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Orthogonal Polarization spectral imaging (OPS) Measurements of Microcirculatory Changes in Term Newborns with Suspected Infection

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A. Introduction

A. 1 Epidemiology

Infection is one of the major problems in the medical care of neonates. Throughout the world, 30 million infants contract an infection in the neonatal period, and 1 - 2 million of these die (1;2). Although most of these deaths take place in developing countries, where neonatal mortality from sepsis may be as high as 60%, even in the developed world the incidence of neonatal infection is still of 1.1 - 2.7 % of all live births (3). These numbers may vary from country to country, from nursery to nursery and have been changing over the years.

The relative immunodeficient state of neonates taken together with the increasing survival of progressively more premature infants contribute to the high mortality and morbidity associated with neonatal infection (1). Sepsis may affect up to 16 % of low birth weight infants (501-1500g) in the neonatal intensive care unit with a mortality rate of 15 - 50% (4;5).

One of the most common challenges in newborn medicine is the diagnosis of infection. The early diagnosis of neonatal infection based on the clinical criteria alone is difficult and laboratory values might lag. Yet rapid progression of untreated infection may greatly increase morbidity or mortality.

The incidence of early onset sepsis has diminished in the past years due to intrapartum antibiotic prophylaxis, whereas the incidence of late onset sepsis has increased. This is due to the better survival of very low birth weight infants who need considerable mechanical and nutritional support and remain hospitalized for long periods (6-8).

A. 2 Definitions and Classification of Neonatal Infection

There is no uniform definition of neonatal infection or sepsis, mainly since blood cultures are not very frequently positive.

For this study we used the definitions resulted from the International Sepsis Definitions Conference 2001(9) and the International Pediatric Sepsis Consensus Conference 2005 (10;11)

Infection

The International Pediatric Sepsis Consensus Conference in 2005, defined infection as: **"**A suspected or proven (by positive culture, tissue stain, or polymerase chain reaction test) infection caused by any pathogen OR a clinical syndrome associated with a high probability of infection" (10). Evidence of infection includes positive findings on clinical exam, imaging, or laboratory tests (e.g., white blood cells in a normally sterile body fluid, chest radiograph consistent with pneumonia, petechial or purpuric rash, or purpura fulminans).

The symptoms that result from these infections may be caused by a wide variety of bacterial and viral pathogens, but their clinical manifestations are very similar (1).

Sepsis

The International Sepsis Definitions Conference 2001 defined sepsis as "a systemic inflammatory response syndrome (SIRS) in presence of a suspected or proven infection" (9). This definition was later accepted for pediatric cases (10;11). A bloodstream infection during the first month of life is called neonatal sepsis.

Severe sepsis

Severe sepsis is defined as sepsis plus one of the following: cardiovascular organ dysfunction or acute respiratory distress syndrome or two or more other organ dysfunctions (10).

Septic shock

Septic shock is a sepsis that causes cardiovascular organ dysfunction (10) resulting in hypotension despite adequate fluid resuscitation (12).

Severe bacterial infections can also be classified as early- and late-onset sepsis.

Early-onset sepsis

Early-onset sepsis (EOS) can be acquired through vertical transmission by ascension from the lower genital tract of the mother, through transplacental transmission after maternal bacteremia or by passage through the birth canal. Early-onset sepsis becomes clinically evident within the first few days of life, and has been

defined in most reports as occurring within the first week or the first 72 hours of life (6;13-15).

Late-onset sepsis

Late-onset sepsis (LOS) presents thereafter, with an upper limit of the 28th, 30th or 90th day of life (6). “Late-onset infections may be acquired intrapartum during the passage through the birth canal, through horizontal spread within the hospital settings, or from maternal or other sources in the home or community” (13).

A. 3 Risk Factors for Neonatal Infection

Several factors such as maternal, environmental, and host factors determine which infants exposed to potentially pathogenic organisms will develop invasive bacterial infections. The presence of any of the following factors can be associated with a 10-fold or greater increased risk of developing systemic infection (5):

- *Chorioamnionitis* as evidenced by intrapartum fever (core temperature of ≥ 38ºC before delivery), uterine tenderness, maternal leucocytosis (White Blood Cells ≥ 18000 leukocytes /µl) or elevated C-reactive protein (≥ 1mg /dl). Infants born to mothers with chorioamnionitis have a sepsis incidence of 1-5% (16) Odds Ratio (OR) = 6.43 (17).
- *Prolonged rupture of membranes* (PROM) ≥ 18 hours before delivery, OR = 64 (17). After delivery 3 - 5% these infants will present with an infection (3).
- *Stained amniotic fluid* (3)
- *Preterm delivery* OR = 4.83 (17)
- *Maternal Group B Streptococcus (GBS) colonization at delivery*: OR = 204; Light colonization: OR = 97.1; Heavy colonization: OR = 247 (17)
- *Previous infant with invasive GBS* (18)

A. 4 Microbiology of Neonatal Infections

The agents responsible for early-onset sepsis are mostly those found in the maternal birth canal (5). The incidence of these pathogens has varied over the past 60 years. Before the introduction of the sulfonamides and penicillin in the 1940s, gram-positive cocci, particularly group A streptococci, were responsible for most cases of neonatal sepsis (16). After the introduction of antimicrobial agents, gram-negative enterics, in particular *E. coli*, were predominant causes of serious bacterial infections of the newborn. An increase in serious neonatal infection caused by group B streptococci was noted in the early 1970s. Since then, *Streptococcus agalactiae* or B ranks as leading cause of early-onset sepsis followed by *E. coli* in the United States and Western Europe. Despite universal screening for B Streptococcus this has not changed. In developing countries *S. aureus* and gram-negative bacilli, specially *Klebsiella* are the most common isolated agents (6;8;13;19) (tab. 1).

Typical Early-onset Pathogens and its Predominance
▪ Group B streptococci (GBS): 1.3-3.7 per 1000 live births (1). ▪ *E. coli*: 1 per 1000 live births (1) (5) (18). ▪ *S. aureus* ▪ *Klebsiellen* ▪ *Enterocci* ▪ Other *Streptocci* ▪ *Lysteria monozytogenes* ▪ Anaerobic Bacteria *(Bacteroides fragilis)*

Table A.1: Early onset pathogens in neonatal infections

A. 5 Pathogenesis of Neonatal Infection

Pathogens can be transmitted from mother to fetus by diverse modes (EOS, LOS). The state of development of the immune system of the newborn plays a key role in neonatal infections.

A. 5. 1 Ways of Acquiring an Early Onset Infection

The developing fetus is protected from the microbial flora of the maternal genital tract. Before labor and membrane rupture, amniotic fluid is nearly always sterile (8). Initial colonization of the newborn and of the placenta usually occurs after rupture of maternal membranes. If delivery is delayed after membranes rupture, the vaginal micro flora may ascend and in some cases produce inflammation of fetal membranes, umbilical cord and placenta. Fetal infection may also result from aspiration of infected amniotic fluid. Even if delivery follows shortly after rupture of the membranes, the infant may be colonized during passage through the birth canal (5).

Before rupture of fetal membranes, organisms in the genital tract may invade the amniotic fluid and produce infection of the fetus. These organisms can invade the fetus through microscopic defects in the membranes, particularly in devitalized areas overlying the cervical os (19). A usual route by which the fetus may become infected is the transplacental spread after maternal infection and invasion of the blood stream.

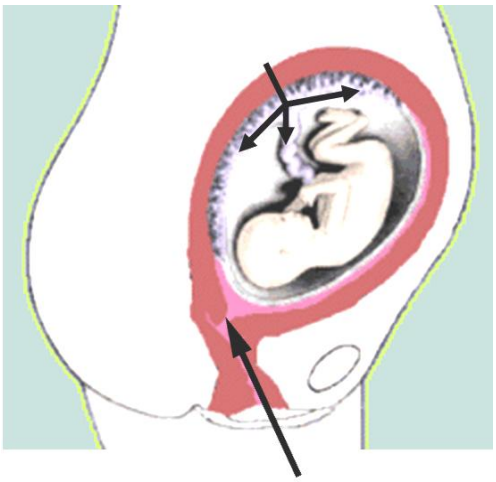


Figure A.1: Ways of acquiring infection in the fetus. The fetus is shown inside the uterus. Uterus and Vagina are shown in red. The branching arrow represents the transplancental spread of bacteria. The ascending arrow represents the ascending infection from the vagina.

Initial colonization of skin and mucosal surfaces of the newborn infant include the nasopahrynx, oropharynx, conjunctivae, umbilical cord and external genitalia. In most infants the organisms proliferate at these sites without causing symptoms. Few infants become infected by direct extension from the sites of colonization (e.g., sinusitis and otitis from nasopharyngeal colonization). Alternatively, invasion of the bloodstream may ensue, with subsequent dissemination of infection. The umbilical cord is a particularly common portal of entry for systemic infection because devitalizated tissues are an excellent medium for bacterial growth and because the recently thrombosed umbilical vessels provide direct access to the bloodstream (5;19).

A. 5.2 The Immune System of the Newborn

The susceptibility of the neonate to a wide variety of microbes results from limitations of both innate and adaptive (antigen-specific) immunity (20). During the second half of pregnancy the fetus develops the ability of cellular and humoral defense. However, the immune system is inhibited to avoid reject-reactions between mother and fetus. The adaptation of the immune system takes place after birth. Hence, immune effector-systems are less powerful in newborn and especially in preterm infants as in adult (21) There are also deficiencies in processes through which the innate immune system facilitates and directs the development of protective antigen-specific immunity. For example, neonatal serum has been shown to be inefficient in killing *E. coli* because of a deficiency of non-immunoglobulin G serum components (6). Many parts of the defense system are relatively immature in the healthy neonate.

The particular differences in the immune system of newborns compared to adults are:

- **T cells** that help for B cell differentiation have an impaired function with a decreased cytokine production and delayed antigen-specific responses of CD4+ T cell. The ability to stimulate B cells is also decreased (21).
- **B cells and immunoglobulin**. The newborn is protected from infection by passive IgG antibodies, predominantly transferred during the latter third of pregnancy. Fetal IgG concentrations are equal or higher than maternal

concentrations after 34 weeks of gestation. The lacking ability of the neonate to produce antibodies in response to polysaccharides, limits its defense against bacterial pathogens to which the mother has developed little or no IgG antibodies. The antibody response to new antigens is delayed but it improves rapidly after birth (5;8). On the other side IgM antibodies are deficient in the newborn. B-lymphocytes transport on their surface polyreactive IgM antibodies with a low affinity to specific antigens (21). This may explain its susceptibility to gram-negative infections (22).

- **Natural killer cells** in neonates are decreased in number and activity against their target cells, including virus-infected target cells, compared with adult (8).
- **Phagocytes**, whose deficiencies include a reduced adhesiveness with impairment of chemotaxis and motility of polymorph nuclear cells (PMNs) and mononuclear phagocytes as well as a diminished neutrophil storage pool (20). Tissue macrophages from neonate animals have a reduced phagocytic and microbicidal activity.
- **Humoral mediators of inflammation and opsonization**. Neonates have moderately diminished alternative and classic component pathway activity and decreased in some terminal complement components. Fibronectin is also lower (8). Cytokine production is deficient and may be responsible for the decreased inflammatory response seen in newborns (22). Thus, neonatal sera are less effective in antibody-independent opsonization when concentrations of antibody are low. These deficiencies contribute to delayed inflammatory responses and impaired bacterial clearance (5).

A. 5.3 The Neonatal Inflammatory Response

“Despite similar clinical presentations, the molecular and cellular processes that elicit the sepsis response differ depending on whether the organism is gram-negative, gram-positive, fungal, or viral in nature. The sepsis response to a gram-negative organism is provoked by the release of lipopolysaccharide (LPS), an endotoxin from within the cell wall of the gram-negative bacteria, which is released during lysis. Gram-positive, fungal, and viral organisms, however, initiate a sepsis response that begins with the release of exotoxins and cellular

antigenic components" (23). Both responses trigger the sepsis cascade, which begins with the release of primary inflammatory mediators such as tumor necrosis factor and interleukin-1β. This initial step in the cascade of events will lead to inflammation and tissue destruction. Once bacteria cross the mucosal epithelium and enter the tissues, phagocytes and opsonins become the critical elements of defense. Antibody and complement opsonize the bacteria for phagocytosis and killing by neutrophils and macrophages (5).

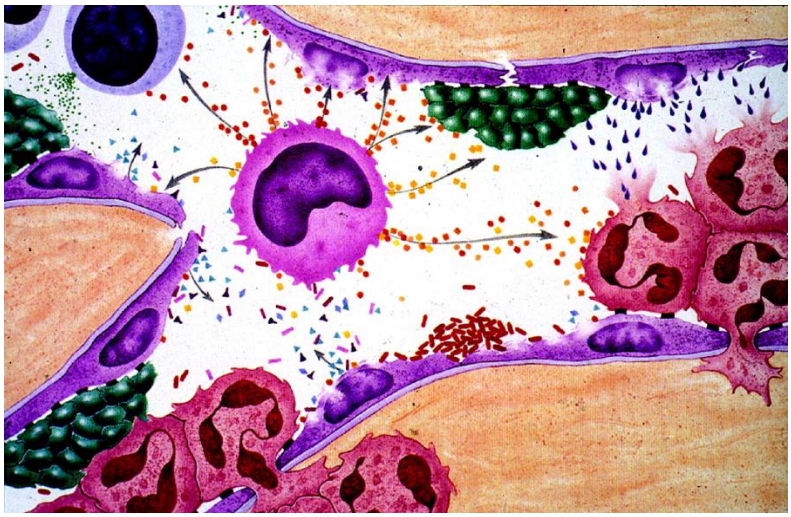


Figure A.2: Endothelium and inflammatory response. The platelets (green bodies), endothelial cells (purple), macrophages (light blue cell with dark centers), polymorphonuclear cells (pink with 3 inner lobes) and cytokines (small pellets) are inflammatory mediators (24).

The absence of type-specific antibodies and the subtle but cumulative deficits of other host defense mechanisms contribute probably to the neonate susceptibility to infection (8). Limitations in the production of chemotactic factors or reduced chemotactic responses of neonatal neutrophils, may end in delayed response of neutrophils to sites of infection. Secondarily, neonatal neutrophils may kill bacteria less efficiently due to reduced amounts of opsonins or because the local bacterial density has reached high levels. Severe infections can deplete the limited marrow neutrophil reserve and worsen the problem (8).

Despite lack of type-specific antibodies and a multitude of subtle deficits in neonatal defenses, relative few infants acquire infections (5).

A. 6 Diagnosis of neonatal infection

Early diagnosis of neonatal bloodstream infection remains one of the biggest challenges to neonatologists. It might be based on pregnancy history and certain risk factors might raise suspicion. Observation of clinical signs, laboratory tests and particularly the intuition and clinical experience help the neonatologist to establish the diagnosis of infection (4).

Clinical signs

The newborn infant responds to harmful stimuli with limited stereotyped reactions (8). As a result clinical signs of bacterial infection in the neonate are inexplicit and may be associated with infectious or with non-infectious disorders. The presence of any of these signs alone or in combination is an indication for complete evaluation to rule out sepsis. Any neonate who is not doing well, without an ostensible reason, is a priori suspect of infection (25). The major signs and symptoms of infection relate to:

- **Abnormalities of temperature regulation** in the form of hyperthermia, or less commonly, hypothermia.
- **Respiratory distress** manifested as tachypnea, grunting respirations, cyanosis, intercostals and substernal retractions, and apnea.
- **Hemodynamic signs** like tachycardia (> 180 beats/min) or bradycardia (< 100 beats/min), hypotension, capillary refill time > 3 seconds, gray skin color.
- **Gastrointestinal findings**, including poor feeding, weak suck, regurgitation, vomiting.
- **Neurological deficiencies** evidenced as diminished awareness, lethargy, tonus abnormalities, irritability and seizures.
- **Cutaneous findings:** Purpura, eruptions.

