zwangere vrouwen in hun derde trimester en vergeleken met niet-zwangere vrouwen (Yan, 2013). Het traceren van de isotopische verrijkingen van choline metabolieten bij zwangere vrouwen liet zien dat, conform onze hypothese, meer betaïne

wordt gebruikt als methyl-donor, maar dat daarnaast minder van het geconsumeerde choline geoxideerd wordt tot betaïne (Yan, 2013). Dit verklaart de lagere plasma betaïne gehaltes bij zwangere vrouwen (Yan, 2013) en is in overeenstemming met de door ons gedocumenteerde daling van het plasma betaïne tijdens de zwangerschap. Choline uit de voeding is hoognodig voor PC synthese via de zogenaamde "CDP route", die noodzakelijk is voor VLDL biosynthese en de daaropvolgende export uit de lever; een proces dat sterk is toegenomen tijdens de zwangerschap. Niet-zwangere vrouwen met verschillende SNPs in het folaat metabolisme, zoals MTHFR C677T vertoonden vergelijkbare veranderingen in de dynamiek van choline zoals die zijn waargenomen tijdens de zwangerschap, namelijk, het hogere gebruik van betaïne voor de methionine synthese en het meer partitioneren van choline naar de CDP route (Ganz, 2016). Bij zwangere vrouwen was er geen verschil in de choline-dynamiek tussen de genotypen. Blijkbaar is het 1-koolstofmetabolisme bij zwangere vrouwen gereprogrammeerd om beter om te kunnen gaan met verminderde folaat beschikbaarheid voor gebruik als methyl-donor in de homocysteïne remethylering.

Toegenomen gebruik van betaïne voor homocysteïne remethylering draagt bij aan het lage homocysteïne gehalte tijdens de zwangerschap, naast bijvoorbeeld de homocysteïne opname door de placenta. Verlagen van de homocysteïne/methionine ratio via remethylering (de methionine cyclus) dient diverse belangrijke maternale methylerings-reacties. Een van deze reacties is de successievelijke methylering van fosfatidylethanolamine (PE) naar PC. Deze reactie wordt gekatalyseerd door PEMT en is de geprefereerde route voor de productie van DHA-verrijkt PC (dit in tegenstelling tot de CPD route). Na de incorporatie van PC in de fosfolipide fractie in VLDL (en mogelijk ook HDL) wordt DHA-verrijkt PC uit de lever geëxporteerd, waardoor dit DHA uit de lever beschikbaar komt voor andere weefsels, de hersenen incluis. In zwangere ratten is PEMT ge-upreguleerd en levert DHA-verrijkt PC een grote bijdrage aan het toegenomen maternale plasma DHA gehalte (Chalil, 2018). Oestrogenen zorgen voor een up-regulatie van PEMT (Ressequie, 2007) en waarschijnlijk ook van BHMT, en zwangere vrouwen gebruiken meer betaïne in de PEMT route *in vivo* (Yan, 2013). Een dergelijke dubbele up-regulatie mobiliseert DHA uit de maternale lever om hiermee in de hoge foetale (hersenen) DHA behoefte te voorzien. Selectieve overdracht van PEMT-afkomstig PC naar de navelstreng ten opzichte van CPD-afkomstig PC steunt dit concept in sterke mate (Yan, 2013).

Pre-eclamptische vrouwen hebben een hoger circulerend homocysteïne zoals gemeld door vele onderzoekers, waaronder wijzelf (**Hoofdstuk 4.2.2**). Een recente meta-analyse bevestigde het verband tussen toegenomen plasma homocysteïne en pre-eclampsie (Gaiday, 2018). Onze bevindingen van een hoger plasma homocysteïne in pre-eclampsie en met name de sterke stijging van het plasma homocysteïne bij een laag plasma folaat/vitamine B_{12}, suggereert dat pre-eclamptische vrouwen het belang van betaïne om als methyl-donor te functioneren hebben verloren, indien vergeleken met gezonde zwangere tegenhangers. De afwijkende hormonale status in pre-eclampsie is een waarschijnlijke verklaring voor dit verlies. Een hieruit voortkomend gevolg kan zijn dat bij pre-eclamptische zwangerschappen minder DHA uit de maternale lever ter beschikking komt

voor placentaire opname en de daaropvolgende depositie in de foetus, met name als ook de folaat en vitamine B_{12} statussen laag zijn.

