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Genetic basis for necrotizing enterocolitis - risk factors and their relations to genetic polymorphisms

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1. ABSTRACT

Necrotizing enterocolitis (NEC) is a common, life-threatening neonatal gastrointestinal disease; it affects approximately 11% of extremely premature neonates. The etiology of NEC is multifactorial. Risk factors may roughly be grouped into four main categories: prematurity; transient ischemia of the intestine; local/systemic inflammation predisposing the bowel to injury, and therapeutic interventions. Recent studies have shown that carrier state of genetic polymorphisms may be associated with perinatal morbidity, including NEC. In perinatal disorders, the significance of genetic variants of cytokines, the renin-angiotensin-aldosterone system, and surfactant proteins have been investigated most widely. Positive findings indicate the implication of genetic polymorphisms of proinflammatory cytokines in premature birth; angiotensin converting enzyme in perinatal adaptation and angiotensin type 1 receptor in the closure of ductus arteriosus; surfactant proteins A and B in respiratory distress syndrome; interleukin (IL)-6 in sepsis, and IL-4-receptor α chain and IL-18 in NEC. This review provides an insight into the genetics of NEC and summarizes genetic data in light of pathologic processes leading to NEC.

2. INTRODUCTION

Necrotizing enterocolitis (NEC) is a common neonatal gastrointestinal disease that affects approximately 11% of premature neonates, born with a birth weight ≤ 1500 gram (very low birth weight, VLBW). The average mortality rate of NEC ranges from 20% to 40% (1). With improving care at the end of the presurfactant era, the incidence of NEC declined briefly, but increased after surfactant use became a standard of care (2). This phenomenon has mainly been attributed to the fact that most VLBW babies are able to overcome a number of previously fatal health problems and survive, and thus, render themselves susceptible to NEC. At least 80% of patients are preterm and/or VLBW. The incidence of the disease is inversely proportional to the gestational age (3,4). Infants of extremely low birth weight (<1000 g) and those 28 weeks or less of gestational age are at exceptionally high risk for NEC.

The most common signs of NEC include abdominal distension, vomiting, increased gastric residuals, lethargy, apnea, bradycardia, or guaiac-positive stools. NEC has been staged according to the characteristics and

severity of symptoms (5). In stage I, there are no clear radiological signs, and these nonspecific manifestations suggest the disease but give no indication of the status of the bowel or the prognosis. In stage II, the diagnosis is clearly established, with the appearance of pneumatosis intestinalis or free air in the portal vein. Stage III indicates more advanced disease, as manifested by shock, disseminated intravascular coagulation, acidosis, thrombocytopenia, and sometimes, intestinal perforation.

Although extensive research concerning the etiology of this devastating disease has taken place, it has not yet been fully elucidated. The main risk factor of NEC is prematurity (6). The incompletely innervated, poorly organized, relatively permeable epithelial barrier is vulnerable to bacterial colonization and pathogenic overgrowth (7), while the underdevelopment of immune system raises the possibility of uncontrolled release of pro-inflammatory cytokines. The risk of transient bowel ischemia also increases due to vasoregulation disturbances and increased risk of thromboembolism in VLBW infants.

Presumably, the initial insult in the chain of events leading to NEC could be perinatal hypoxia or a mild postnatal infection, either of which results in mild mucosal damage (8). Once bacterial translocation or toxin absorption have occurred, the production of local inflammatory mediators is likely to be a key step in the pathogenesis of NEC (7). The consequently occurring inflammatory response consists of leukocyte adhesion and activation, complement activation, release of cytokines, reactive oxygen species, and nitric oxide, and results in areas of focal necrosis of the intestine (9). This injury may stimulate excessive pro-inflammatory cytokine production in the intestinal wall (10,11). Complement activation is also a key step for blood clotting and the local mesenteric thromboembolism is another factor leading to intestinal ischemia. Thrombosis may also develop as a result of therapeutic interventions such as placement of an umbilical artery catheter.

Based on the above outlined pathomechanisms, the risk factors of NEC may roughly be grouped into four main categories: prematurity *per se*; transient ischemia of the intestine; local/systemic inflammation predisposing the bowel to injury, and therapeutic interventions (see Figure 1). However, one should consider that this grouping is highly subjective and there are numerous interconnections between the different pathogenetic factors.