Clinical manifestations of the group B streptococcal early-onset syndrome may be present at birth or may appear at any time within the first 72 hours of life. For *E. coli* infections the start is typically after 2-3 days (3). Onset can be sudden and followed by a fulminant course, with the primary focus of inflammation in the lungs.

The diagnosis of neonatal infection is difficult to establish based on the clinical criteria alone and the results of microbiological isolates from sterile body fluids need

at least 3 days to be obtained. Therefore clinicians have to rely on laboratory tests to rule out an early onset infection (26).

Laboratory tests

Complete blood counts and white blood cell ratios: Total leukocyte and neutrophil count, total non-segmented neutrophil count and neutrophil ratios are neither specific nor sensitive for the diagnosis of neonatal infection (5;25). Ottolini et al. reported a sensitivitiy of 41% and a specificity of 73 % in diagnosing neonatal infection by abnormal values of white blood cell (WBC) count.

Acute-phase reactants: C - reactive protein (CRP). CRP is a hepatic protein produced within the 6 - 8 hours as part of an immediate inflammatory response to infection or tissue damage. The acute-phase-response is effective in the newborn. CRP concentration is elevated in umbilical cord blood and in newborns blood after bacterial and fungal infections (27). Its increase in plasma is due to inflammatory cytokines such as Interleukin 6 (27).

Acute infections caused by gram negative pathogens as enterococci cause a strong increase in CRP. The bacterial endotoxin stimulates directly the macrophages (CRP > 100mg /l).

Acute infections caused by gram positive bacteria, parasites and fungi cause a moderate increase in CRP (CRP < 100mg/l). On the other side, virus, like Adenovirus cause no or just light CRP increase (CRP < 30mg/l) (28).

With appropiate antibiotic therapy, CRP will decrease (15;29). As it does not cross the placenta, CRP is an excellent marker for neonatal bacterial infection; however, it is not useful for early diagnosis. The increase in CRP response to neonatal bacterial infection occurs about 12 – 36 h after the onset of clinical signs. The sensitivity at the beginning of sepsis is only 60 % (29). Therefore, a negative CRP at the initial evaluation does not rule out serious infection, whereas a positive CRP suggests that more than 12 – 36 h have elapsed since the onset of the inflammatory response (26). CRP can be considered as a “specific” but “late” marker of neonatal infection. False positives can be due to meconial aspiration, fetal asphyxia, shock, cerebral hemorrhages, tissue necrosis and recent vaccination such as Hepatits B (29-31). CRP is also a good parameter to evaluate the efficacy of the therapy (27).

Proinflammatory cytokines such as **Interleukin 6 (IL-6), Interleukin 8 (IL-8)** and **tumor necrosis factor-α** are increased in serum 1 – 2 h after exposure to bacterial products and precede the increase in CRP. A number of studies reported a cytokine overproduction from the umbilical cord cells when exposed to endotoxines. Cytokines such as the interleukins IL-1β, Il-6, and IL-8 and TNF-α are endogenous mediators of the immune response to inflammation including that caused by bacterial infections. IL-6 is involved in many aspects of the immune response. It is elevated in the presence of chorioamnionitis and delivery room intubation, yet depressed in the presence of pregnancy-induced hypertension. Several studies suggest that elevated levels of IL-6 detected after birth may provide an early and sensitive parameter for the diagnosis of neonatal bacterial infection. IL-8 is predominantly produced by monocytes, macrophages and endothelial cells. Its kinetics are similar to those from IL-6 (26). However, “because of variations in study design and methodology, estimates of diagnostic sensitivity and specificity for IL-6 and IL-8 levels range from 60% to greater than 95% for each one” (8).

Procalcitonin (PCT): is one of the calcitonin precursors. Its release is stimulated by microbial infections. Monocytes and hepatic cells are believed to be the producers. Serum concentrations of PCT begin to rise 4 h after exposure to bacterial endotoxins, peak at 6-8 h, and remain raised for at least 24 h (29). Unfortunately, false positives have also been found in neonates with respiratory distress syndrome, intracranial hemorrhage, pneumothorax, severe trauma and hemodynamic failure without having a bacterial infection. The value of PCT as an early marker for bacterial infection is also complicated by a physiological increase of procalcitonin during the first days of life. However, Chiesa and colleagues (32) studied the accuracy of IL-6, CRP and PCT in all perinatal events and concluded that the “physiological” increase is relative small when compared to the magnitude of PCT response to bacterial infection. Both the sensitivity and specificity of PCT were greater than those of CRP and IL-6.

Procalcitonin may be an important valuable marker for the detection of early neonatal infection when reference values, the clinical condition, and the administration of antibiotics are taken into account (29;30).

A. 7 Treatment of neonatal infection

Successful management of neonatal bacterial sepsis depends on early diagnosis and prompt initiation of appropriate antimicrobial therapy and supportive measures. If untreated, infections in newborns can rapidly become severe and life-threatening.

If the physician considers a newborn to be septic, cultures should be obtained and treatment with antibiotics be started immediately. Currently, the drugs most frequently used and recommended to treat suspected severe neonatal infections, in both developed and developing countries are a combination of penicillin or ampicillin and aminoglycosides (usually gentamicin) (5;33).

In general, initial therapy must include coverage for gram-positive cocci, particularly group B streptococci and gram-negative enteric bacilli. Penicillin is the choice for gram-positive cocci. Choice for therapy for gram-negative infections depends on the current pattern of antibiotic susceptibility in the local hospital. Most experts prefer ampicillin and gentamicin for therapy of presumptive sepsis and ampicillin and cefotaxime for presumptive bacterial meningitis (19).

Combining two or more antibiotics is the usual clinical practice when initiating therapy for presumed systemic bacterial disease (*e.g.*, ampicillin and aminoglycoside are combined to treat suspected early-onset septicemia or meningitis before identification of the pathogen). After a bacterium has been identified and its susceptibility to various antimicrobials is determined, the most appropriate drug or drugs should be selected (16).

A. 8 Microcirculation

A. 8. 1 Definition

The term microcirculation concerns the blood flow through blood vessels smaller than 100 µm (i.e. arterioles, capillaries and venules). The most purposeful function of the circulation occurs in the microcirculation, specifically in the capillaries (6-12µm). The biggest part of interchange of nutrients and cellular excreta between circulating blood and tissue cells takes place here. The microcirculation of each organ is organized specifically to ensure adequate oxygen and nutrients delivery to meet the demands of every cell. The typical arrangement of the capillary bed is not found in all parts of the body; however, some similar arrangement serves for the same purposes.

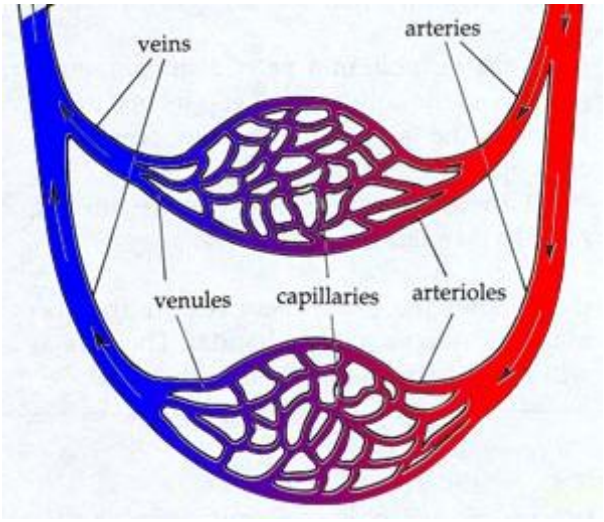


Figure A.3: Schematic drawing of a capillary bed. Red colored vessels contain arterial, violet mixed and blue vessels contain venous blood. Arrows indicate the direction of the blood flow. The capillary network is the site of OPS measurements.

The local conditions of the tissues can cause direct effect on the vessels in controlling local blood flow and metabolic demand in each tissue area thanks to the metarterioles and precapillary sphincters (34;35).

A. 8.2 Blood supply in the epidermis of newborns

At birth, the blood supply of the skin shows relative little variation. It is richly supplied by a dense subepidermal plexus, samples of which look more or less the

same wherever they are examined. Middle and deep dermis are richly endowed with vasculature (36).

The mature pattern of capillary loops and of the subpapillary venous plexus is not present at birth but develops as a result of the absorption of vessels at some sites and capillary budding with migration of endothelium at other sites. Several studies have observed the morphology changes of capillaries during the first weeks of life. Perera et al. (37) investigated the changes that take place in the upper dermis obtaining the following results:

- At birth the skin shows no papillary loops except for the palms, soles and nail folds. At the same time skin shows a disorderly capillary network.

- By the end of the first week of life the capillary network assumes a more orderly network pattern and papillary loops begin to appear as a small superficial dilatations or buds in the second week.

- Defined loops begin in the 4^{th} or 5^{th} week in at least one area of the skin but are observed over all in the 14^{th} to 17^{th} week. The development of order with a distinct horizontal plexus is a gradual process, first apparent during the second week but it is not characteristic of all areas until the 14^{th} to 17^{th} week. The development of an orderly sub papillary plexus and papillary loops is greatly delayed in skin creases (Fig.A.4).

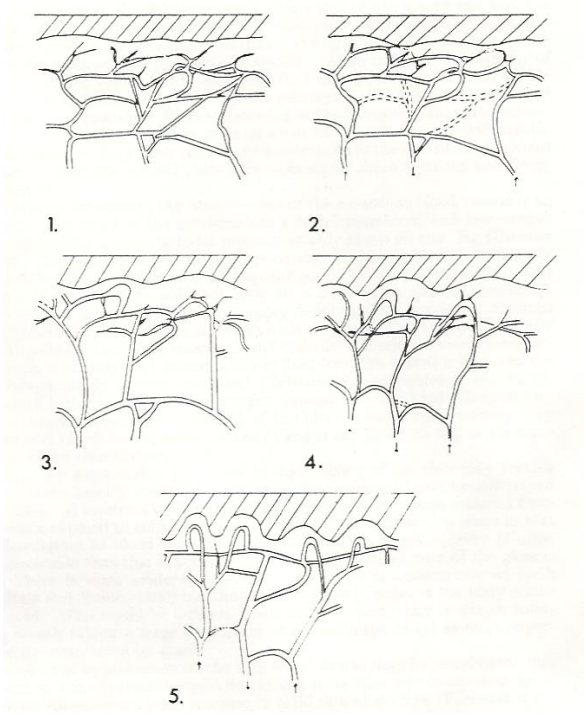


Figure A.4: Development of Microvascular skin perfusion from Perera et al. (37). It demostrates how capillary loops develop over time and in this process the capillary network becomes more organized and less abundant.

Other investigators have suggested that a mature pattern depended more on the weight of the child than on the time since birth. The microvasculature of the skin continues to develop during the first 3 months of life with some areas being faster than others. Several investigators have suggested that it may be due to cold and blood stasis in those areas (36).

A. 8. 3 Techniques to Observe the Microcirculation *in vivo*

Since the microcirculation plays a critical role in many disease states, such as diabetes, hypertension and sepsis, knowledge of its structure and function in humans is of great importance (38).

The direct observation of the microcirculation in disease has long been the aim of many scientists. Investigation on the capillary circulation began soon after the microscope came into use. Malpighi, for example, observed blood flow in the mesentery and bladder of the frog in 1686. Most of the observations were made on animals and upon translucent tissues. In 1874, Hueter by means of reflected light observed the vessels on the inner border of the lower lip, calling his method cheiloangioskopie. Weiss standardized in 1916 a method for the observation of the skin capillaries at the ends of the fingers and toes (39).

Laser Doppler Fluxometry (LDF)

In LDF monochromatic laser light is emitted into the tissue and scattered back giving an enlarged signal which is process to a linear output. LDF uses the Doppler shift to give a value of the mean erythrocyte flux found in the investigated tissue. Is very easy to apply and requires few minutes to obtain readings (40).

The electromagnetic waves from the laser light are emitted into the tissue. The reflected parts are captured with a photo-detector. When the light hits an object in movement the frequency changes which produces the “Doppler-effect”.

Intravital microscopy

Intravital microscopy allows visualizing the interaction of blood components with the endothelium (e.g. leucocytes and thrombocytes) and the leakage of macromolecules into the tissue. Intravital microscopy usually requires a fluorescent

dye to enhance contrast which has restricted its use primary to animal experiments (40). Measurements of the human microcirculation can be made through the capillary nailfold bed without the use of fluorescent dyes, but its value may be limited by the extreme sensitivity of nailfold microcirculation to external temperature or vasoconstrictive agents. The capillary microscopy setup consists of an intravital microscope, which restricts its use in humans to the skin and other easy accessible sites like the lip and the bulbar conjunctiva (41). Applications in perioperative and intensive care have not yet entered into routine due to the awkward handling of the devices and the limited number of vascular beds that can be visualized (40).

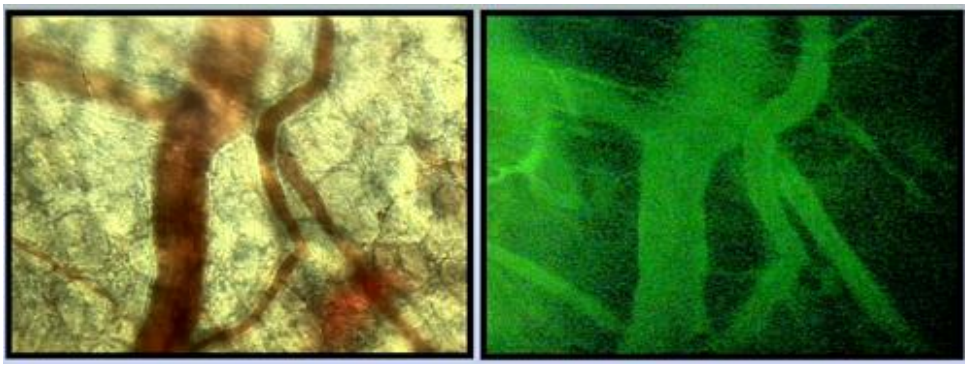


Figure A.5: Image of mouse cremaster muscle: without (left) and with (right) fluorescent dye using intravital microscopy (42).

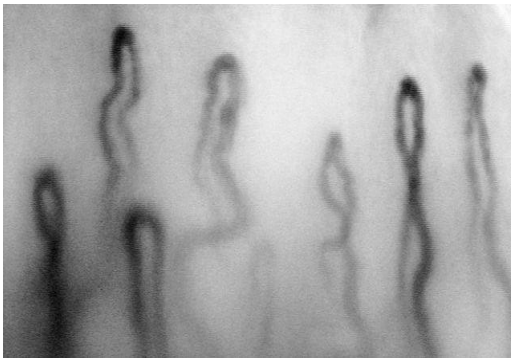


Figure A.6: Nail fold capillary microscopy (43).

Orthogonal Polarisation Spectral (OPS) imaging

OPS imaging was invented while developing a video microscope to obtain high contrast images of blood in the microcirculation using reflected light. It offers clinically relevant perspectives since it allows an easy and portable access to a variety of

vascular beds without the need of dyes for contrast enhancement (40). This makes also an applicability on organ surfaces possible, where not only capillaries but also complete microcirculatory networks can be studied (41).

OPS imaging makes the direct observation of the microcirculation in real time possible.

Several validation studies comparing OPS imaging against standard intravital fluorescence microscopy in animal models (38;44;45) have demonstrated that it is possible to make quantitative measurements of relevant parameters (vessel diameter, red blood cell velocity and functional capillary density) from these images. Further studies have also showed a possible microcirculation monitoring at the patient bedside using semi-quantitative analysis of the images (46-48). For more details see B.3.

Sidestream Dark-field (SDF) imaging

It is an actual improvement of OPS imaging that also allows a microcirculatory bed side monitoring.