Concluderend is het duidelijk geworden dat er een intieme relatie bestaat tussen DHA en het 1-koolstofmetabolisme met belangrijke rollen voor choline en zijn geoxideerde metaboliet betaïne. Mobilisatie van maternaal DHA uit de lever via de PEMT route is een mechanisme om de beschikbaarheid van DHA te vergroten en daarmee de hoge foetale behoeften af te dekken, vooral in het derde trimester (zie LCP). Dit proces is waarschijnlijk aangetast in pre-eclampsie. De afhankelijkheid van de folate/vitamine B_{12} status tijdens pre-eclampsie en in de niet-zwangere situatie, en de verschuiving naar een afhankelijkheid van choline/betaïne tijdens gezonde zwangerschappen, benadrukken het belang van nutritionele interacties en hun variaties onder verschillende (hormonale) omstandigheden.

EPILOOG

Obesitas, lifestyle in het algemeen, en insuline sensitiviteit in het bijzonder, behoren tot de weinige modificeerbare risicofactoren ten behoeve van de preventie van pre-eclampsie, met name pre-eclampsie van het "late-onset type". De huidige obesitas-epidemie in Westerse samenlevingen voorspelt dat de incidentie van dit type pre-eclampsie in de komende decennia aanzienlijk zal gaan stijgen. Modificatie van het obesitas-gerelateerde risico op pre-eclampsie zou bij voorkeur (lang) voor de conceptie moeten beginnen. Het stimuleren van een gezonde levensstijl is niet alleen belangrijk voor vrouwen met obesitas, maar voor iedere vrouw met een zwangerschapswens, maar eigenlijk voor ons allemaal.

Het is van uiterst belang dat zwangere vrouwen geen leefstijl adopteren waarbij het "eten voor twee" of "veel rust nemen" wordt aanbevolen. Ze dienen betrokken te blijven bij de normale dagelijkse activiteiten en deel te nemen aan het arbeidsproces zolang dat in overeenstemming is met hun fysieke conditie. Echter, in onze huidige hightech Westerse samenleving vermindert de dagelijks deelname aan lichamelijke activiteiten, zowel gedurende de werkuren als in onze vrije tijd, waarbij laatstgenoemde deels is vervangen door andere activiteiten zoals het in contact blijven via de sociale media en internet winkelen. Dagelijkse lichamelijke activiteiten hebben een substantiële impact op ons energieverbruik, maar het behouden of het verbeteren van insuline-sensitiviteit en metabole flexibiliteit zijn waarschijnlijk het belangrijkst. Het bestaan van "gezonde obesitas" suggereert dat "fitness" wel eens belangrijker kan zijn dan "fatness". Zelfs subtiele onvolkomenheden in onze dagelijkse energiebalans zullen op den duur impact hebben, waarbij het surplus opgeslagen wordt in het witte vetweefseldepot. Dit compartiment heeft een opmerkelijke, ogenschijnlijk onbeperkte, capaciteit om uit te dijen, wat tot op zekere hoogte nodig is in de eerste helft van de zwangerschap; i.e. gedurende de expansiefase voor de opbouw van vetreserves die nodig zijn in de latere zwangerschap. Gedurende deze periode is lichamelijke activiteit van groot belang, met

name voor vrouwen met obesitas. Wandelen is goedkoop en kan in bijna alle gevallen gemakkelijk worden geïmplementeerd in de dagelijkse routine.