While NEC is mainly a disease of preterm neonates, it still affects only a minority of this vulnerable population, which suggests an individual susceptibility toward NEC and its risk factors. Genetic polymorphisms may be an important factor in this individual susceptibility. The capability of individuals to secrete proteins (cytokines, enzymes, receptors etc.) that may be involved in the pathogenesis of disease varies greatly. The biosynthesis of those proteins is under strict genetic control. Encoding genes have been cloned and sequenced and several genetic polymorphisms have been uncovered. Many of them are single nucleotide polymorphisms (SNPs) with the transition of one nucleotide to another one. The majority of genetic variants are present in the non-coding intron regions. In these cases, the variants bear only an indirect relevance, if any, to gene function. Only about 2-3 percent of the genetic variants affect the promoter or the encoding regions (exons) of the gene. Variants in the promoter region may influence the speed of gene transcription, while those in the exons may lead to the alteration of the amino acid sequence of the encoded proteins. As a result, the quantity or the quality of the protein produced may change.

This review summarizes those genetic polymorphisms that have been suggested to contribute to the development of NEC and its risk factors grouped by prematurity, ischemia, and inflammation.

3. PREMATURITY

Prematurity is the only factor consistently found in epidemiologic studies to be an independent determinant of NEC. The increased susceptibility is attributed to an immature mucosal barrier and barrier response (6,7). Furthermore, the humoral and cellular immune responses are also impaired in the premature infant. Specifically, secretory IgA deficiency in the terminal ileum and colon facilitates bacterial translocation, while inadequate T-lymphocyte activity compromises response to foreign antigens (12, 13).

Premature birth may be the result of several pathogenic pathways. However, 70-80% of cases are attributed to maternal infection and chorioamnionitis (CA), and 15% to pre-eclampsia (PE). The genetic background of both disorders is the subject of extensive studies.

3.1. Chorioamnionitis

An increase of amniotic fluid and placental cytokine concentrations, such as interleukin (IL)-1β, IL-6, and IL-8 has been reported with term labor (14,15). In tissues from women who have delivered preterm, the levels of these cytokines show an even further increase, regardless of their apparent infection status (16). This supports the general assumption that inflammatory mediators play a crucial role in human birth and in the pathogenesis of premature birth. Pro-inflammatory cytokines have an impact on events associated with the initiation of labor (17). IL-1β and TNF-α levels are strongly correlated with the production of prostaglandins which contract the uterus (18). IL-1β receptor, an anti-inflammatory cytokine, counteracted these effects of IL-1β (19). Cytokines may facilitate the induction of preterm birth on the fetal side as well (20). Matrix metalloproteases, which are responsible for the breakdown of fetal membranes, are activated in chorioamnionitis, probably by cytokines such as TNF-α and IL-6 (21).

Data support the implication of cytokine genetics in spontaneous preterm birth (sPTB) risk. Simhan *et al.,* found that IL-6 $^{-174}$CC genotype occurs less frequently among women with sPTB less than 34 weeks, indicating that this SNP may reduce the risk of sPTB (22). The prevalence of TNF-α G^{-308}A alleles was also investigated