SDF imaging consists of a light guide (Fig. 3), surrounded by 530nm light-emitting diodes (LEDs), a wavelength of light that is absorbed by the haemoglobin of red blood cells, allowing their observation as dark cells flowing in the microcirculation. The LEDs at the tip of the guide are optically isolated from the inner image-conducting core, and pump light deep into the tissue, illuminating the microcirculation from within.

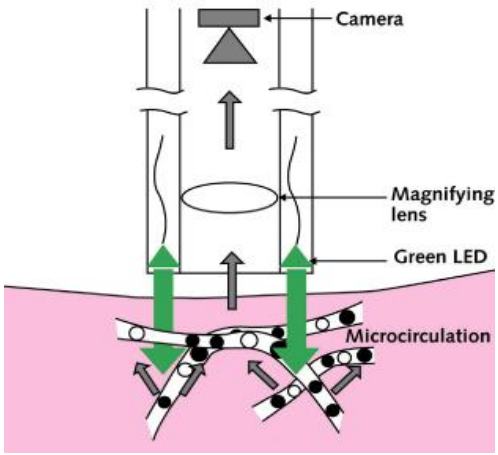


Figure A.7: Sidestream Dark-field imaging (49). This image shows the principals of SDF technique. Green light is emitted via LEDs. Only scattered light is detected by the camera after passing through a magnifying lens.

This dark-field illumination applied from the side completely avoids tissue surface reflections, giving clear images of microcirculatory structures and red, as well as white, blood cell flow. A magnifying lens projects the image onto a video camera. It is expected that SDF imaging will improve the imaging modality for the microcirculation, especially for capillaries (50). Until then, validation and comparative studies need yet to be done.

A. 8. 4 Microcirculation Dysfunction and Sepsis

Microcirculatory dysfunction during sepsis is characterized by heterogeneous abnormalities in blood flow with some capillaries being underperfused, while others have normal to abnormally high blood flow (49;51;52).

An intact and correctly functioning microcirculatory system is essential for efficient tissue oxygen delivery, yet, in sepsis, mediators of the inflammatory response impair microcirculatory function. The precise mechanisms involved remain to be elucidated. They include (53):

- a reduction in the number of perfused capillaries (so-called reduced functional capillary density),
- reduced red blood cell (RBC) deformability. Microvascular RBC flow is passively distributed throughout the capillary networks according to local vessel resistance (diameter and length) and hemorheologic factors (blood viscosity and RBC deformability).During sepsis the mechanical properties of the RBC, including membrane deformability and shape recovery are progressively altered such that the RBC becomes less deformable (54).
- endothelial cell dysfunction with increased permeability and apoptosis. Injury to the endothelium due to primary inflammatory mediators release results in expression of adhesion molecules on the cell surface which produce increased rolling, strong adherence, and transmigration of leukocytes into underlying tissue. Activated endothelial cells attract platelets, monocytes, and neutrophils - cells that are capable of initiating or amplifying coagulation. Thus, damaged endothelium induces a net procoaulant phenotype with the result of microthrombi formation and impaired fibrinolysis (12;23).
- altered vasomotor tone as consequence of activated endothelium.

- increased number of activated neutrophils with more neutrophil - endothelial interactions due to increased endothelial expression of surface adhesion molecules and release of inflammatory cytokines.
- activation of the clotting cascade with fibrin deposition. Inflammation and coagulation are closely linked in sepsis. In response to microbial invasion, macrophages release primary inflammatory mediators that results in the activation of the coagulation system and the complement system (23). Inflammatory mediators generate tissue factor expression and initiate coagulation through activation of the extrinsic pathway. This results in increased coagulation, and simultaneous depression of the inhibitory mechanisms of coagulation, and suppression of the fibrinolytic system results in a procoagulant state that may lead to the formation of microvascular trombi (55). The formation of thrombin from activated coagulation stimulates the activation of proinflammatory mediators. Thrombin has multiple effects on inflammation and also helps to maintain the balance between coagulation and fibrinolysis. Thrombin has a proinflammatory effect on endothelial cells, macrophages, and monocytes, resulting in the release of TF, platelet activating factor, and TNF-α. The cytokine response contributes to platelet activation and aggregation (23).

Even in the absence of sepsis, newborns and premature infants have a predisposition toward hypercoagulation. Neonatal plasma concentration of protein C and protein S is reduced. During sepsis, this hypercoagulability is further exacerbated by decreasing amounts of inhibiting coagulation factors (antithrombin (AT), protein C, protein S, reduced thrombomodulin, plus inhibition of fibrinolysis by plasminogen activator inhibitor-1 (PAI-1)) (23).

The clinical presentation is similar to adults; hypercoagulability leads to a prothrombotic state with resulting organ dysfunction (23).

It has been demonstrated that microcirculation plays an important role in the pathogenesis of shock and organ dysfunction, especially in sepsis (49). The introduction of orthogonal polarization spectral imaging allowed the first clinical observation of the microcirculation in human internal organs under the state of sepsis and septic shock. Recent studies using this technique have demonstrated the crucial role of microcirculatory abnormalities in defining the severity of sepsis and predicting

its outcome, a condition not revealed by systemic hemodynamic or oxygen-derived variables (46;48;53).

De Backer *et al*. investigated the sublingual microcirculation by 50 patients with severe sepsis using orthogonal polarization spectral imaging. They observed reduced vascular density and a smaller proportion of perfused small (< 20 µm) vessels in septic patients compared to control patients (52). Sakr *et al*. monitored the microcirculation on a group of 49 patients suffering from septic shock from the onset of septic shock until its resolution. They concluded that microcirculatory alterations improve rapidly in septic shock survivors but not in patients dying with multiple organ failure (46).

Based on these observations we hypothesized that similar changes should be seen in term neonates.

B. Goals of the dissertation

1) Primary question:

Are there changes in microcirculation of the skin very early in infection in term newborns?

2) Secondary questions:

1. What is the ideal site to observe non-invasively the microcirculation with OPS in term infants?
2. How to reduce movement or pressure artifacts of the images
3. Adapt the image analysis to the images obtained in neonates

C. Material and Methods

C.1 Study Design and Study Group

The microcirculation was observed prospectively on newborns on their 1st, 2nd and 3rd day of life after parental consent. No blood samples were taken due to the study. The investigator was blinded to clinical status and laboratory parameters of the infants. Microcirculation of the skin was analyzed in all newborns that fulfilled the inclusion criteria (see below).

Newborns were excluded according to the exclusion criteria (see below).

General Inclusion criteria

- Birth weight > 1500g.
- Gestational age between 36 and 41 weeks.
- Age < 12 hours at the first measurement
- Written consent from the parents (see addendum (G)).

Inclusion criteria for the control group

- No risk factors for infection (see A.3)
- Repeated clinical examination without pathological findings.
- Normothermia.
- APGAR-score ≥ 8 at 1st, 5th or 10th minute.
- CRP values ≤ 0.5 mg / dL on the 3 first days of life.

Inclusion criteria for the study group: "neonatal infection risk"

- Rupture of fetal membranes > 18 hours
- Maternal fever (> 38,5ºC)
- Elevation of maternal C-reactive Protein
- Fetal Tachycardia
- Stained amniotic fluid
- Lethargy or diminished awareness without a peripartal infection risk

Exclusion criteria

- Congenital defect of cardiovascular system, urogenital system, central nervous system, lungs, gastrointestinal system and skin.
- Congenital TORCH- infection
- Anemia (Hct <35%)
- Polycythemia (Hct >65%)
- Phototherapy
- Severe perinatal asphyxia
- Chromosomal anomaly
- Missing of parental consent

C. 2 Diagnosis of Infection

C. 2. 1 Clinical Evaluation

On the 1st and 3rd day of life a paediatrician obtained a physical exam of the neonates. Nurses examined the newborns several times a day and if something abnormal was seen, the physician was immediately informed. Clinical signs for infection are described in detail in A.6.

C. 2. 2 Laboratory Testing

In case of suspect findings on the physical exam or risk factors for infection in the maternal history, blood samples were obtained. Total blood counts as well as CRP and IL-6 were measured. This is part of the routine care for all infants. If CRP and IL-6 levels were below 0.5mg/dl and below 50pg/ml respectively, and the clinical status of the infant remained well, no more blood samples were obtained. If the infant continued to have positive clinical signs or laboratory tests for infection, CRP values were controlled during the next days.

Infection was defined as clinical suspicion in combination with increased C-reactive protein (>0.5 mg /dL) and/or increased IL-6 (>50 pg /mL) during at least one of the 3 days. Elevated CRP and IL-6 serum levels lead to the assignment into the infection group. An elevated IL-6 alone without any elevation of the CRP values

during the next days was considered false positive (29;56) and the infant was assigned to the control group.

IL-6 and CRP were analyzed in all infants with signs or risks for infection on the first day of life. If both were negative and the clinical status of the infant remained good no more blood samples were taken. If the infant began or continued to have clinical signs of infection and/or IL-6 was positive, CRP was controlled during the next days.

C - reactive protein: It was quantitatively determined through a turbidimetric immune test using the Olympus System CRP Latex- Reagent. The system can measure CRP down to very low concentrations.

Interleukin 6 was calculated with the IL-6 EASIA-CB assay in the laboratories of the department for clinical chemistry, Klinikum Grosshadern. The IL-6- EASIA-CB is a solid phase Enzyme Amplified Sensitivity Immunoassay (EASIA) performed with beads. The assay is based on a sandwich assay in which monoclonal antibodies directed against distinct epitopes of IL-6 are used. The minimum Detectable Concentration is estimated to be 1, 5 pg/ml.

C. 2. 3 Microbiological Testing

Blood culture

When by the clinical history or symptoms or laboratory findings, an infection was suspected, a blood sample for bacterial culture was taken. The quantity of the sample was at least 1 ml and was sent for aerobic culture to bacteriology department of the Max-von-Pettenkofer Institute in Munich.

Cerebrospinal fluid culture

If infants appeared sick and the CRP level was above 2 mg /dl prior to antibiotic therapy, a spinal tap was performed and spinal fluid was sent for culture and laboratory exam.

C. 2. 4 Treatment

In case of a presumed systemic bacterial infection the infant was treated intravenously for at least 3 days with antibiotics or until CRP values returned to normal levels (≤0.5 mg/dl). The infants received Ampicillin at a dose of 150 mg per kilogram body weight per day and Cefotaxim at a dose of 100 mg per kilogram body weight per day. When bacterial meningitis was suspected, the dosage was increased to 300 mg per kilogram body weight per day for Ampicilin and 200 mg per kilogram body weight per day for Cefotaxim. During treatment with antibiotics Nystatin 0.5 I.U. was given twice a day to prevent fungal superinfections.

C. 3 Defining Infection and Group Assignment

Infection was defined as elevated C-reactive protein (>0.5 mg /dL) with or without increased interleukine-6 (>50 pg /mL) during at least one of the 3 days of measurement. Elevated CRP levels lead to the assignment into the infection group. An elevated IL-6 alone without any elevation of the CRP values during the next days was considered false positive (29;56) and the infant was assigned in the control group.

After discharge, prior to image analyzes, infants were classified as following:

- Non Infected if CRP < 0.5 mg /dl during the 3 days of measurement.
- Infected if CRP > 0.5mg /dl at least one of the 3 days of measurement.

C. 4 Visualization of Microcirculation with Orthogonal Polarization Spectral (OPS) Imaging

C. 4. 1 Technical background of OPS imaging

To obtain images polarized incident light is projected through a beam splitter into the tissue. To image blood, a wavelength region, centred at an isosbestic point of oxy- and deoxyhemoglobin (548 nm), is chosen. Most of the superficially reflected light retains its polarization. In deeper layers of the tissue, a multiple scattering

occurs producing depolarized scattered light. A second orthogonally oriented polarizer is used to reject the light reflected from tissue surfaces with unchanged polarization before visualization. Thus, only light which has penetrated the tissue and has undergone scattering is depolarized and therefore detected. Scattered light works as a virtual source of light illuminating the objects from behind (fig. C.1) (57-59).

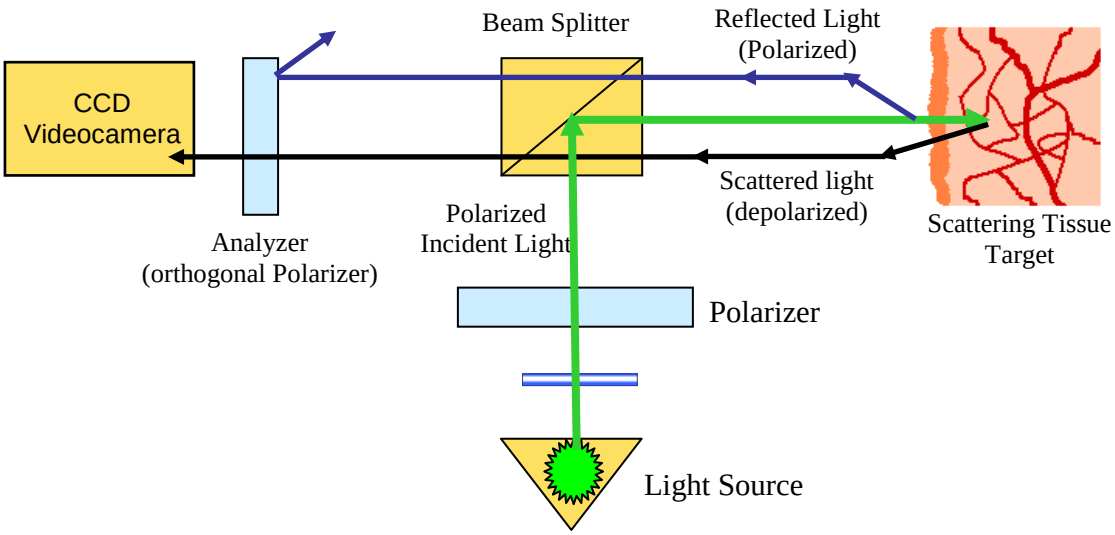


Figure C.1: Optical schematic of the OPS imaging probe adapted from Groener et al. (59). Polarized green light is sent via a polarizer and a beam splitter into the tissue. Two kinds of light are reflected. Polarized reflected light, which is absorbed by the analyzer, and depolarized scattered light, which is recorded by the CCD video camera

C. 4. 2 Cytoscan®

Cytoscan® is a small and portable handheld device which has OPS imaging technology incorporated (60) (fig. C.2.).

Figure C.2: Cytoscan® A/R imaging device

For our study we used the Cytoscan® A/R (Cytometrics, Inc. Philadelphia, PA, USA). It is equipped with a mini-CCD (charged-coupled device) camera of 5 x objective (Costar CV-M536 CCIR, JAI, Japan) (see fig.C.2). The 5 x objective lens gives a magnification at the camera face of 1 µm per pixel with a field of view of approximately 0.7 mm x 0.8 mm.

Figure C.3: Parts of the device: Probe (right), light source (metallic box) and CCD camera (black box).

The main parts of the device are the base unit (left side) with a holster for the probe on the top of it. The probe (right) is connected to an external light source (left on top of the base unit) via a guide cable and emits green light of a wavelength of 548nm during the measurement. The CCD camera connector cable (at the rear of the probe) is plugged into the base unit (fig. C.3). Via a PAL - Video recorder (Video Cassette Recorder, AG-7350, Panasonic, Osaka, Japan) and a monitor (PVM-1442 QM diagonal 33cm Sony, Munich, Germany), online imaging as well as recording of the images could be performed (fig. C.4). Images were recorded in standard VHS - Videocassettes (FUJI E -240 and SONY 240 DXF).

Figure C.4: Measurement Set with Cytoscan® devices and monitor on top, and video recorder underneath.