Een gebalanceerde voeding is de tweede pijler van een gezonde levensstijl, zowel voor als tijdens de zwangerschap. Het voldoen aan voedingsaanbevelingen, zoals die voor de LCPω3 en de nutriënten betrokken bij 1-koolstofmetabolisme (folaat, vitamine B_{12}, vitamine B_6, betaïne, choline) zijn belangrijk voor het behoud van respectievelijk een anti-inflammatoire/pro-inflammatoire balans en de talloze belangrijke methylerings-reacties. Pre-eclampsie belemmert de normale aanpassingen die in de zwangerschap plaatsvinden in bijvoorbeeld de LCP en 1-koolstofmetabolismen, wat de speciale behoefte aan deze voedingsstoffen versterkt, zowel voor het welzijn van de moeder als haar baby. Omdat het ontstaan van pre-eclampsie in vrouwen met obesitas van inflammatoire aard is, kan een optimale LCPω3/ω6 balans van grote waarde zijn. Een hoge kwaliteit voeding met vis, vers fruit, groenten en vezels, in combinatie met het vermijden van snelle suikers, sterk bewerkte voeding en "junk food", kan een voordeel hebben boven voedingssupplementen vanwege de talrijke nutriënt interacties. Zorgvuldigheid moet worden betracht om de bovenste limieten van PCB, dioxine, kwik en vitamine A niet te overschrijden, terwijl de huidige aanbevelingen voor foliumzuur en vitamine D supplementen dienen te worden opgevolgd. De benodigdheden aan kritische nutriënten in vele Westerse gemeenschappen, zoals vitamine B_{12}, jodium en selenium, behoeven mogelijke dekking door middel van supplementen, of de, tot nu toe weinig onderzochte, consumptie van schelpdieren en zeewier.

De momenteel meest effectieve preventiestrategie met geneesmiddelen voor het reduceren van het pre-eclampsie risico in hoog-risico populaties is de behandeling met lage dosis aspirine ("aspirine for evidenced-based preeclampsia prevention trial"), bij voorkeur startend voor de 16de zwangerschapsweek (ACOG Comité, 2018). Het is intrigerend dat aspirine tegenwoordig beschouwd wordt als een zogeheten "caloric-restriction mimetic"; i.e. een farmacologisch middel dat autofagie induceert vergelijkbaar met "hongeren". Autofagie geïnduceerd door calorie-beperking genereert energie door het kataboliseren van endogene bronnen, waarbij beschadigde en bijna beschadigde organellen/proteïnen in cellen worden opgeruimd (het "schoonvegen van cellen"). Autofagie wordt beschouwd als een "anti-verouderingsresponse". Fibroblasten van honderdjarigen zien eruit alsof zij op een "natuurlijke manier calorie-beperkt" zijn. Het optimaliseren van de opruimcapaciteit van de placenta en haar vermogen om met stress om te gaan vormen mogelijk een preventief mechanisme waarmee aspirine het risico op pre-eclampsie vermindert. Overdadige voeding remt autofagie, en het behoud van energiebalans door het respecteren van het eenvoudige advies om s' nachts te vasten kan beschermend werken tegen een dergelijk remmend effect.

Er is epidemiologisch bewijs dat kinderen van pre-eclamptische moeders een verhoogd risico hebben op toekomstige cardiovasculaire en metabole ziekten. Een genetische component in de efficiëntie van adaptieve stressresponsen dicteert een vergelijkbare efficiëntie in de placenta's syncytiotrophoblast (ST) en de nakomelingen. Indien de ST niet goed is omgegaan met doorgemaakte stress, dan zullen de pre-eclamptische

nakomelingen mogelijk ook niet goed om gaan met toekomstige stress. De toekomstige risico's verbonden met een geboorte na een pre-eclamptische zwangerschap worden tegenwoordig onderkend. Het kind wordt niettemin blootgesteld aan de verleidingen van de huidige overvloedige samenleving en een gezonde levensstijl dient het primaire advies te zijn. Zulke aanbevelingen gelden ook voor hun moeders, die ook een verhoogde kans hebben op bovengenoemde ziektes.

Pre-eclampsie vertoont opmerkelijke overeenkomsten met ouderdom-gerelateerde ziektes zoals neurodegeneratieve aandoeningen. De pre-eclamptische placenta zou de uitscheiding van toxische amyloïd eiwitten gemeen kunnen hebben met neurodegeneratieve neuronen, wat de verspreiding van deze eiwitten in respectievelijk de moeder en de neuronale omgeving kan veroorzaken. Fundamenteel vergelijkbare pathofysiologische cascades zouden deze overeenkomsten kunnen onderbouwen, zoals een aangetast lysosomaal compartiment. Het delen van inzichten, ontdekkingen en nieuwe strategieën kan voor beide onderzoeksvelden voordelig zijn.