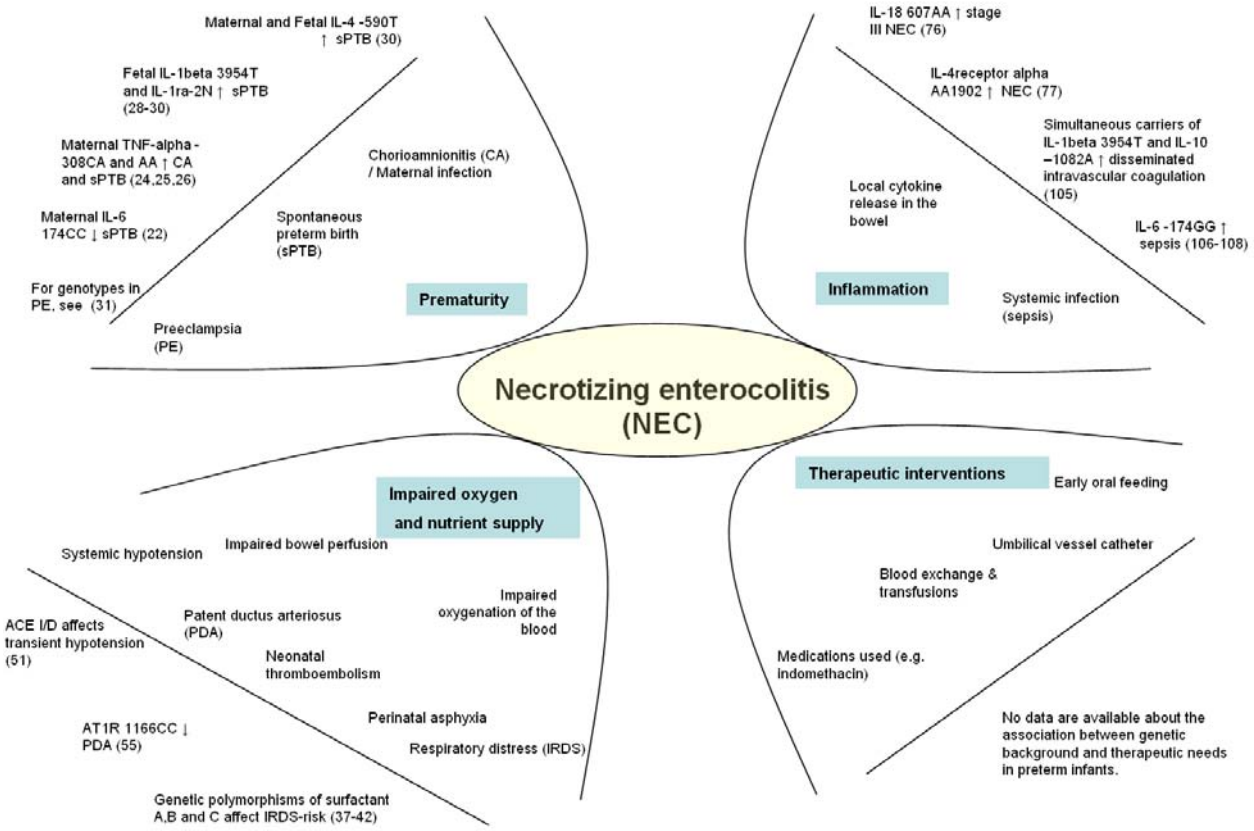

Figure 1. Schematic figure of risk factors grouped according to prematurity; impaired oxygen and nutrient supply of the bowel; inflammation; and therapeutic interventions. Figure cites only those studies (ref. in parentheses) that observed associations between perinatal morbidity and genetic polymorphisms. Please, note that there are numerous interactions between risk factors, perinatal disorders and necrotizing enterocolitis.

in women with sPTB. Dizon-Townson found no difference between TNF-α genotypes of sPTB and control women (23). To the contrary, others found tha carriers of TNF-α $^{-308}$A allele are at an increased risk for CA (24) and sPTB (25). Maternal homozygous carriage of the TNF-α C^{-863}A polymorphism was associated with preterm delivery and adverse neonatal outcome in a recent study of women with preterm labor before 34 weeks of gestation. However, neither maternal nor fetal carriage of the TNF-α G^{-308}A polymorphism was associated with adverse neonatal outcome (26).

These studies tested the association between maternal genotype and pregnancy outcome. In CA, however, the fetus is the major source of increased pro-inflammatory cytokines. Until now, however, only a few studies have been done to describe the association between fetal cytokine genotype and preterm birth. The fetal carriage of IL-1β^{3954}T alleles (that are associated with increased IL-1β activity) presented an increased risk for sPTB (27), while the presence of (interleukin-1 receptor antagonist) IL-1ra-2N alleles in the fetus had no impact on sPTB (28). Two recent findings also support the significance of fetal homozygosity for IL-1ra-2N allele in prematurity (29, 30).

These results suggest that both maternal and fetal cytokine genetic variants may determine risk for premature birth. While a strong genetic relationship exists between the mother and offspring, it would be of interest for those studies that enroll both mother and offspring to establish the independent contribution of their genetic background to pregnancy outcome. The importance of the common genotyping of the mother and offspring may be highlighted by the results of Kalish *et al.*, who observed that maternal and fetal carriage of the IL-4 $^{-590}$T allele – that leads to increased IL-4 levels - but not IL-10 A^{-1082}G, IL-1β C^{3954}T variant and another IL-1ra variant than 2N, were associated with the risk of sPTB in multifetal pregnancies (30). Therefore, the interaction between maternal and fetal genotypes should be taken into account when establishing the risk of premature birth in future studies.

3.2. Preeclampsia

Another frequent cause of premature birth is preeclampsia (PE). The genetic background of PE has been extensively investigated and several SNPs have been suggested to play a role in its pathogenesis, including the polymorphisms of the renin-angiotensin-aldosterone system (RAAS), cytokine polymorphisms, genes influencing blood clotting etc. (reviewed in (31)). However, review of data

investigating the association of genotype with the risk of PE is beyond the scope of this paper.