C. 4.3 Development of a Holding Device to Fixate the Cytoscan® Probe

During first test measurements it became increasingly clear, that it is very difficult to maintain the Cytoscan® camera device manually in a position which is stable enough to record good quality images. Due to magnification of the image, minimal movements of the hand resulted in wiggly sequences with pressure bias. In order to reliably evaluate the capillary microcirculation of a newborn, at least a few seconds of steady images are needed. Therefore it was necessary to develop a tool that would help to maintain the position of the Cytoscan® stable during the measurements. In collaboration with the department for technical assistance of the Frauenklinik Maistrasse we built a camera holding device with two aluminium bars and 2 articulations. The new device helped to adjust the position of the camera. The use of this prototype immediately improved the image quality by helping to achieve a stabile camera position (fig. C.5).

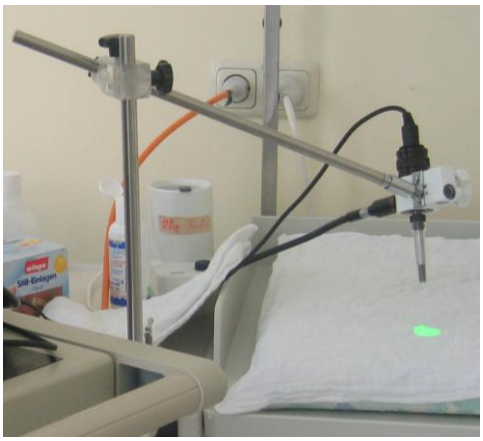


Figure C.5: First holding Device for the Cytoscan® imaging probe.

Figure C.6: Definitive holding Device for the Cytoscan® imaging probe.

The major disadvantage of the first prototype was its rigidity. To be able to react faster to the unpredictable movements of the newborn infants and to be able to adjust the camera position more accurate, we developed additional models of the camera holding device up to the actually used prototype (fig. C.6). It has a wide range of motion in three spatial dimensions, is stabile and very precisely adjustable. Additionally it is small enough to be used inside of incubators for measurements on premature infants. The holding device not only increases the quality of our images, it also reduces the time needed to study the microcirculation of infants from approximately 30 minutes to around 5 minutes. Especially in cases of restless newborns this is a substantial advantage.

C. 4. 4 Optimal Site for OPS Measurements

Based on the observations of Genzel-Boroviczèny and co-workers (61;62) the measurements were performed on the skin of the inner upper arm. The skin of term newborns is thicker than the skin of premature infants. This increased thickness leads to overall lower quality of the images. The light can not penetrate as deep into the tissue as it does in premature infants with their not yet finally developed skin. Additionally, the OPS recordings of term infants' microcirculation show a smaller number of microvessels in the inner upper arm as compared to the recordings obtained from premature infants.

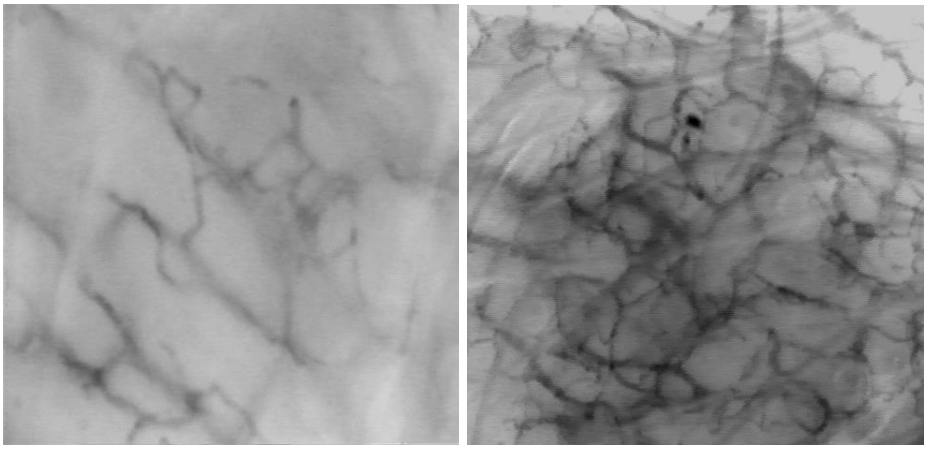


Figure C.7: Differences between the images obtained on upper arm from a mature (left) and premature (right) infant.

In the search for a better image recording area, measurements of virtually all areas of the skin of term newborns were done. We found that images obtained at the ear conch, had a better quality and showed a larger heterogeneity in microvessels than those obtained in inner arm. The ear conch is also free from lanugo and has a good vascularization. Finally, the newborns were not at all bothered by the measurement as the access is very easy and undressing is unnecessary (fig. C.8).

Figure C.8: Obtaining OPS images from the ear conch.

C. 4. 5 Duration of Measurements

The infants were measured in a calm moment, usually when they were asleep or after breastfeeding. Thus, we tried to avoid movement artefacts caused by a restless

child and vascular changes caused by a possible increase of blood pressure, which was observable when the children were crying. To perform the measurements the infant was placed on a changing table at room temperature. The ear which was exposed was chosen for the measurement. Using the holding device (see C.4.3.), the Cytoscan probe was slowly lowered to the skin until an image appeared on the monitor. A drop of water was placed between the skin and the probe to enhance the quality of the images. Once the image on the monitor was focused, the recording of the sequences began. We attempted to obtain 10 sequences of 10 seconds of duration each on the ear conch. For each sequence a different capillary network was searched. The same procedure was used in the inner side of upper arm. Measurements on the ear conch were performed on the first, second and third day of life.

In general, the recording was finished after 20 minutes but it always depended on how quiet the infant was. If the infant began to get agitated the measurement was postponed till after the next breastfeeding.

C. 4.6 Analysis of the OPS Sequences

The videos obtained were recorded on VHS - Videocassettes. After digitalization and coding using the record function of CapiScope® computer Program (KK Technologies, UK), the OPS sequences were analyzed off-line and blinded to clinical status of the infant. A six digit code was assigned to each infant to blind the observer to group assignment when evaluating the images. For the analysis a semi-quantitative method based on the works of De Backer (52) and Boerma (47) was used.

For that purpose a grid with three equidistant horizontal and three equidistant vertical lines was drawn and placed on the monitor showing the image. Two different parameters were analyzed:

Functional Vascular Density (FVD)

- Analysis with a semi quantitative method

The functional vascular density was calculated as the number of vessels crossing the lines on the grid placed over the image, divided by the total length of the lines. Vessels were identified by watching the recordings of the OPS measurements. In cases where an artefact such as hair, skin or water impeded the vision of the vessels

crossing a line, the length of this line was subtracted before dividing it by the total length of the line.

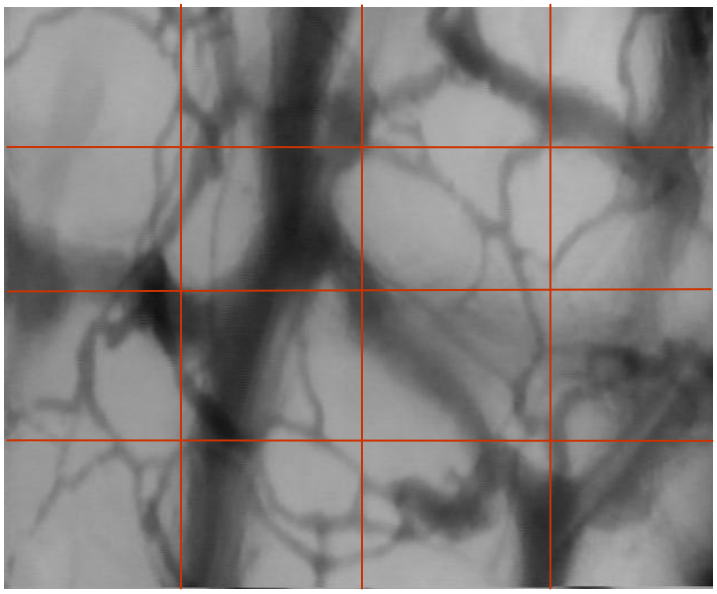


Figure C.9: OPS image obtained at the ear conch of a term infant with grid. The red lines over the image represent the grid used for the semi quantitative method of FCD determination.

- Quantitative analysis using the CapiScope® software

To calculate the Functional Vascular Density (FVD) with CapiScope® we traced the perfused capillaries within a defined area. CapiScope® calculates the length of the marked capillaries and divides this by the area of observation.

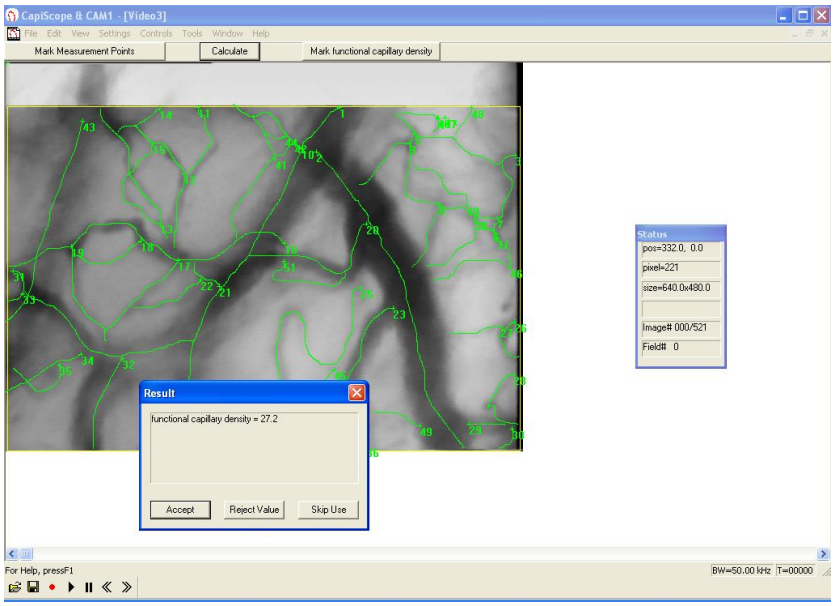


Figure C.10: Screenshot during FVD determination using the CapiScope® software.

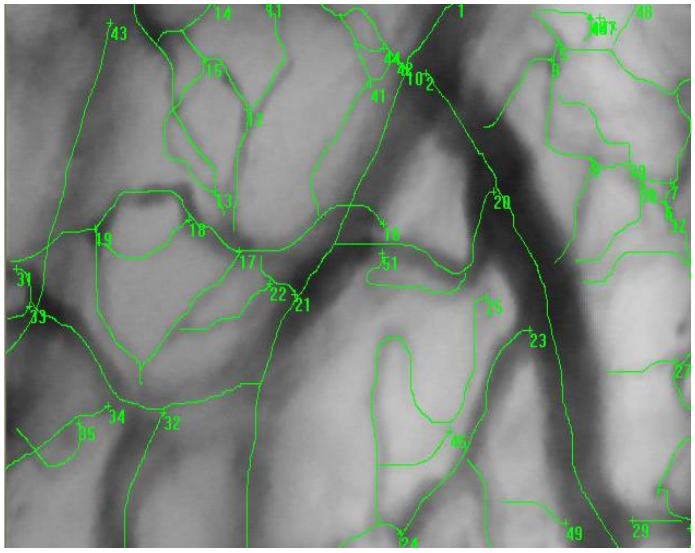


Figure C.11: More detailed image of FVD determination via CapiScope®.

Blood Flow analysis with a semi-quantitative method

To determine the blood flow the researcher watched repeatedly the recordings of the OPS measurements. Similarly to the calculation of the FVD only capillaries that crossed the lines of the grid were evaluated. The evaluated vessels were divided into two groups according to their diameter. Small vessels were defined as vessels with a diameter < 20 µm and large vessels were defined as those with a diameter between 20 – 100 µm. Vessels with a diameter larger than 100 µm were not seen.

The type of intravascular blood flow was classified for each vessel as no flow, intermittent flow, sluggish flow and continuous flow.

All vessels that did not have a continuous flow were classified as pathologic. The final read out of the analysis of the capillary blood flow was the percentage of vessels with a continuous flow.

C.5 Statistical evaluation

The Mann Whitney U test was used to compare microcirculatory parameters of the 2 groups and Apgar score values.

T-Tests were performed to compare the clinical data of the groups. Values are given as mean ± standard deviation (SD) if parametric, otherwise as median with 95% confindence interval (CI). Level of significance was set at $p < 0.05$.

D. Results

D. 1 Patient Data

D.1.1 Characteristics of the Study Subjects

A total 47 newborns were included into the study. Eighteen of the included infants were female (38%). 29 (62%) infants did not have any risk factor for an infection. Out of all infants, 16 (34%) developed an infection (Infection group) and the rest, 31 (66%) newborns, remained well (No Infection group) (see fig.D.1).

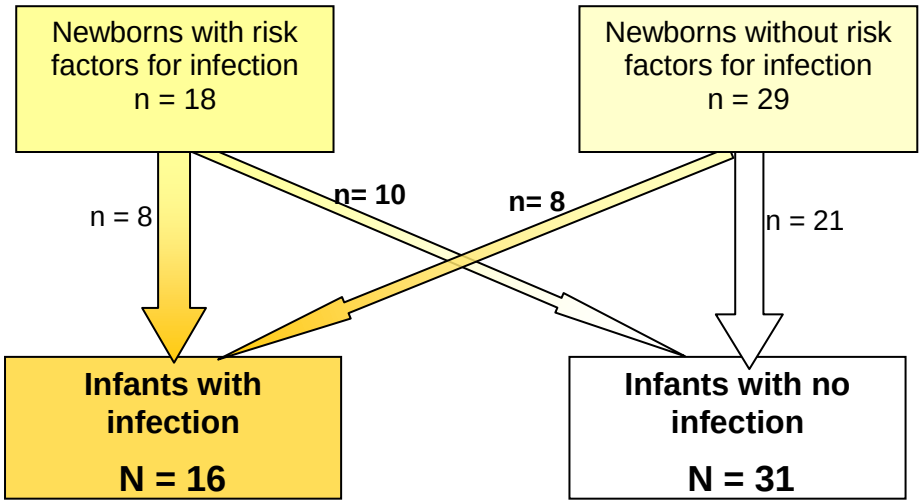


Figure D.1: Study population and final assignment to the study groups

		Infection Group n = 16	No Infection Group n = 31
Number of newborns		16 (33%)	31 (67%)
Sex	Male	14 (87%)	15 (48%)
	Female	2 (13%)	16 (52%)
Mean birth weight ± SD (gr.)		3526± 490	3311± 456
Mean gestational age ± SD (weeks)		39.3± 1.2	38.9± 1.2

Table D.1: Demographic data of the infants in both groups.

No statistical differences were observed in birth weight and gestational age between both groups. However, the percentage of males was noticeably larger in the infection group (tab. D.1).

D.1. 2 Birth Modus and APGAR-score

The types of birth included: spontaneous delivery, caesarean section, vacuum extraction and forceps extraction. In both groups the majority of infants were delivered spontaneously. There was no statistical significant difference on birth modus (tab. D.2).

The APGAR-score of the infants was determined at the first, fifth and tenth minute of life as it is standard of care in the department of neonatology. The APGAR-scores did not significantly differ between both groups (tab. D.3).

The pH in the umbilical cord and the base excess (BE) in the umbilical cord were slightly lower in the infection group compared to the no infection group. This difference did not reach statistical significance (tab. D.3).

	No Infection	Infection
Spontaneous Delivery	22 (68.7%)	7 (43.7%)
Caesarean section	5 (15.6%)	5 (31.2%)
Vacuum Extraction	3 (9.3%)	3 (18.7%)
Forceps Extraction	1 (3.1%)	1 (6.2%)

Table D.2: Birth modus

	No Infection			Infection		
	1min	5min	10min	1min	5min	10min
Median APGAR-score	9	10	10	9	10	10
Min. APGAR-score	7	10	10	7	10	10
Max. APGAR-score	9	10	10	10	10	10
pH in umbilical cord ± SD	7.32 ± 0.08			7.29± 0.09		
BE in umbilical cord ± SD(mmol/L)	-4.6 ± 3.19			-4.27 ± 3.7		

Table D.3: APGAR-scores and pH and BE in umbilical cord

D.1.3 Body Temperature

The rectal temperature of the infants included in the study was measured daily by the nurses. The results of temperature measurements showed no statistical difference between the infants with infection and the infants without it (tab. D.4).