Vroege opsporing van ziekte is een belangrijk aandachtspunt in pre-eclampsie onderzoek. Een veelbelovend en tamelijk nieuw onderzoeksveld is de uitscheiding van vesikels (micro-vesikels, exosomen) in het extracellulaire compartiment, zoals momenteel wordt onderzocht in de oncologie. Aangetoond is dat de placentaire ST op levendige wijze exosomen uitscheiden. De ladingen van micro-vesikels en exosomen worden geselecteerd en beïnvloed door de fysieke conditie van de uitscheidende cel. Deze partikels zouden functies kunnen hebben in de "surveillance" in diverse pathologische situaties, en idealiter vroege veranderingen in deze toestanden kunnen weergeven. Ze kunnen derhalve in de toekomst gebruikt worden als vroege bio-markers voor pre-eclampsie. Spannend is de mogelijkheid om de lading van deze deeltjes te modificeren. Met informatie over de juiste adreslabels kunnen zulke "tags" geneesmiddelen of bepaalde ladingen (zoals RNA species) bezorgen bij aangewezen doelcellen. Implementatie van technologische innovaties zal waarschijnlijk realiteit worden en daarmee een toenemend onderdeel gaan uitmaken van de huidige klinische praktijk.

Onderzoek naar de preventie en behandeling van pre-eclampsia, en feitelijk naar vele andere ziekten, kan meer dan ooit profiteren van "ruimdenkendheid" door het adopteren van inzichten uit verschillende wetenschappelijke velden. De ontwikkeling van farmacologische middelen, en de implementatie van hightech innovatieve oplossingen zijn belangrijk, maar uitsluitend indien ze samengaan met het propageren van een gezonde leefstijl.

Dankwoord, curriculum vitae, list of publications

DANKWOORD

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CURRICULUM VITAE

Franciska Verena Aarts werd geboren op 28-10-1959 te Den Haag, en groeide op in de aangrenzende gemeente Voorburg. Na haar middelbare school (Openbare Dalton Scholengemeenschap Voorburg Leidschendam; ongedeeld VWO) studeerde ze Fysiotherapie aan de Haagsche Academie voor Lichamelijke Opvoeding. Zij behaalde in 1983 haar diploma, en begon aansluitend aan haar studie Geneeskunde aan de Rijks Universiteit te Leiden. In maart 1990 behaalde zij haar artsendiploma en van april 1990 tot januari 1992 was zij werkzaam als arts-assistent niet in opleiding (AGNIO) op de afdeling gynaecologie/obstetrie van het Bronovo ziekenhuis te Den Haag. Na de geboorte van haar eerste zoon Jeroen in mei 1992 was zij nog enkele maanden werkzaam op de obstetrie afdeling van het Diaconessenhuis te Voorburg. In januari 1993 trouwde ze met Eric Velzing en in maart 1993 vertrok ze met haar gezin voor enkele jaren naar Curaçao. Hier heeft ze gewerkt aan de evaluatie van een nieuw perinataal registratiesysteem voor verloskundigen (GGD Curaçao) en nachtdiensten gelopen op de afdeling obstetrie van het Sint Elisabeth Hospitaal te Curaçao (Anna Paviljoen). Zij begon aan haar promotie onderzoek, met een LCPω3 suppletiestudie onder gezonde zwangere vrouwen, begeleid door haar promotor prof. dr. F.A.J Muskiet (Universitair Medische Centrum Groningen, UMCG). In februari 1994 beviel ze van haar tweede zoon Jasper en in december 1996 keerde het gezin terug naar Nederland. De ernst van de zwangerschapscomplicatie pre-eclampsia onder Curaçaose zwangeren heeft ertoe bijgedragen dat deze zwangerschapscomplicatie een prominente plaats in haar proefschrift kreeg onder begeleiding van prof. dr. A.J. Duits (bloedbank Curaçao) en prof. dr. S.A. Scherjon. In de loop van de jaren 2000 zijn de benodigde publicaties behaald. Het lange verdere verloop van de promotie had te maken met de fascinatie voor en gedegen studies over deze zwangerschapscomplicatie en gerelateerde onderzoeksvelden.

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