4. COMPLICATIONS POSSIBLY LEADING TO DISTURBED OXYGENATION OF THE BOWEL

The physiologic characteristics of the newborn intestinal circulation are unique when compared with the adult condition (32). Most importantly, intestinal vascular resistance across newborn intestine is low. The low vascular resistance decreases the capacity of this vasculature to respond to systemic circulatory perturbations, such as hypotension and arterial hypoxemia.

Neonatal bowel may become ischemic via several mechanisms. The early perinatal life is frequently complicated with transient hypoxia due to immaturity of the lung such as respiratory distress syndrome (RDS). Furthermore, neonates are extremely prone to vasoregulatory disturbances such as patent ductus arteriosus (PDA) or transient hypotension that are characterized by local and/or systemic hypotension. The hypotension may affect the gastrointestinal tract as well. Thromboembolic events may also occur and induce bowel ischemia. Bowel ischemia may invariably lead to ischemic necrosis, inflammation and bacterial overgrowth, all present in varying degrees and severity in NEC.

4.1. Poor oxygenation

4.1.1. Respiratory distress syndrome

The most characteristic feature of RDS is surfactant deficiency. Surfactant deficiency in RDS is mainly caused by immaturity and a lack of differentiation of the alveolar epithelial cells involved in surfactant synthesis and secretion. There are four main forms of surfactant proteins. SP-A has roles in surface activity and regulatory roles, particularly in innate immunity (33,34); SP-B is essential for the processing of surfactant and for the surface activity (35); SP-C has roles in surfactant metabolism and function; the regulatory roles of SP-D mainly pertain to innate immunity (36).

Several studies support the importance of genetic polymorphisms in surfactant production. Both SP-A and SP-B associate with susceptibility to RDS. A Finnish study of 688 premature infants additionally showed that the SP-A and SP-B gene variations associated interactively with the risk of RDS. Other studies also support that the SP-A alleles $6A^2$ and $1A^0$ were associated with an increased, and the $6A^3$ with a decreased risk of RDS, these associations being determined by the SP-B Ile131Thr genotype together with the degree of prematurity (37-42). The association between genotypes and RDS is, however, largely influenced by the degree of prematurity, and antenatal glucocorticoid therapy. The genotypes of SP-A, SP-C and SP-D also associate with several other inflammatory lung and airway diseases. Rare mutations in SP-B or SP-C cause serious, often fatal lung diseases in the neonate.

4.2. Circulatory disturbances

The predominant anatomic lesion of NEC is coagulative or ischemic necrosis. Ischemia in NEC accounts for the necrosis, but the mechanism remains unresolved. An atavistic "diving reflex" has been hypothesized in neonates who experience severe anoxic episodes, during which blood is diverted preferentially to the heart and the brain, to the detriment of the abdominal organs (43). Other extrinsic regulatory mechanisms, such as the participation of the renin-angiotensin axis in bowel ischemia deserve serious attention as well (44).

4.2.1. Transient systemic hypotension

Transient systemic hypotension (TrSH) increases the susceptibility of the VLBW neonate to intestinal necrosis through intestinal ischemia. Main causes of TrSH include underdeveloped vasoregulation, hypoxia and infection (45). RAAS is one of the main neuro-endocrine components of vasoregulation during neonatal adaptation. Studies suggested that blood pressure is strongly influenced by RAAS activity in newborn infants (46).

Following hypoxic stress, increased RAAS activity mediates the rise in blood pressure and cardiac output. At the same time, it contributes to the profound redistribution of organ blood flow by increasing blood flow to the brain, myocardium and adrenal glands at the expense of gastrointestinal and renal circulation. Developmental stage has a major impact on RAAS activity. The neonate's angiotensin II (AII) production is low, but sensitivity to AII is high, as the result of the low availability of angiotensin converting enzyme (ACE) (47) and the high expression of AII type 1 receptor (AT1R) (48).

There are inherited factors that also affect the individual variability of RAAS activity. There is evidence showing that expression of ACE molecules is affected by the insertion/deletion (I/D) polymorphism of ACE gene of up to 40 per cent (49). This polymorphism has been associated with adult cardiovascular disorders in a large number of studies. An association was observed between AT1 receptor gene $A^{1166}C$ polymorphism and high blood pressure in adults (50).

Considering this, it can be supposed that these genetic variations in the activity of RAAS alter the risk for TrSH in VLBW neonates. Harding *et al.* found that ACE DD genotype may adversely influence the early health of preterm infants, citing an association with increased severity of illness (51). Our recent results (Nobilis *et al.* in press), however, showed that subjects with ACE DD genotype are protected against TrSH, while no impact of $AT1RA^{1166}C$ genotype on TrSH risk was detected. This finding suggests that the association between RAAS genotype and vasoregulatory disturbances depends on the maturity of the neonate.