	No Infection	Infection
1st day (mean ± SD)	37 ± 0.30 ºC	37 ± 0.49 ºC
2nd day (mean ± SD)	37.1 ± 0.16 ºC	37.3 ± 0.37 ºC
3rd day (mean ± SD)	37 ± 0.13 ºC	37 ± 0.13 ºC

Table D.4: Rectal temperature in grades Celsius measured during the first 3 days of life.

D.1.4 Clinical Symptoms for Infection

Infants were seen daily and their clinical status assessed. Twelve of the newborns (38%) in the no infection group showed abnormal clinical symptoms including lethargy, problems with temperature homeostasis, capillary refill time being larger than 3 seconds and grunting respirations in the first day of life. None of these symptoms persisted on the following days (tab. D.5).

In the infection group seven (44%) newborns showed clinical symptoms of infection during their first day of life. Two of the infants had an impaired temperature homeostasis on their second day of life. No pathological findings were reported for the third day of life (tab. D.5).

	No Infection			Infection		
	1st	2nd	3rd	1st	2nd	3rd
Lethargy	2	0	0	1	0	0
Problems with temperature regulation	7	0	0	3	2	0
Capillary refill time ≥ 3 seconds	2	0	0	2	0	0
Grunting respirations	2	0	0	1	0	0

Table D.5: Main clinical signs observed among the infants which could indicate infection.

D.1.5 IL-6 Values

IL-6 values were obtained from a total of 23 newborns in their first day of life because of suspected infection. From the infection group 8 out of 12 examined newborns had positive IL-6 values for an infection with a mean value of 507.3 pg /mL (fig. D.1) and a median of 99.6 pg /mL.

In the no infection group, two from a total of 11 had positive IL-6 values for an infection. The IL-6 mean value of the no infection group was 32.5 pg /mL (fig. D.2). The median was 29.4 pg /mL.

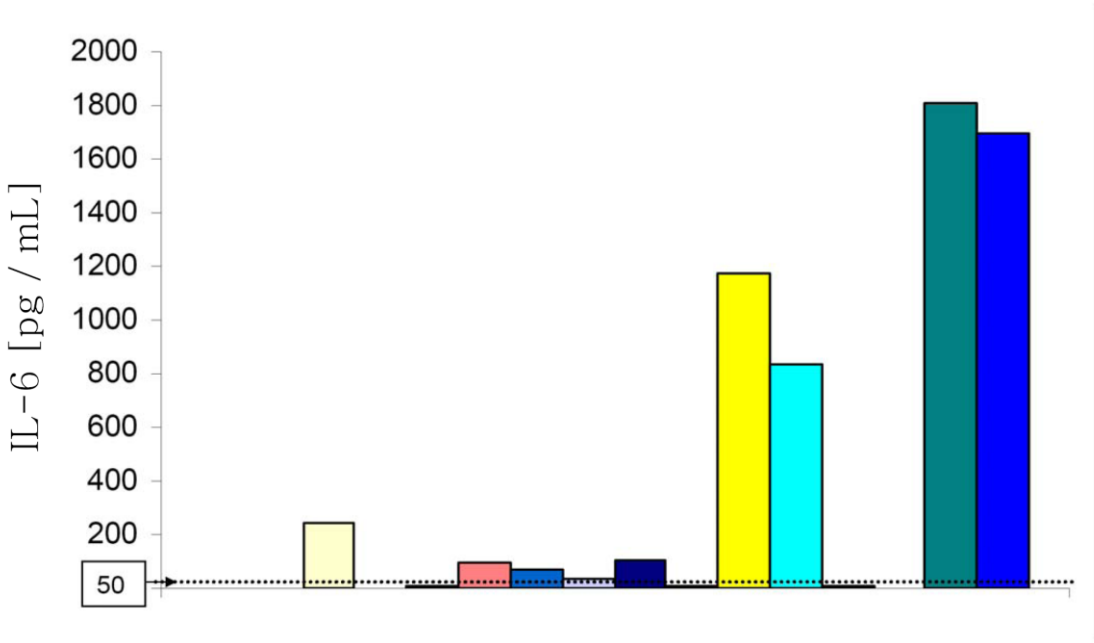
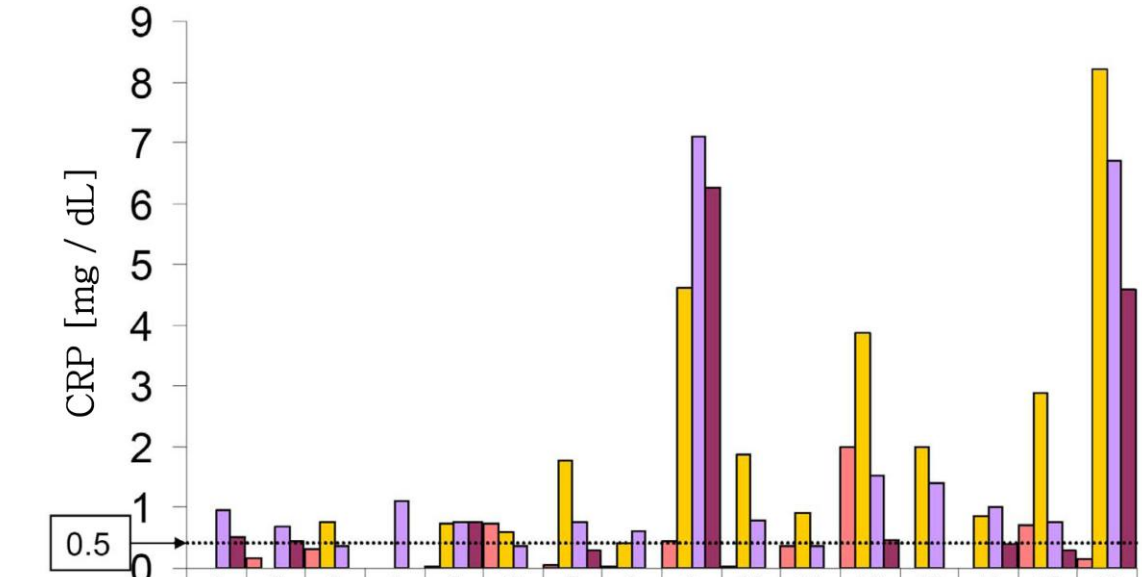


Figure D.2: Measurements of IL-6 obtained from the infection group on the first day of life. Each column indicates the IL-6 value of one individual patient. The dotted line indicates the limit for a positive IL-6 test

D.1.6 CRP Values

During the second day of life, in thirteen of the newborns CRP was analyzed. During the third day, in all infants of the infection group, and during the fourth day of life, in nine cases the levels of CRP were determined. The strongest increase of the CRP value was seen from the first to the second day of life. The majority of CRP serum levels declined after the second day, which was probably due to immediate start of antibiotic treatment (chapter C.2.4.). CRP levels were determined more than once in all infants in the infection group and in 2 infants in the no infection group. Figure D.3 shows the CRP serum levels of the infection group sorted by the

corresponding child. Most of the CRP serum levels are below 2 mg /dL. Few values show a dramatically elevated CRP indicating a severe infection (fig. D.3).



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1st day of life		0,18	0,32		0,02	0,74	0,05	0,02	0,45	0,02	0,38	2	0		0,72	0,14
2nd day of life			0,77		0,73	0,58	1,77	0,43	4,61	1,87	0,91	3,88	2	0,87	2,89	8,2
3rd day of life	0,97	0,68	0,36	1,1	0,76	0,36	0,76	0,62	7,1	0,8	0,38	1,53	1,4	1,01	0,76	6,71
4th day of life	0,52	0,45			0,76		0,3		6,26			0,47		0,4	0,3	4,58

Figure D.3: Course of CRP of the infection group sorted by individuals. The dotted line indicates the limit for a positive CRP test. The days of life are coded in colours. The CRP values are included in a table below, sorted by individual and day of life.

D.1.7 Blood Pressure and Heart Rate

For the majority of the included infants the blood pressure was measured. No significant differences between the infection and the no infection group were found (tab. D.6).

	No Infection			Infection		
	1st day n=21	2nd day n=11	3rd day n=11	1st day n= 9	2nd day n= 6	3rd day n=7
Systolic blood pressure [mmHg]	68± 9	69± 6	72± 8	69 ± 12	67± 6	65± 9
Diastolic blood pressure [mmHg]	40± 12	40± 7	41± 6	37± 8	40± 13	40± 7
Mean blood pressure [mmHg]	48± 6	48± 8	52± 6	47± 10	48± 10	48± 7

Table D.6: Mean values and standard deviation of blood pressure

D.1.8 Therapy

Thirteen infants out of the total of sixteen infants in the infection group (81.25%) and one infant (3.2%) out of the no infection group received treatment with antibiotics and Nystatin for three days. One of the newborns from the no infection group received prophylactic treatment due to long rupture of membranes, 62 hours, before delivery. Three out of the thirteen newborns with infection that received antibiotics, received a treatment for presumed bacterial meningitis for 5 days (see D.2.4.). Four newborns of the infection group did not receive antibiotic treatment despite increased CRP-values because parents refused any treatment.

One of the children in the infection group was treated with tincture of opium for 6 days for methadone withdrawal. One of the no infected newborns received a vaccine for Hepatitis B virus in its first day of life and another child from these group received calcium 10% orally due to low serum calcium levels

D. 1.9 Microbiology Results

A blood culture was obtained from all infants that received antibiotic treatment. None of them was positive. Two newborns had also a liquor culture, one of them was positive for *Staphylococcus aureus*.

D. 2 Optimal Location for the OPS Measurement

As described in C.4.4. the skin of term newborns is more developed, has more layers and therefore is thicker than the skin of premature infants. These circumstances made it necessary to find a, in regard to previous work (61;62), new location for OPS measurements in term newborns. Extensive searches lead finally to an optimally suited area, the ear conch (see C.4.4.)

Figure D.4 shows an OPS image of an infant from the control group. The relatively thin skin at the ear conch and the superficial course of the capillaries enables an easy scanning and recording of sharp images of the capillary net. A wide variety of micro vessels with distinct diameters that are close up to a capillary network is seen.

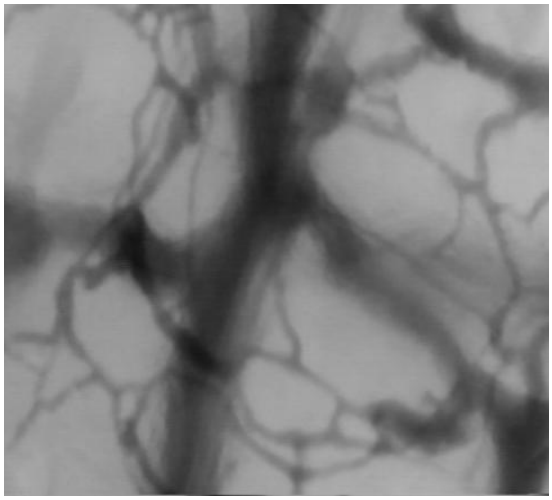


Figure D.4: OPS image of the micro vessels of the ear conch of an infant from the control group.

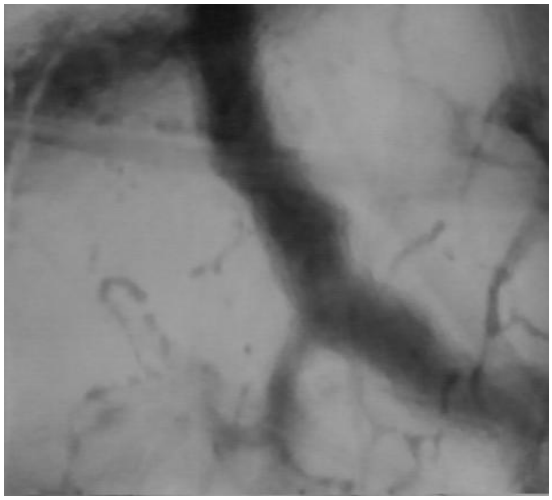


Figure D.5: OPS image of the micro vessels of the ear conch of an infant from the infection group.

Besides the differences in the structure of the capillary bed, OPS images recorded at the upper arm of a term newborn from the no infection group, show a poorer quality than those obtained from ear conch. Capillaries are predominant and no arteriole or venule can be seen. White curtain or smears like artefacts, caused by the skin folds, or lanugo, appear frequently on the images of the arm. It is very difficult to achieve an image with a good quality over the full size of the image field (fig. D.6).

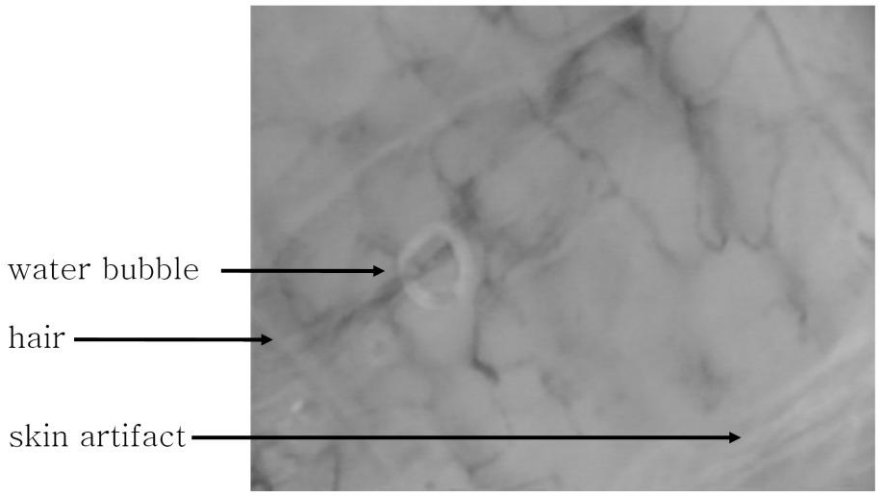


Figure D.6: OPS image of the micro vessels of the upper arm of an infant from the control group. The curtain or smear like artefacts are caused by skin, the double contrasts are hair artefacts.

The differences between recordings from ear and upper arm seem even more impressive, if images recorded at the upper arm and ear conch of an infant from the infection group are compared. Even on the non moving picture, in comparison to an image recorded from a healthy newborn, multiple gaps between the erythrocytes and the pronounced decrease in the number of visible vessels can be seen (figs. D.4 and D.5). On the images recorded from the upper arm of an infant from the infection group, the same findings like gaps between the erythrocytes in one capillary and decreased number of vessels can be observed, but the difference to the healthy control is far less pronounced (figs. D.6 and D.7). In the moving picture, gaps between erythrocytes can be observed as alterations in microvascular flow, such as no flow, sluggish flow, and intermediate flow. Decreased number of vessels represents a decrease in functional vascular density (see C.4.6).

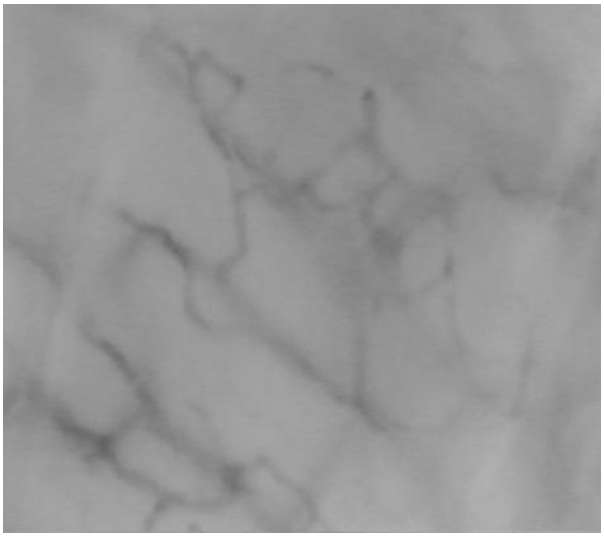


Figure D.7: OPS image of the micro vessels of the upper arm of an infant from the infection group.

