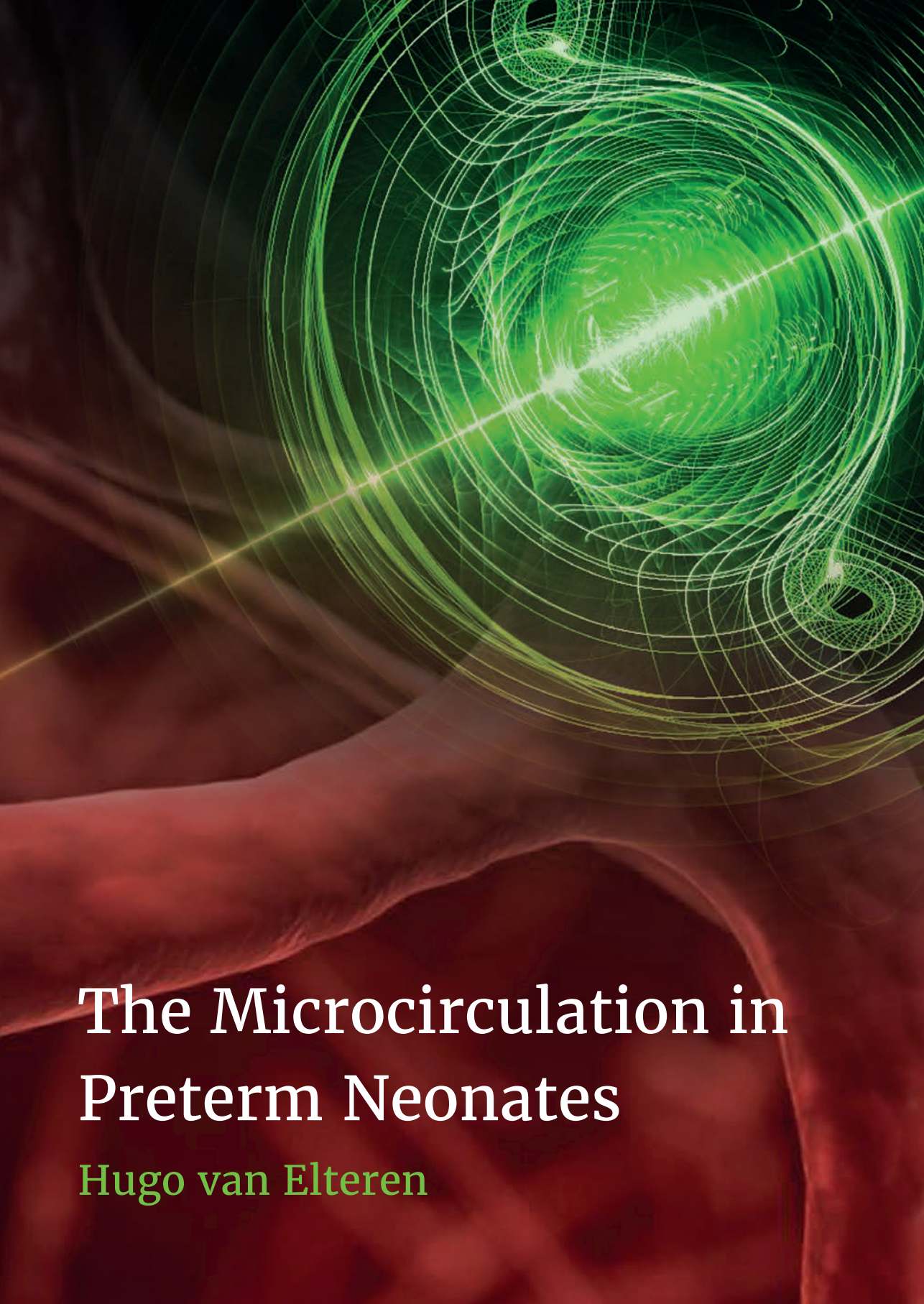

The Microcirculation in Preterm Neonates

Hugo van Elteren

THE MICROCIRCULATION IN PRETERM NEONATES

De microcirculatie in preterme neonaten

Hugo Adriaan van Elteren

Colophon

The studies described in this thesis were performed at the neonatal intensive care unit and maternity ward of the Erasmus MC - Sophia Children's Hospital, Rotterdam, The Netherlands.

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Hemodynamic adaptation to hypoxia in neonatal critical care

H.A. van Elteren, C. Ince, I.K.M. Reiss

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INTRODUCTION

Hypoxia is a condition that is not only observed in critically ill patients admitted to the intensive care unit. In fact, every human being has experienced extreme hypoxic conditions in utero. The fetus can survive and grow under these conditions because of decreased metabolic demands and altered cardiovascular circulation. Naturally, if oxygen delivery (DO_2) does not meet oxygen consumption (VO_2), the fetus will suffer and will eventually show signs of fetal distress. For this condition, the term dysoxia is appropriate, as it describes the condition in which the need for oxygen to sustain cellular respiration (mitochondrial oxygen requirement for fueling oxidative phosphorylation to produce ATP needed for the energy requirements of cells to live and function) exceeds that being supplied by the microcirculation (1). It can be argued that the holy grail of critical medicine is the identification of dysoxia and its adequate treatment. In neonatal intensive care, this quest is confounded by the changing metabolic requirement of the neonate as its growth progresses.