4.2.2. Patent ductus arteriosus

Another frequent complication of the perinatal period of the VLBW infants is PDA. PDA, through decreased enteral blood flow, is also considered as a risk factor of NEC. The genetics of PDA is poorly understood.

Published data indicate that AngII is a potent vasoconstrictor of pulmonary arteries (52). Preventing the production of AngII, ACE inhibitor therapy leads to

pulmonary hypotension in adults (53). Indirect data also support the significance of the RAAS in closure of the PDA. ACE inhibitor treatment of pregnant women was associated with PDA in the neonate (54). Therefore, we recently tested the association between RAAS genetics and PDA in VLBW neonates (55). We showed that in addition to immaturity and RDS, the AT1R CC^{1166} genotype might play an independent role in the development of PDA. Speculatively, there are two possibilities. From one hand, AngII might have a direct effect on the PDA itself. (However, data are not available about whether AT1R is expressed on smooth muscle cells of the ductal wall at all). On the other hand, the indirect effect of AngII on the patency of the PDA is more reasonable. Independently of the underlying mechanism, the enhanced efficacy of AT on AT1R in subjects with AT1R CC^{1166} genotype may contribute to the closure of ductus arteriosus in VLBW neonates.

4.3. Neonatal thromboembolism

Increased thrombotic activity can lead to focal ischemia in the microvasculature of the gastrointestinal tract. Thus, it is reasonable to postulate that in some cases neonatal thromboembolism may play a role in NEC.

One possible candidate for the linkage between inflammation and thromboembolism is platelet activating factor (PAF), which has been suggested to be a central component of NEC (56-58). PAF is an endogenous phospholipid mediator and produced by inflammatory cells, endothelial cells, platelets and bacteria of the intestinal flora. The role of PAF in NEC has been highlighted by a number of studies: a PAF challenge induces systemic hypotension, but, locally in the intestine, increases gut mucosal permeability and through triggering thromboembolism, causes intestinal necrosis. This event precedes cell necrosis, and occurs at doses below that causing necrosis. Several functional polymorphisms of PAF receptor and PAF-metabolizing PAF-acetylhydrolase exist (59, 60). However, none of them have been investigated in the pathogenesis of NEC. Further research should shed light on the role of PAF polymorphisms in the pathogenesis of NEC.

Various genetic prothrombotic defects, affecting the physiologic anticoagulant systems, (such as antithrombin-, protein C, and protein S- deficiency, the mutation of coagulation factor V ($G^{1691}A$) (FVL), and the prothrombin gene variant ($G^{20210}A$) ($FIIG^{20210}$)) have been well established as risk factors for thrombotic events of the adult. For detailed review see Ref. (61). In addition, metabolic diseases such as homocystinuria, moderate hyperhomocysteinemia caused by homozygous methylene-tetrahydrofolate reductase (MTHFR) polymorphism $C^{677}T$ have been described (62). Theoretically, these inherited prothrombotic conditions might play a role in the pathogenesis of thromboembolic ischemic gastrointestinal events, including NEC. However, recently Kenet *et al.* investigated the FVL, MTHFR, $FIIG^{20210}A$ polymorphism in 166 preterm infants and could not demonstrate any association between the risk perinatal morbidities (including NEC) and genotype (63).

5. INFLAMMATION AND NEC

The local and systemic host response to a wide variety of stimuli involves a cascade of humoral mediators that provoke, sustain, and moderate the process of inflammation. Inflammatory reaction is the final common method by which the body deals with invading organisms, toxins, trauma, and ischemia-reperfusion injury (11). Animal models have demonstrated that the intestine can be a major source of these circulating cytokines (64, 65). Cells within the gastrointestinal system also elaborate a wide variety of cytokines when involved in inflammatory bowel diseases or in experimental inflammatory conditions.

Elevated concentrations of different cytokines are often found in patients with symptoms of sepsis, shock, or systemic inflammatory response syndrome (66). As expected, therefore, several investigators have found elevated cytokine concentrations in newborn infants with NEC (10, 67).

5.1. Local inflammation

In the immature or mildly damaged mucosa, the close proximity of bacteria and intestinal epithelial cells may facilitate transcellular penetration of inflammatory mediators. This increases the intestinal epithelial permeability *in vivo*, leading to focal mucosal "leak" and local entry of bacteria or bacterial products (8). The activation of polymorphonuclear cells, adhesion to venular endothelium, induction of proinflammatory cytokines leads eventually to vasoconstriction, ischemia, and subsequent reperfusion.