D.3 Microcirculation Parameters

D.3.1 Microvascular Blood Flow

The microvascular blood flow was recorded on the upper arm and ear conch for all infants on their first, second and third day of life as described in C.4.4.-5. The evaluation was performed as described in C.4.6. All kinds of flow that were not conti-

nuous were rated as pathologic flow (see C.4.6). The percentage of vessels with continuous flow was significantly decreased in the infection group (p = 0.0003) (fig. D.8).

We also compared the flow quality between both groups in small vessels (diameter ≤ 20 micrometers) and large vessels (diameter ≥ 20 micrometers). In each group the percentage of the total of vessels with normal flow was significantly (p < 0.05) lower in the infection group compared to the no infection group. The proportion of normal flow in small vessels was 89 % in the no infection group whereas in the infection group was 66% (fig. D.9).

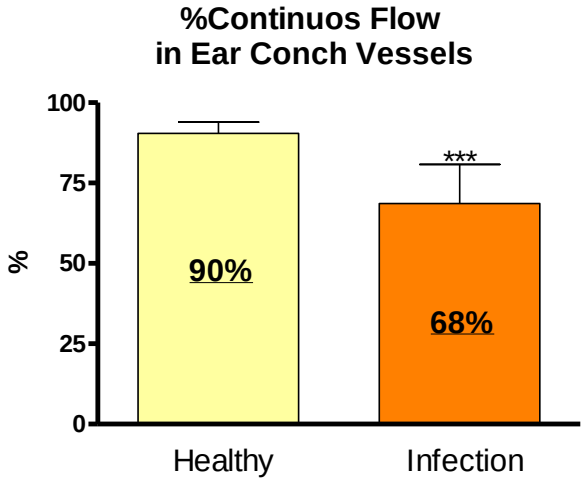


Figure D.8: Comparison of continuous flow between the no infection (mean = 90% and 95% CI [87-94]) and the infection groups (68% [56-81]) in all vessels seen on images recorded at the ear conch. The difference is highly significant (p = 0.0003).

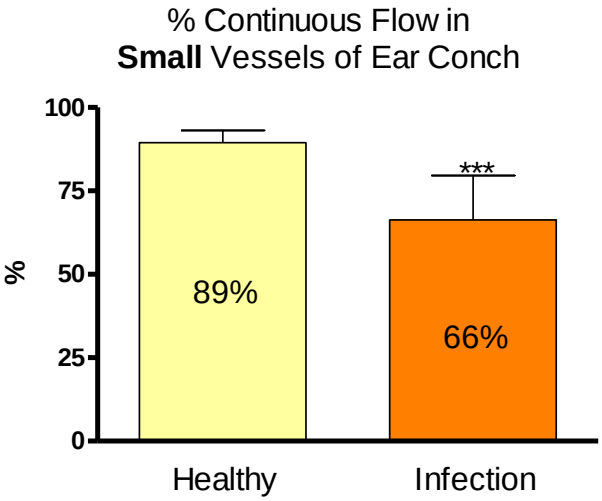


Figure D.9: Comparison of continuous flow between the no infection (89% [86-93]) and the infection groups (66% [53-78]) in small vessels seen on images recorded at the ear conch. The difference is highly significant (p = 0.0009).

These proportions were similar for the large vessels, with 70 % of continuous blood flow in the large vessels of the infection group and 92 % in the large vessels of the no infection group (fig. D.10). Hence, the quality of the bloodflow between Infection and Non Infection groups was significantly different for small and large vessels.

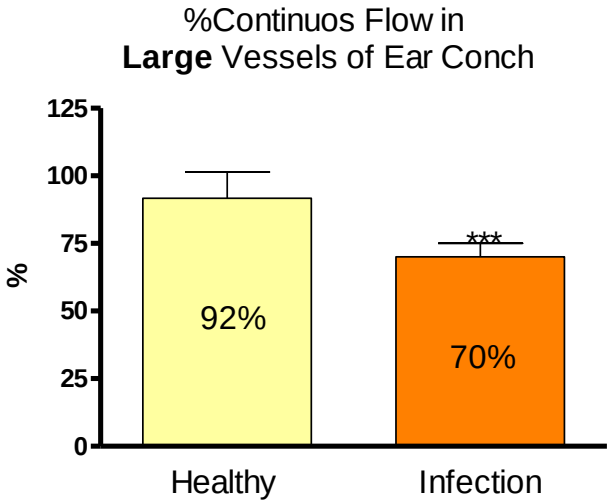


Figure D.10: Comparison of continuous flow between the no infection (92% [88-95] and the infection groups (70% [59-80)] in large vessels seen on images recorded at the ear conch. The difference is highly significant ($p < 0.0001$).

Images obtained from the upper arm showed a similar trend towards a lower proportion of continuous flow in the infection group, but in these measurements no statistical significance was reached (fig. D.11).

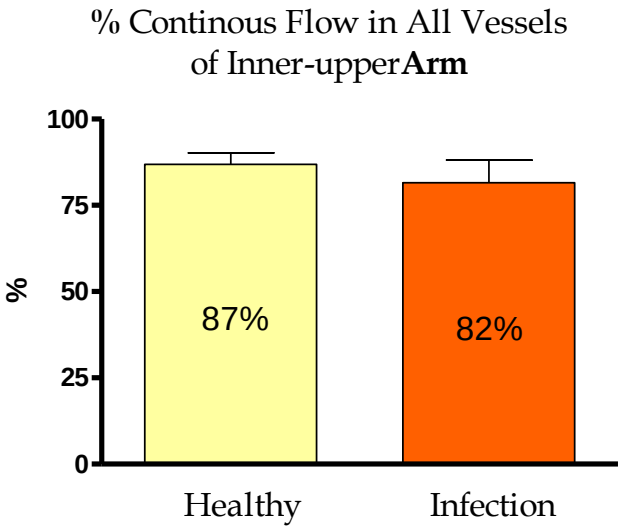


Figure D.11: Comparison of continuous flow between the no infection (87% [83-90]) and the infection groups (82 % [75-88]) in all vessels seen on images recorded at the upper arm. No statistical significant difference between the groups.

D. 3. 2 Correlation between laboratory values and microcirculatory changes

There was no correlation between CRP and /or IL-6 values with the degree of pathological microcirculatory flow.

D. 3. 3 Functional Vascular Density (FVD)

As described previously, the functional vascular density was determined by the number of vessels that crossed the lines of a superimposed grid divided by the total length of these lines (see C.4.6). Comparing the FVD of images recorded at the ear conch of newborns included in the no infection group to those of the infection group, a tendency to a lower FVD in the infection group was determined. However this difference did not reach statistical significance (fig. D.12). Evaluation of the pictures recorded at the upper arm showed a comparable tendency, which also lacked statistical significance (fig. D.13).

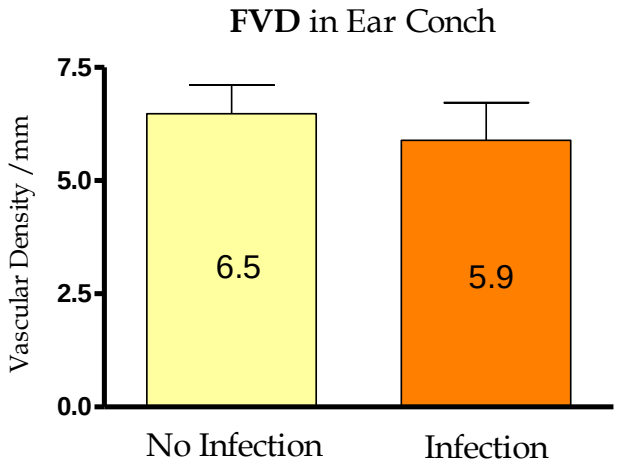


Figure D.12: Comparison of functional vascular density between the no infection (6.5 [6.2-6.8] /mm) and the infection groups (5.9 [5.5-6.5] /mm) in all vessels seen on images recorded at the ear conch. No statistical difference was reached.

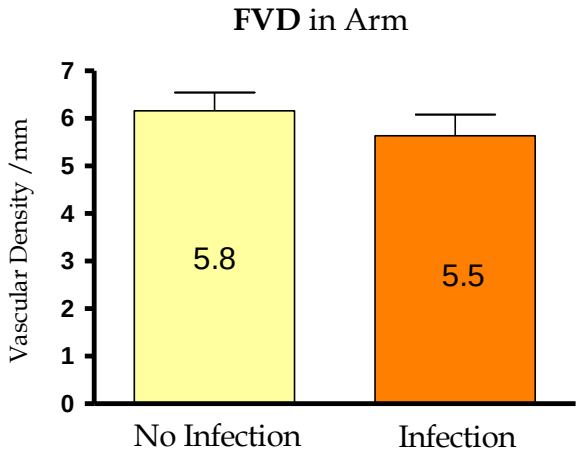


Figure D.13: Comparison of functional vascular density between the no infection (5.8 [5.5-6.2] /mm) and the infection groups (5.5 [5.0-6.1] /mm) in all vessels seen on images recorded at the upper arm. There is no statistical significant difference between the groups.

D. 3. 4 CapiScope® Program versus Semiquantitative method for quantifying Functional Vascular Density.

To see if there was a correlation between the semi-quantitative method (grid) and the quantitative method (Capiscope® software) we selected scans from both groups of infants in which no artefacts were seen. A total of 42 scans were analyzed using both methods. Values were compared using the Pearson's correlation coefficients. Results showed an extremely high correlation between both methods with a Spearman rank correlation coefficient of 0.64 ($p < 0.0001$) (fig. D.14).

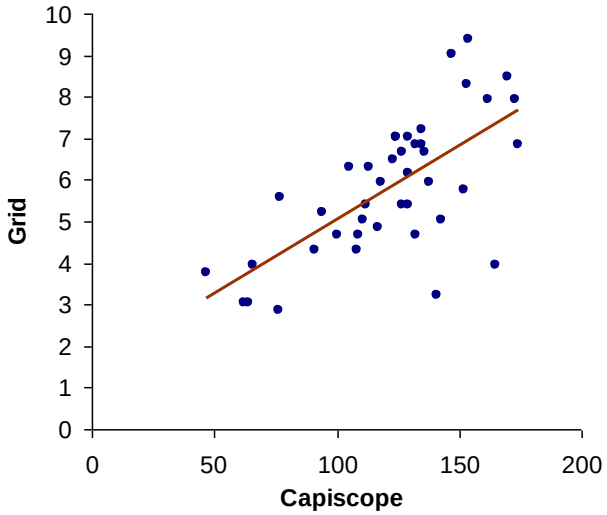


Figure D.14: Correlation of the functional vascular density calculated using the semi-quantitative method (grid) and the CapiScope® software. Independent calculation of FVD using both methods was performed. Dots represent the values obtained using the semi-quantitative method (Y-axis) and the CapiScope programme (X-axis).

E. Discussion

E. 1 Defining and Diagnosing Infection

There is no universal agreement on the definition for neonatal septicaemia and particularly a definition for neonatal infection with negative blood, urine or spinal fluid cultures is not well established. Therefore, finding a definition of infection for the study was a challenge. Positive blood and spinal fluid cultures are considered for adult and even older children the gold standard (4) in the diagnosis of bloodstream infections (11). However, cultures in newborns are often false negative, and therefore not useful as gold standard. They can be falsely sterile as suggested in *post mortem* cultures. *In vivo* blood cultures might be false negative due to the low yield caused by insufficient sample volumes (11) or intermittent or low-density bacteraemia due to inhibition of bacterial growth by earlier (i.e. intrapartum) antibiotic administration (63). In our unit just 1 mL of blood is sampled for blood culture. Fischer et al. estimated that if 1 ml of blood is sent for culture (in practice these volumes are often smaller), the sensitivity of this test is only between 30-40% whereas if 3 ml are sent for culture, the sensibility rises to 70-80% (64). With the low sensitivity of the test in neonates and the time required for final results, neonatologists do not rely on positive blood cultures for the diagnosis of infection or sepsis.

In the presented study (probably due to the low sensitivity of blood cultures, small volume of blood submitted for culture and the frequent use of intrapartal administration of antibiotics to the mother to prevent infection) in just 1 infant spinal fluid culture was positive (representing 6.2 % in the infection group) and no positive blood cultures were found. Serial determinations of C-reactive Protein levels have been shown to be useful in the diagnostic evaluation of neonates with suspected infection (15;15;29;29;63;65;66).

Neonatal infection was defined as a CRP serum level of more than 0.5 mg /dl with or without an IL-6 serum level > 50 pg /ml. Infants showing an elevated CRP were included into the infection group of the study (see C.2 and C.3). We were aware that this proceeding would most likely lead to an over diagnosis of infection and lead consequently to antibiotic treatment of infants with no infection. However, as this proceeding was applied in clinical practice, we also adopted it for the presented study. In 4 infants with presumed infection, microcirculation appeared normal with

more than 85% of the vessels with a continuous microcirculatory blood flow. These infants were described as clinically well without signs of infection and their CRP levels were between 0.62-2 mg/dl.

E. 2 Patient population

Male predominance in the infection group

Interestingly, 80% of newborns with infection were male newborns. It has been previously described that male newborns are at higher risk for neonatal infections (67) although the cause of this is still unknown. Our data seem to confirm this elevated risk of male newborns for infection.

E. 3 OPS Measurements

Several validation studies comparing OPS imaging with standard intravital fluorescence microscopy in animal models have demonstrated that it is possible to make quantitative measurements of relevant parameters (vessel diameter, red blood cell velocity and functional capillary density) from these images (45;59). Further studies have also shown a possible microcirculation monitoring at patient bedside using semi-quantitative analysis of the images (46-48;52). The use of OPS in newborns has been already validated (61).

OPS-imaging has some disadvantages. With this technique, only those tissues with a thin epithelial layer can be explored. The direct visualization or study of organs can only be made during surgery. The study of the microcirculation during infection in newborns can only be conducted on the skin. This does not allow the observation of changes occurring in central organs. Movement- or pressure- artefacts as well as the presence of blood, saliva or hair can affect the quality of the images obtained and thus the resultant data. Therefore, investigator movements or pressure were avoided by using a holding device for the microscan camera. Moreover, any type of dirt on the skin must be removed before the measurement. In our measurements the most frequently problem was the movement of the babies. The only possibility to obtain good quality images was to record only when the newborns were asleep which made sometimes the recordings time demanding.

E. 3. 1 Holding Device for OPS Measurements

The quality of the images obtained with OPS is very sensitive to movement artefacts. For the evaluation of the microcirculatory status of an infant it is necessary to record sequences of at least 10 seconds duration without virtually moving the camera device. Stabile images of ten seconds duration are the basic requirements for off-line video analysis using CapiScope® (KK Technology, Colyton, Devon, UK). Up to a certain extent it is possible to stabilize the images with the automatic movement correction software tool of CapiScope®. Although this is very helpful for correcting small movements in the sequence, movements of wider amplitude may result hard and very often impossible to correct (68).

In this context the development of the holding device for the camera was a major improvement for the image quality (see C.4.3). The holding device not only stabilized the image, but also reduced dramatically the time needed to achieve the sequences. This new invention has had a great impact on the overall image obtaining and evaluation quality.

E. 3. 2 Applicability of OPS for Microcirculation Recordings in Term Infants

The applicability of OPS in the study of the microcirculation in term newborns was essential for our experiments. Like in the studies performed on preterm infants (61;62) we can conclude that using OPS in newborns is possible (see D.2.). Until now, most of the studies done in humans have examined the microcirculation under the tongue (47;48;52;69) or in internal organs during surgery(44;70;71). We used the ear conch and the upper internal part of the arm as measuring points due to the fact that measurements under the tongue in term infants are not possible, since any object introduced in the mouth of a healthy newborn stimulates the sucking reflex.