The pathogenic role of increased proinflammatory cytokine production in NEC has been suggested by previous data. TNF-α in NEC has been most extensively studied. In addition to causing shock, TNF-α induces gastrointestinal necrosis when administered parenterally to adult rats (57). These data are in line with those demonstrating higher than normal plasma TNF-α levels in neonates with NEC (68). Histologically, increased TNF-α expression was observed in Panneth cells, lamina propria eosinophils, and infiltrating macrophages in infants with NEC (69). Viscardi *et al.* showed that expression of TNF-α in the full-thickness of the surgical specimens of intestine was higher in 29 neonates with acute NEC compared to infants with congenital intestinal malformations (70). Other human blood results, however, did not confirm the clinical significance of altered TNF-α production (71). Other data suggest that the level of both pro- and anti-inflammatory cytokines can be altered in NEC and the pro- and anti-inflammatory balance of cytokines might be disrupted. Viscardi et al, in the above mentioned paper, showed that IL-1β mRNA levels were higher in bowel specimens of NEC patients than in control infants (70). Weeks *et al.* reported that umbilical cord blood IL-6 levels were elevated in 133 preterm neonates who developed subsequently NEC (72). In a study by Harris *et al.* IL-6 levels were five- to tenfold higher in infants with bacterial sepsis complicated with NEC at the onset of disease than in infants with bacterial sepsis alone, and in control infants (73). In a study by Edelson *et al.*

Table 1. Polymorphisms investigated in association with necrotizing enterocolitis and its risk factors

Factor	Type of polymorphism	Site of polymorphism	Effect Of The Polymorphism (Reference)
Interleukin-1 receptor antagonist	86 base pair variable number of tandem repeat	Intron 2	Higher Levels Of IL-1ra In The Case Of IL-1ra-2n (28-30)
Interleukin-1 beta exon 5	Single base C→T	Exon 5, position 3954	Higher IL-1 Beta Expression In The Presence Of T (104)
Interleukin-4 receptor gene alpha chain	Single base A→G	Position 1902	Enhanced Signal Transduction In The Presence Of G (77)
Interleukin-6	Single base G→C	Position -174	Low IL-6 Production In The Presence Of C (105)
Interleukin-10 promoter	Single base G→A	Position -1082	Low IL-10 Production In The Presence Of A In Vitro (105)
Interleukin-18 promoter	Single base C→A	Position -607	Higher IL-18 Expression In The Presence Of C (76)
TNF-alpha promoter –308	Single base G→A	Position –308	Enhanced TNF-Alpha Production In The Presence Of A (75)
TNF-alpha promoter -238	Single base G→A	Position -238	Decreased TNF-Alpha Production In The Presence Of A (75)
ACE-gene I/D polymorphism	Insertion/deletion	Intronic region	Lower ACE Activity In The Presence Of I (49)
Angiotensin-receptor 1	Single base A→C	Position 1166	Enhanced Systemic Vasoconstriction Leading To Hypertension Int He Presence Of C^{1166} (50)
Surfactant protein A	Splice variants (5′UT exons that splice in different configurations)	5′ÚT	$6A^2$ And $1A^0$, Are The General Higher-Risk RDS Alleles (33, 34)
Surfactant protein B	Single base C→T	~120 bp upstream of the Δi4 polymorphism	Ile131Thr Variation Affects A Putative N-Terminal *N*-Linked Glycosylation Site Of Prosp-B. Ile131Thr Polymorphism Is A Determinant For Certain *SP-A* Alleles As Factors Causing Genetic Susceptibility To RDS ($6A^2$, $1A^0$) Or Protection Against It ($6A^3$, $1A^2$) (35)
Coagulation factor V	Single base G→A	Position 1691	Predisposition To Thrombosis Due To Activated Protein C Resistance Int He Presence Of A^{1691} (63)
Prothrombin gene variant	Single base G→A	Position 20210	Associated With Elevated Levels Of Factor II In Plasma, Increases The Risk Of Developing Venous Thrombosis (63)
Methylene-tetrahydrofolate reductase (MTHFR)	Single base C→T	Position 677	Decreased Enzyme Activity, Hyperhomo-Cysteinemia, And Increased Risk For Thromboembolism (63)

circulating concentrations of proinflammatory cytokines IL-1β and IL-8, and counterinflammatory cytokines, including IL-1 receptor antagonist (IL-1ra) and IL-10 were higher at the onset of NEC compared to weight-, gestation-, and age-matched controls (10). In 7 premature neonates Romagnoli *et al.* reported higher IL-10 levels than in healthy preterm neonates, this was associated with mortality (74).