Previous studies in premature infants have used the inner arm as measurement location for OPS recordings (61;62). During our first assays in term infants we tried to assess the quality of the scans obtained in different body parts of term newborns. The result was the discovery of a new possible measure point, the ear conch. It has the great advantage of being an ease accessible location without the disadvantage of having to remove clothing. Thus, the baby is not disturbed and the scans can be easily obtained.

Life-threatening infections cause disturbances in the microcirculation of central organs reducing their blood perfusion and lead to organ failure and death (53). The ear conch is perfused by terminal branches of the external carotid artery and thus connected to central blood circulation. It can be hypothesized that alterations of perfusion observed at the ear conch are more relevant to the overall health status of the infant than changes of perfusion seen in the arm which resembles peripheral circulation.

When comparing the results obtained in the study, we observed that the microcirculatory changes that can be seen during infection in newborns are easily recognizable in the scans from ear conch. In fact ear conch scans have some advantages over arm scans:

- They are easier and faster to obtain (to get images of the arm we have no need to undress the baby).
- The least pressure artefact is immediately noticed in the ear conch since the blood flow suddenly appears to be slower or even disappear. On the other side, the image quality of scans from arm appears to improve with slight pressure since more vessels are seen. This makes measurements at the arm more prone to bias caused by pressure (fig. E.1).
- The ear conch is a very small area in newborns. Due to its size the measurements are all executed on virtually the same spot, which results in similar scans during the different measurement times. We have observed that the differences of scans obtained with just a centimetre of distance in inner arm can present extremely different capillary nets which result in very different data obtained from the same newborn during the same sets of measurements.

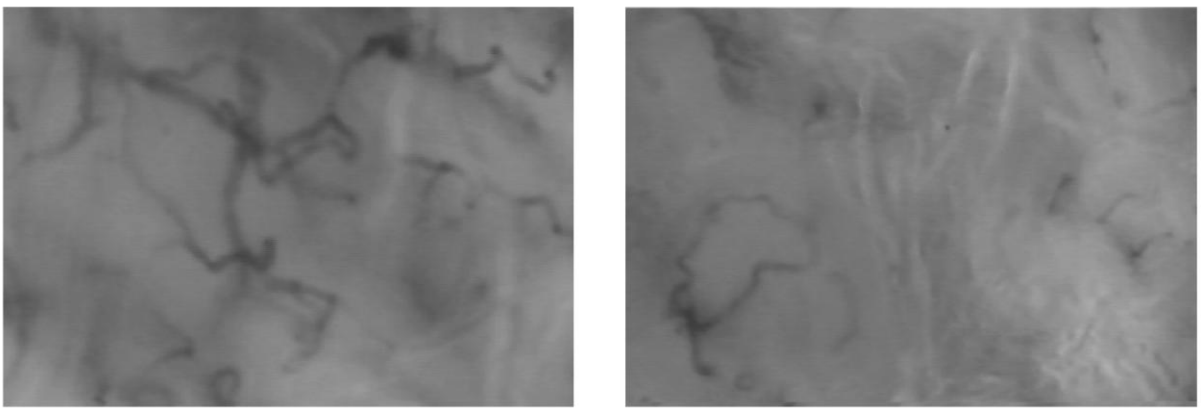


Figure E.1: Images taken from the inner-upper arm from the same infant with (right) and without (left) pressure. Pictures were taken at the same point of measurement.

E. 4 Quantifying Microcirculatory Parameters with CapiScope® Software versus the Semiquantitative Method.

Previous studies (61;62) in newborns have used the CapiScope®, a validated analysis software (72) to quantify microcirculation. It provides automatic red blood cell velocity measurements and calculates the capillary density. The program is very sensitive to low image quality and highly dependent on exact marking of central axis of the vessel. Especially the latter makes the image evaluation process extreme time-consuming.

The fact that the grid method correlates well with the CapiScope® calculated vascular density led us to accept the grid method as a reliable method to quantify the vessel density in microcirculatory scans. With the semi-quantitative method it is possible to determine most of the parameters involved in tissue perfusion, including vascular density and quality of perfusion. Reproducibility of the semi-quantitative score has been described by De Backer et al. as been very good, having an intra-observer variability between 2.5 and 4.7 % for vessel density and 0.9 to 4.5% for vessel perfusion. The inter-observer variability is slightly higher (52). On the other side, it is not possible to determine the velocity of red blood cells as precisely as CapiScope® software does.

E. 5 Microcirculatory changes in neonatal infection

It has been shown that microcirculation is altered during infection (39;49;55;58;69;73). Studies in adults with sepsis have demonstrated that a pathological flow can be clearly observed *in vivo* with scans obtained using OPS and SDF image techniques (47;49;52;53;73). The impact of microcirculatory changes on the outcome of patients suffering from sepsis has been shown by Sakr et al. (46). Until now microcirculatory alterations during infection have never been observed in vivo on term newborns via OPS.

The results obtained in the present work show, that OPS imaging is a helpful tool to observe and analyze microcirculatory changes in newborns with infection. From the total of 16 newborns of the infection group, 11 (69%) presented a pathological, not continuous, flow in more than 20 % of the vessels at least one of the three days

of measurements (see D.3.1). Previous studies in adults have shown such results on patients suffering from severe sepsis. None of our infants were severely septic. The highest CRP-value was 8.2 mg/dl which did not correlate with severe sepsis symptoms. There was no correlation to see between the degree of pathological microcirculation and the CRP value.

The fact that microcirculatory changes could already be observed in situations of moderate infection can be explained taken into account:

- the hypercoagulable state of the newborn infant (26)
- and the overproduction of cytokines compared to adults which has been reported in several studies (74-76).

In 4 of these 11 infants a pathological microcirculatory flow was detected at the same time as the CRP was elevated. These patients had elevated CRP levels already on the first day of life. In 6 neonates this pathological microcirculatory flow was even seen a day before the increase of CRP.

An early initiation of antibiotic treatment is crucial for the outcome of newborns with infection and so is the need of tools that allow an early diagnosis of infection. The observations of this study indicate that OPS measurements might be a useful complementary tool helping to establish a diagnosis of infection in term newborns. The fact that half of the patients that developed microcirculatory alterations, showed these a day before laboratory changes were measurable, should lead to further investigation. Microcirculatory alterations seem to occur before CRP levels are increased in serum.

Peculiar measurements in the infection group:

Four infants (25%) in the infection group had a normal flow with < 20% of vessels with disturbed flow. Looking in detail into the data of these patients some possible explanations for these findings became apparent.

- Two of the four had only borderline elevated levels of CRP of 0.74 mg /dL and 0.62 mg /dL, respectively. In these cases it might well be that they actually did not have an infection. Taking into account that almost all of the literature written about the sensitivity and sensibility of CRP in newborns is done with laboratory values of 1 mg /dL or above (15;32;65), this could be explained as false positives. Maybe this should lead to thinking about if the clinical practice is correct or if we

are being too cautious when considering a neonatal infection with CRP levels greater than 0.5 mg /dL.

- An additional case reached a CRP value of 2 mg /dL on the second day of life. The baby was born via caesarean section and so, had theoretically no traumatic delivery. Among the three days of measurements the child showed no clinical signs or symptoms for an infection. Could it be a false positive? No obvious explanation could be found to explain the CRP elevation in this case.
- The last of these four cases presented on the first day of life a CRP of 8.2 mg /dL together with signs and symptoms for an infection. Again the blood culture and the liquor puncture showed no growth. The microcirculatory changes observed in this infant where very interesting. Instead of the sluggish flow predominance, seen on the other infants, we observed a mixture of flows and a new one until now not observed. No flow or stagnant flow was seen in almost all capillaries with a smaller diameter than 10 µm. The rest showed a continuous flow or even a faster flow than usual. Probably the child had already entered in a hyperdynamic phase and due to the fact that we have defined pathologic flow as not continuous we missed this pathological finding. From this situation could be deduced that for following studies a “hyper dynamic” flow should be included in the classification of flows for the semi-quantitative method.

When comparing the microcirculation of this newborn with that of the only other newborn who reached high levels of CRP in blood (7.1 mg /dL), we saw a big difference. The second one presented a day before CRP reached its maximum, a pathologic flow in 82% of all its small vessels. From these differences arise further questions:

- Are such microcirculatory differences due to the inter-individual inflammatory system responses? There are some studies which report a genetic polymorphism in bactericidal reactions of neonates which correlates with the severity and outcome of neonatal sepsis (77;78).
- Is it due to the nature of the pathogen? Mohamed *et al*. (76) observed that the response of neonatal blood cells in secreting pro-inflammatory cytokines was different to different organisms.

Probably both factors affect these changes on microcirculation. If the differences in these turn out to be secondary to the nature of the pathogen, one could speculate,

that OPS might get even stronger impact on helping in the diagnosis and treatment of neonatal infections in the future. However, these are only single observations and cannot take to definitive conclusions, but should be subject of investigation in larger scaled studies using OPS by neonatal infection.

E. 6 Further Interesting Observations Using OPS

OPS in dark pigmented children.

At the beginning of the study we tried to observe the microcirculation in some black children. On their first day of life the images obtained could show the microcirculation but on the next days an overall melanin dots made these observations almost impossible. Melanin has a broad spectrum of absorption of wavelengths of 250 to 1200 nm which increases steadily toward shorter wavelengths (79). OPS light has a wavelength of 550 nm which is absorbed firstly by the melanin located in the epidermis. At the same level, the haemoglobin contained in blood vessels also absorbs this light. Thus, it is difficult to distinguish clearly the real path of the erythrocytes among the vessels and therefore the analysis of the microcirculation.

E. 7 Correlation between Laboratory Values and Microcirculatory Changes

There was no correlation between the levels of elevated CRP and/or IL-6 values with the degree of pathological capillary perfusion. However microcirculatory flow was compromised in infants with elevated CRP levels. We hypothesize that the degree of altered perfusion correlates with the degree of organ damage caused by infection. Thus, microcirculatory changes show the direct effect of infection on tissue function. In contrast, CRP and IL-6 elevations are surrogate markers of inflammatory response as they do not resemble direct organ damage but a secondary immune response to infection.

E. 8 Impact of these data

The fact that changes on the microcirculation are easily observed on the ear conch of newborns suffering from a moderate infection may enable the use of OPS imaging as a complementary diagnostic or control tool in infections of newborn in the future. To make that possible the recruitment of more data and patients is needed to be able to analyse the results with enough statistical power.

Further studies should also try to be able to solve the question if distinct pathogens show different changes in microcirculation blood flow and if the changes are reliably visible one day before laboratory testing becomes positive.

Since different software tools are available to date, it might be even possible to develop an automatic evaluating tool in future, so that trained experts for the evaluation of the microcirculation of newborns, might become superfluous in future, which could, in case larger studies show a sensitivity and specificity for this test, lead to OPS as an easy bedside test for infection.

F. Summary

The early diagnosis of neonatal infection remains a challenge. Recent studies using Optical Polarized Spectral (OPS) imaging techniques, have reported microcirculatory alterations in adult patients with severe sepsis and septic shock. The objective of this study was to compare microcirculatory flow and vascular density between healthy controls and sick newborns with neonatal infection.

OPS images were obtained prospectively from the vascular bed of ear conch and upper arm of 47 newborns on their 1st, 2nd and 3rd day of life. Infection was defined as IL-6 >50pg /mL and CRP >0,5 mg /dL. OPS sequences were analyzed off-line, blinded to clinical status of the infant with a semi-quantitative method based on the works of Sakr (46) and Boerma (47). Flow was examined differentiating between no flow, intermittent, sluggish and continuous flow. All vessels that did not have a continuous flow were classified as pathologic and their percentage was determined. Vascular density of the sequences was also calculated.

Sixteen infants developed an infection. Most episodes of infection were not severe. Microcirculatory data was analyzed and tested with a non parametric test. The images obtained from ear showed a better image quality and an easier performance than those from upper arm in term newborns (infection: 32%, 95% confidence interval [19-44] vs. healthy: 10% [6-13]; p= 0.0003). Despite a tendency to higher percent of impaired flow, the microcirculatory flow in arm did not reach a statistical significance when comparing both groups (infection: 19% [16-25] vs. healthy: 13% [10-11]; p= non significant). A trend to lower vascular density was observed in the sequences obtained from ear in the infected group but not in those obtained from arm. Both places did not show a statistical difference.

It can be concluded that ear conch is a better site to study the microcirculation in term newborns than the arm. In the ear conch even in infants with mild to moderate signs of infection, microcirculatory flow is impaired in a large proportion of vessels early in the course of disease. Since these changes can be easily recognized at the external ear, an on-line evaluation of microcirculatory flow could be an important screening tool for infection in the future.

G. Zusammenfassung

Die frühe Diagnostik neonataler Infektionen bleibt eine Herausforderung. Neue Studien mit *Optical Polarized Spectral Imaging* (OPS) haben bei Erwachsenen mit schwerer Sepsis und septischem Schock Veränderungen der Mikrozirkulation gezeigt. Das Ziel der präsentierten Arbeit war es den mikrozikulatorischen Blutfluss und die funktionale Gefäßdichte zwischen gesunden Neugeborenen und Neugeborenen, die an neonatalen Infektionen erkrankt waren, zu vergleichen.

Die OPS Bilder wurden prospektiv an den subcutanen Gefäßbetten der Ohrmuschel und dem Oberarm von 47 Neugeborenen an ihrem ersten, zweiten und dritten Lebenstag aufgenommen. Infektionen wurden als IL-6 > 50 pg /mL und CRP > 0,5 mg /dL definiert. Die OPS Sequenzen wurden off-line und verblindet mit einem semi-quantitativen Verfahren basierend auf Arbeiten von Sakr (47) und de Boerma (48) analysiert. Der Blutfluss wurde dabei in die Kategorien „kein Fluss“, „intermittierender Fluss“, „stagnierender Fluss“ und „kontinuierlicher Fluss“ eingeordnet. Alle Blutgefäße, die keinen kontinuierlichen Blutfluss zeigten, wurden als pathologisch gewertet und der prozentuale Anteil der pathologischen Gefäße wurde ermittelt. Auch die Gefäßdichte der Sequenzen wurde berechnet.

Sechzehn der untersuchten Kinder entwickelten eine Infektion. Die meisten Infektionen verliefen nicht schwer. Die Mikrozirkulationsdaten wurden mit einem nicht parametrischen Testverfahren analysiert. Die Bilder von der Ohrmuschel zeigten eine bessere Bildqualität und waren leichter aufzunehmen als die des Oberarms. Die Analyse der Aufnahmen am Ohr zeigten eine signifikant erhöhte Anzahl an Blutgefäßen mit gestörtem Blutfluss in der Gruppe der Kinder mit Infektion (Infektionsgruppe: 32%, 95% Konfidenzintervall [19-44] vs. Kontrollgruppe: 10% [6-13]; p= 0.0003). Abgesehen von einer Tendenz zu einer erhöhten Rate an Gefäßen mit gestörtem Fluss, erreichten die Mikrozikulationsmessungen am Oberarm im Vergleich beider Gruppen keine statistische Signifikanz (Infektion: 19% [16-25] vs. Kontrollen: 13% [10-11]; p= nicht signifikant). Ein Trend zu einer geringeren funktionalen Gefäßdichte wurde in den Ohrsequenzen der Infektionsgruppe jedoch nicht in den Armsequenzen beobachtet. An beiden Messorten wurde keine statistische Signifikanz erreicht.