The presence of genetic variants may contribute to the inter-individual variance of cytokine response to inflammatory stimuli. The association between genetic variants and cytokine production has been verified for several cytokines in the adult (for details, see Table 1). In our previous reports (75-77), we evaluated whether functional polymorphisms of cytokine encoding genes (namely TNF-α (78, 79), IL-1β (80), IL-4 receptor α chain (81), IL-6 (82), IL-10 (83), IL-18 (84), see Table 1) might affect the risk and complications of NEC in VLBW infants. We have reviewed the clinical course of 136 VLBW neonates. NEC was diagnosed in 46 neonates according to a classification system of Bell et al (5). Stage I NEC was identified in 17 infants; stage II NEC was present in 21 infants; stage III NEC was present in 8 patients with perforated bowel. The 90 control gestational age matched VLBW neonates were free of NEC. Remnant dried blood spot samples were used to obtain DNA, than polymerase chain (PCR) reaction and subsequent restriction fragment length polymorphism (RFLP) were applied to determine genetic variants of cytokines enlisted in Table 1. We analyzed the association of cytokine genotypes with the risk and severity of NEC.

We found that the prevalence of any of the investigated TNF-α, IL-1β, IL-6, IL-10 mutant alleles showed no difference in infants with or without NEC (75, 77). Furthermore, the presence of these genetic variants was not associated with NEC severity or outcome. This finding indicates that carrier state of these alleles does not influence the risk of this complication. A serious limitation of these retrospective studies is the lack of serial TNF-□, IL-1□, IL-6, and IL-10 measurements and that we did not study the correlation between genotype and cytokine levels. Therefore, based on our results, we can only hypothesize that similar to the observation of Weitkamp et al. (85), these genetic variants might have minor, if any, impact on the actual TNF-□, IL-1□, IL-6, and IL-10 levels in VLBW population. Further investigation of genotypes with the serial measurement of cytokine levels in a larger number of infants with NEC should be done to elucidate this issue.

Interestingly, we found that while the risk of NEC is not associated with the investigated IL-18 genotypes, the frequency of IL-18 ^{607}AA genotype was significantly higher in infants with stage 3 NEC compared to that established in infants with NEC stages 1 and 2 (76). IL-18 plays a key role in the host defense by inducing interferon-gamma, amplifying the Th1 cytokine production and stimulating CD8+ and NK cells' cytotoxicity and IL-8 accumulation (86). A pathogenic role of altered IL-18 levels in extensive inflammatory processes affecting the intestinal wall has been suggested by data obtained in Crohn's disease patients (87). Assuming that IL-18 levels contribute to the intestinal inflammation and the A^{-607} polymorphism is associated with altered IL-18 mRNA production (84), our results indicate that the presence of an AA genotype might adversely affect the outcome of NEC in VLBW babies through altered IL-18 levels.

Presumably, IL-4 also plays a central role in neonatal gut immunology (88). It inhibits human

macrophage colony formation, monocyte-derived H_2O_2 production (89), and release of inflammatory mediators such as TNF-α and IL-1β (90, 91). IL-4 has powerful anti-inflammatory actions both in vivo and in vitro. IL-4 inhibits Th1-cell proliferation and opposes the effects of inflammatory cytokines on macrophages. Although data are not available about its importance in NEC, isolated lamina propria mononuclear cells from inflamed intestine expressed IL-4 mRNA and secreted this cytokine in lower amounts than control cells (92).

Interestingly, the prevalence of the mutant variant of the IL-4 receptor α gene was lower in neonates with NEC compared with those without NEC, suggesting that this mutation might protect against the development of NEC in VLBW infants (77). The investigated variant of IL-4 receptor α gene does not influence IL-4 levels, but its presence is associated with enhanced transduction of IL-4 signals. Because enhanced IL-4 transduction shifts the development of lymphocytes to a more pronounced Th2 state (93), it is tempting to speculate that the elevated number of Th2 cells in carriers of this genetic polymorphism is a protective factor against the development of NEC. Because homozygous carrier state of IL-4 receptor α wild type allele (ie, IL-4 receptor α AA^{1902}) presents a greater risk to NEC (odds ratio: 2.51 in our study), based on these data it can be postulated that infants with this genotype should be monitored more carefully during the first few postnatal weeks. Further studies are needed to be carried out to determine the mechanism of the protective effect of the IL-4 receptor α G^{1902} variants.

5.2. Systemic inflammation

Bacterial colonization and sepsis have long been considered as important risk factors for the pathogenesis of NEC. The increased susceptibility of the neonate to bacterial infections is the result of a variety of factors. CA is considered to be the primary cause, but delayed maturation of immune response may also play a role. The results from previous studies have suggested that the preterm neonates may not have a fully-developed capacity to secrete cytokines in vitro (94, 95). Recent reports, however, have indicated that in vivo plasma levels of TNF-α, IL-1β, IL-4, IL-6, IL-8 and IL-10 are enhanced in septic neonates, and high levels of IL-6 and IL-8 are considered to be helpful in diagnosing sepsis (73, 74, 96-99). Primary proinflammatory cytokines such as TNF-α and IL-1β induce secondary pro- and anti-inflammatory mediators such as IL-6 and IL-10. These cytokines have been shown to contribute substantially to the primary response of the host to infection. Both TNF-α and IL-1β are capable of inducing the same symptoms and the same severity of septic shock and organ dysfunction as endotoxin in experimental settings and in human subjects.

Genetic variations in pro-inflammatory TNF-α and IL-1β genes and anti-inflammatory IL-1 receptor antagonists are of major interest with respect to genetically determined differences in the response to bacterial infections. Indeed, an association has been found between sepsis- related complications and some genetic variants of IL-1 receptor antagonist and TNF-βin adults (100, 101). The prevalence of septic shock and meningitis were higher in carriers of TNF-α $^{-308}$A and IL-10 $^{-1082}$A variants, while IL-1β C^{3954}T was not shown to have any impact on the risk of sepsis (reviewed in (102)). So far, only a few studies have been carried out to test the association between cytokine SNPs and the risk of sepsis and related complications in neonates. Weitkamp *et al.* (85) did not observe any impact of TNF-βgene variants on risk of sepsis. Bessler *et al.* showed also similar prevalence of IL-1 receptor antagonist SNPs in preterm infants with or without early onset sepsis (29)). Hedberg *et al.* showed that the TNF-α G^{-308}A allele did not affect the development of sepsis in ventilated premature infants but the presence of this allele increased neonatal mortality once sepsis developed (103). We also found no association between the carrier state of TNF-αG^{-308}A, IL-1β C^{3954}T, IL-6 G^{-174}C, and IL-10 G^{-1082}A SNPs and the risk of early onset sepsis (104).

Our analysis has also shown that the majority of sepsis-related complications (transient systemic hypotension, hepatic failure, multi-organ failure) are independent of cytokine polymorphisms. However, the simultaneous carriage of IL-1β ^{3954}T and IL-10 $^{-1082}$A alleles (possibly leading to high pro-inflammatory and low anti-inflammatory capacities) was more frequently present in those few septic subjects in whom disseminated intravascular coagulation developed. We also investigated whether genetic polymorphisms of cytokine genes leading to a more intense inflammatory response might predispose VLBW infants to the development of acute renal failure (ARF) in severe infection. In our study we found that the constellation of TNF-α and IL-6 genetic variants was associated with ARF (105). In contrast with our findings, Harding *et al.* observed a higher prevalence of the IL-6 $^{-174}$GG genotype among those patients, who developed bacterially confirmed septicemia compared with those who did not develop infection (106). This association remained significant after its adjustment for other well-established predictors for the development of septicemia. Late infection alone was similarly associated with GG genotype. The authors posited that this finding could be a result of altered basal IL-6 production (107). Homozygosity for IL-6 $^{-174}$G alleles was predictive for early onset sepsis in another study, as well (108).

6. CONCLUSIONS

Prematurity; transient ischemia of the intestine; local/systemic inflammation predisposing the bowel to injury, and therapeutic interventions are independent risk factors of NEC. However, recent data suggest that the neonates' genetic background may also contribute to the susceptibility toward neonatal bowel inflammation and its risk factors. The association between perinatal complications and cytokine genetics has been investigated most extensively, but emerging data also support the possible implication of genetic polymorphisms of other system such as RAAS and surfactant proteins in perinatal morbidity. Recently, positive findings indicate the implication of the genetic polymorphisms of IL-4-receptor

α chain and IL-18 in NEC, proinflammatory cytokines in premature birth, IL-6 in sepsis, surfactant proteins A and B in respiratory distress, ACE in perinatal adaptation and AT1R in the closure of ductus arteriosus (Figure 1). Serial measurements of serum cytokine levels and vasoactive factors during the development of NEC along with the determination of genotypes may help to elucidate whether the observed associations between genotype and disease are causal.

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