Aus den Ergebnissen lässt sich schlussfolgern, dass die Ohrmuschel ein besserer Ort als der Arm ist um Mikrozirkulationsmessungen an reifen Neugeborenen durchzuführen. Sogar in Neugeborenen mit leichten Infektionszeichen ist der

mikrozirkulatorische Blutfluss in einem Grossteil der Gefäße früh im Krankheitsverlauf gestört. Da diese Veränderungen an der Ohrmuschel leicht erkannt werden können, könnte die online-Evaluation der Mikrozirkulation in Zukunft ein wichtiges Skreeningverfahren für Infektionen werden.

H. List of Abbreviations

AT	antithrombin
BE	base excess
CCD	charge coupled device
CI	confidence interval
CRP	C- reactive protein [mg / dl]
dL	decilitre
E. coli	Escherichia coli
EOS	early onset sepsis
FCD	functional vascular density
Fig.	Figure
GBS	group B streptococcus
IgG	immune globulin G
IgM	immune globulin G
IL	interleukin
LDF	laser doppler fluxometry
LED	light-emitting diode
LOS	late onset sepsis
LPS	lipopolysaccharides
mg	milligram
ml	millilitre
OPS	orthogonal polarisation spectral imaging
OR	odds ratio
PAI-1	plasminogen activator inhibitor-1
PCT	procalcitonin
pg	picograms
PMNs	polymorph nuclear cells
PROM	prolonged rupture of membranes
RBC	red blood cell
S. aureus	staphylococcus aureus
SD	standard deviation
SDF	sidestream dark-field imaging
SIRS	septic inflammatory response syndrome

Tab.	table
WBC	white blood cell count [leucocytes / ml]
µm	micrometer

I. List of Figures

J. List of Tables

K. Bibliography

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L. Addendum

L. 1 Patient Information Brochure

Untersuchung der Mikrozirkulation bei Neugeborenen mit perinatalem Risiko für eine bakterielle Infektion.

Liebe Eltern,

wir dürfen Ihnen ganz herzlich zu Ihrem Nachwuchs gratulieren.
Wie Sie bereits im Gespräch mit uns erfahren haben, führen wir derzeit eine Untersuchung zur Früherkennung von Infektionen bei Neugeborenen durch. Mit dieser Broschüre möchten wir Ihnen weitere Informationen zur Verfügung stellen, die Sie für die Entscheidung über eine Teilnahme Ihres Kindes an dieser Studie benötigen.

Hintergrund

Bakterielle Infektionen sind eine der häufigsten Erkrankungen (Häufigkeit 1-3%) der Neugeborenen. Die Zeichen einer Infektion sind bei Neugeborenen allerdings unspezifisch, zudem finden sich typische Laborveränderungen oft erst mit einer gewissen Zeitverzögerung. Ein schnelles Erkennen der Krankheit ist jedoch wünschenswert, da ein früher Beginn der Therapie den Krankheitsverlauf entscheidend beeinflusst.

Studie

Um Infektionen bei Neugeborenen möglicherweise schneller erkennen zu können, möchten wir eine nicht invasive Methode etablieren, die Veränderungen der Hautdurchblutung bei

Neugeborenen mit Infektionsrisiko erfasst. Hierbei wird eine Methode angewendet, die für Ihr Kind völlig unbedenklich ist. Die Messungen werden bei Neugeborenen mit Infektionsrisiko durchgeführt, sowie bei gesunden Neu-geborenen als Kontrollgruppe.

Methode, Ablauf und Dauer der Teilnahme

Wir legen Ihrem Baby einen kleinen Messfühler auf die Haut. In dem Messfühler befindet sich eine Lichtquelle, die normales Licht mit einer Wellenlänge von 550 nm aussendet und daher grün leuchtet. Die Lichtquelle ist so konzipiert, dass es zu keiner Erwärmung oder anderen Auswirkungen auf die Haut kommt. Das grüne Licht ermöglicht die Darstellung von Blutgefäßen direkt unter der Haut, indem das reflektierte Licht durch eine kleine Videokamera erfasst und in einem Computer analysiert wird. Dadurch kann die Hautdurchblutung beurteilt werden.

In *Abbildung 1* sehen Sie unser Messgerät.

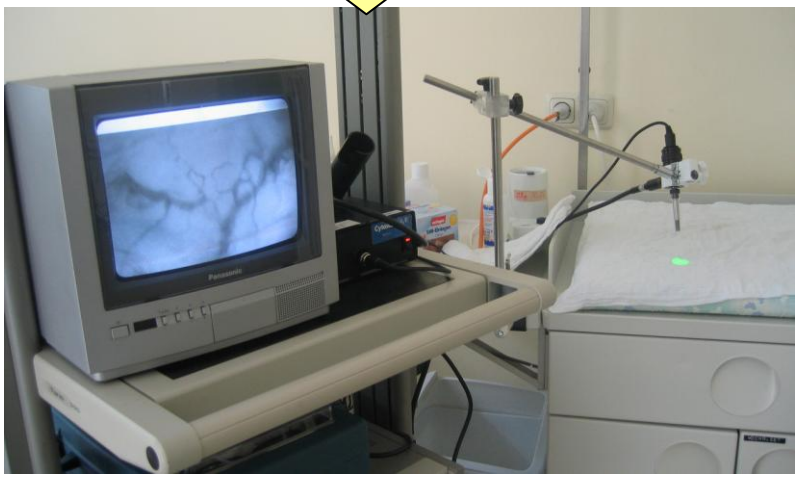

Abb.:1: Messung der Hautdurchblutung bei einem Neugeborenen

Am Ende der Messung werden noch die Körpertemperatur und der Blutdruck Ihres Kindes gemessen.

Die Untersuchung wird am 1., 2. und 3. Lebenstag durchgeführt. Sie ist für Ihr Kind schmerzlos, ohne Risiko und dauert ungefähr 15 bis 20 min. Sie können selbstverständlich gerne bei der Untersuchung anwesend sein, wenn Sie dies wünschen. Bei einer Nichtteilnahme an der Studie entstehen Ihnen und Ihrem Kind keinerlei Nachteile.

Datenschutz:

Nur die Ärzte der Studie haben im Rahmen der gesetzlichen Vorschriften Zugang zu den vertraulichen Daten, in denen Sie namentlich genannt sind. Diese Personen unterliegen der Schweigepflicht und sind zur Beachtung des Datenschutzes verpflichtet. Die Weitergabe der Daten im In- und Ausland erfolgt zu statistischen und wissenschaftlichen Zwecken, und Ihr Kind wird darin nicht namentlich genannt. Die Daten werden zur Auswertung verschlüsselt (d.h. die Daten werden ohne Namensnennung mit einer Nummer versehen).

Die Teilnahme an der Studie ist freiwillig und Sie können jederzeit die Teilnahme Ihres Kindes ohne Angabe von Gründen und ohne Nachteile für die weitere Behandlung, zurückziehen.

Falls Sie weitere Fragen zu dieser Studie haben, wenden Sie sich bitte an uns.

Studienleitung:

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Wir danken Ihnen für Ihr Interesse und wünschen Ihnen und Ihrem Kind alles Gute !

L. 2 Consent Form

Klinikum der Universität München

Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe – Innenstadt
Direktor: Prof. Dr. med. Klaus Friese

Kinderklinik und Poliklinik im Dr. von Haunerschen Kinderspital
Direktor: Prof. Dr. med. Dietrich Reinhardt

Leitung der Neonatologie:
PD Dr. med. Orsolya Genzel-Boroviczény

Klinikum der Universität München • Neonatologie – Innenstadt
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LMU

Ihr Zeichen | Unser Zeichen | Ansprechpartner | Telefon 089-5160 4535 | Telefax -4917 | E-Mail genzel@med.uni-muenchen.de

Einwilligungserklärung

Name des Patienten
(in Druckbuchstaben):.....
Geb. Datum: .....-.....-.....
Patient Nr.:.....
Ich erkläre mich freiwillig bereit, dass mein Kind an der klinischen Untersuchung mit dem Titel:

„Untersuchung der Mikrozirkulation bei Neugeborenen mit perinatalem Risiko für eine bakterielle Infektion“

teilnimmt.

- Ich bin durch den Arzt/ die Ärztin.....in einem persönlichen Gespräch ausführlich und verständlich über die Untersuchung und den Studienablauf sowie über Wesen, Bedeutung und Tragweite der klinischen Untersuchung aufgeklärt worden.
- Ich habe darüber hinaus den Text des Informationsblattes für Eltern/Erziehungsberechtigte und diese Einwilligung gelesen und verstanden.
- Aufgetretene Fragen wurden mir vom Prüfarzt/-ärztin verständlich und ausreichend beantwortet.
- Ich hatte ausreichend Zeit, mich zu entscheiden.
- Ich habe das Recht, jederzeit und ohne Angabe von Gründen meine

Einwilligung zur Teilnahme meines Kindes an der Prüfung zurückzuziehen, ohne dass mir und meinem Kind daraus Nachteile entstehen.

- Ich bin damit einverstanden, dass im Rahmen dieser klinischen Untersuchung erhobene Daten meines Kindes anonymisiert (d.h. ohne Nennung des Namens) aufgezeichnet werden, zur wissenschaftlichen Auswertung der klinischen Prüfung und zur Verwendung für weitere mit der Prüfung dieser Untersuchungen im Zusammenhang stehende wissenschaftlichen Fragenstellungen weitergegeben werden, sowie zur Überprüfung an die zuständigen Überwachungsbehörden oder an die Ethikkommission übermittelt werden.
- Ferner erkläre ich mein Einverständnis, dass die Studienleitung und vorgenannte Fachleute und Beauftragte in personenbezogene Daten meines Kindes Einsicht nehmen. Dies dient einer vollständigen Überprüfung der ordnungsgemäßen Durchführung der klinischen Untersuchung. Beim Umgang mit personenbezogenen Daten werden die Grundsätze des Datenschutzrechtes beachtet.

Datum.....

Unterschrift des 1. Sorgeberechtigten.....

Unterschrift des 2. Sorgeberechtigten.....

Unterschrift des aufklärenden Prüfarztes.....

L.3 Table for clinical information

Ethikett u. Nummer

Geb. Datum…….
Geb.Zeit……
Gewicht……..

Geburtanamnese

Infektionsrisiko

- O Blasensprung ≥18h
- O Mütterliches CrP- Erhöhung
- O Dick grünes Fruchtwasser
- O Mütterliches Fieber (≥38,5°C)
- O Choriamnionitis
- O Procedere:

Klinische Zeichen für Infektion	1.LT	2.LT	3.LT
O Reduzierter Allgemeinzustand			
O Temperaturinstabilität			
O **Hämodynamischezeichen:**			
Tachykardie (>180bpm), Hypotonie (<65mm Hg) grauer Hautkolorit, Rekap.Zeit >3sek,			
O **Respiratorischezeichen**			
Tachypnoe+ Stöhnungen/ Enziehung, Apnoe			
O **Neurologischezeichen**			
Tonus Störungen, niedrieger Fontanelle,Krämpfe….			
O **Haut**			
Purpura, eruption…			
O **Andere**			
TOTAL			

Labor	1.LT	2.LT	3.LT
O Leukozyten=			
O Neutrophile=			
O I:T- Quotient=			
O Thrombozyten=			
O Hb/Hkt=			
O IL-6=			
O CRP =			
O Lactat=			

O …….

Bakteriologie

- O Blutkultur vor Antibiose:
- O Ohrenabstrich
- O Urin
- O Liquor

Blutgasanalyse

BE=___ pCO2=___

pH=___ pO2=___

Medikamente

Bemerkungen

M. Curriculum vitae

Personal data

Name:	Irene Alba Alejandre
Date and Place of birth:	29 March 1981 in Granada, Spain
Marital status:	Married
Nationality:	Spanish

School Education

1985 –1992	School Sagrado Corazón de Jesús, Granada (Spain)
1992 –1993	Victor Mager School. Winnipeg. MB (Canada)
1993 –1999	High school Sagrado Corazón de Jesús, Granada (Spain)

Professional Education

Oct. 1999 – Sept. 2003	Medical studies, University of Granada (Spain).
Oct. 2003 – Jul. 2004	5th academic year, University of Lausanne (Switzerland) (Erasmus scholarship)
Sept. 2004 – Feb. 2005	6th academic year, Ludwig-Maximilian University Munich (European „free mover Program“)
July 2005	Final degree at the University of Granada (Spain)

Doctoral Thesis

2005 -	Frauenklinik Innenstadt, LMU Munich in the group of Professor Orsolya Genzel-Boroviczèny „Orthogonal Polarization spectral imaging (OPS) Measurements of Microcirculatory Changes in Term Newborns with Suspected Infection“

Further Qualifications

Languages	German:	Fluent in written and spoken
	English:	Fluent in written and spoken
	French:	Fluent in written and spoken
	Spanish:	Native language

Practicals

July 2001	Pediatric Orthopedia, Hospital Carlos Haya, Málaga (Spain)
July –August 2004	Gynecology and Obstetrics, Machame Hospital, Moshi (Tansania)
November 2004	Endokrinology, Medizinische Klinik Innenstadt der LMU
February 2005	Cardiac surgery, Klinikum Großhadern der LMU
June –August 2005	Neonatology, 1. Frauenklinik in der Maistrasse der LMU

Scholarships

2003 – 2004	ERASMUS scholarship, University of Granada (Spain)
2005 – 2007	DAAD-La Caixa scholarship for a research project: „Microcirculatory changes in Newborns at risk for early onset Infection“

Residency

Since August 2007:	Residency training program for gynecology and obstetrics, Frauenklinik Innenstadt der LMU, Munich.

Publications

Oral presentation

Europaediatrics 2006, European Pediatric Research Society. Barcelona, 7. –10. October 2006: „Microvascular Flow is compromised early in Neonatal Infection“.

Selected poster presentations

I. Alba-Alejandre, S. Hiedl, A. Bauer, F. Christ, O. Genzel-Boroviczèny, „Mikrozirkulatorische Veränderungen bei neonataler Infektion.“, 32. Jahrestagung der Gesellschaft für Neonatologie und Pädiatrische Intensivmedizin. Viena, 18 –20 Mai 2006.

I. Alba-Alejandre, S. Hiedl, O. Genzel-Boroviczèny, „Microvascular Flow is compromised early in Neonatal Infection.” 24th European Conference on Microcirculation. From Vascular Biology to Clinical Microcirculation. Amsterdam, 30. Aug.-2. Sept. 2006

I. Alba-Alejandre, S. Hiedl, O. Genzel-Boroviczèny, “Microvascular flow is compromised early in neonatal infection”. 23th perinatal German Congress. Berlin, 29 Nov. -1.Dec 2007.

I.Alba- Alejandre, V. Nobis, B. Heindl, F. Kainer, “ROTEM: Hilfreich in der Diagnostik der Fruchtwasserembolie? — Ein Fallbericht”. 23th perinatal German Congress. Berlin, 29 Nov. -1.Dec 2007.

I. Alba-Alejandre, V. von Bodungen, F. Kainer, “ROTEM – Einsatz in der Geburtshilfe”. 82. Tagung der Bayerischen Gesellschaft für Gynäkologie und Geburtshilfe. Augsburg, 29. - 31. May 2008 .

I. Alba-Alejandre, F. Kainer, K. Friese, I. Mylonas, „Hbs-Antigen Nachweis in der Schwangerschaft“ 57th Congress from the German Gynecology and Obstetrics Society. Hamburg, 16-19th Sept. 2008.

I. Alba-Alejandre, S. Hiedl, A. Heger, P.Hantschmann, F. Kainer, „Spontane postnatale Remission bei massiver intrauteriner Dilatation der Darmschlingen“ 24th German Congress for Perinatal Medicine. Berlin, 6-9th May 2009

Original Article

I. Alba-Alejandre, F.Kainer, K. Friese, I. Mylonas, „Hbs-Antigen-Nachweis in der Schwangerschaft”. Geburtshilfe und Frauenheilkunde. Heft 06, Jahrgang 69, Juni 2009.

Munich 16 November 2009

(Irene Alba Alejandre)

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