Both intra- and extra-uterine adaptation mechanisms are known to come into play to avoid hypoxia and cellular dysfunction. Although these mechanisms are protective, they can potentially be pathological, as there are limitations to these defense mechanisms. These mechanisms can further be confounded by treatment modalities that may themselves cause harm. For example, after birth, diseases associated with maladaptation, such as patent ductus arteriosus (PDA) and persistent pulmonary hypertension of the newborn (PPHN), are often treated with excessive amounts of oxygen. However, it has become clear that hyperoxia itself can cause damage by the formation of reactive oxygen species (ROS). Although ROS has prominent signaling properties important in both normal and patho-physiological states of vasodilation(2), ROS can cause membrane lipid peroxidation and DNA fragmentation (3). In neonates, there is direct and indirect evidence that ROS plays a role in a variety of conditions, including bronchopulmonary disease (BPD), retinopathy of prematurity (ROP), periventricular leukomalacia (PVL) and necrotizing enterocolitis (NEC) (4). As technology has advanced, treatment of newborns as young as 24 weeks of gestational age has been realized. In some other western countries, the lower limit is even 22 weeks of gestational age. Unfortunately, this trend has also seen a rise in the incidence of maladaptation in newborns. The understanding of oxygen transport and handling pathways in the developing fetus, as well as methods of monitoring and treating such a developing condition, is the challenge facing the neonatologist today.

Antenatal adaptation to hypoxia

Oxygen transfer to the fetus is mainly regulated by uterine artery blood flow (5). In intrauterine life, the human fetus is subject to a hypoxic environment. Extrapolations from animal studies suggest that under normal conditions, human fetal PaO_2 is between 2.5 and 3.5 kPa (6). Low partial pressure of oxygen also implies that fetal

tissues are more susceptible to a state of oxygen insufficiency. Several maternal and environmental conditions can impede the process of oxygen delivery. The following three forms of antenatal hypoxia have been suggested: 1) pre-placental hypoxia, where both the mother and her fetus become hypoxic (high-altitude, cyanotic maternal heart disease), 2) utero-placental hypoxia, where the maternal oxygenation is normal, but the utero-placental circulation is impaired (pre-eclampsia, placental insufficiency) and 3) post-placental hypoxia, where only the fetus is hypoxic due to fetal disease (7). Most of the data regarding the effects of hypoxia have been acquired from animal studies in which different techniques have been used to mimic chronic hypoxia. However, it is unclear whether these models of chronic hypoxemia result in the same adaptive mechanisms as occur in hypoxemic neonates.

In normal fetal life, metabolism and oxygen consumption are decreased compared to extrauterine life. Many physiological functions are reduced, including respiratory effort, gastrointestinal digestion and absorption and renal tubular re-absorption. There is also no need to maintain thermoregulation because the thermal environment is maintained by the mother. These changes result in reduced tissue oxygen consumption. From an evolutionary perspective, it is assumed that the hypoxic environment is important for development, as it protects the fetus from oxidative damage(8). The hypoxic environment is also critical for lung development, as the hypoxic environment stabilizes hypoxia inducible factor (HIF). HIF is a family of transcription factors that play a central role in cellular adaptation to insufficient oxygen. HIF-1 is activated during fetal development, and it is also active during the normal homeostasis of most tissues. In the human lung at 8 to 13 weeks of gestation, HIF-1a expression is mainly restricted to branching epithelium, whereas HIF-2a appears to be present in the vascular structures of the lung parenchyma (9). Mouse models have identified the different functions of HIF subunits. HIF-1a knockout mice suffer from severe cardiovascular defects and die in utero, whereas HIF-2a knockout mice suffer from postnatal respiratory distress due to insufficient surfactant production and slow heart rate (10).

HIF-1 target genes include those encoding vascular endothelial growth factor (VEGF), erythropoietin, glucose transporters, and glycolytic enzymes (11). Enhanced erythropoiesis results in higher hematocrit and hemoglobin concentrations in cord blood. The proportion of fetal hemoglobin (HbF) in cord blood is generally increased at high altitudes compared to equivalent populations at sea-level (12). The affinity of fetal hemoglobin for oxygen is substantially greater than that of adult hemoglobin. The P50 value for fetal hemoglobin (i.e., the partial pressure of oxygen at which the protein is 50% saturated) is roughly 19 mmHg whereas adult hemoglobin has a value of approximately 26.8 mmHg (13). This phenomenon is a result of decreased binding of 2,3-diphosphoglycerate to HbF. The increase of fetal hemoglobin concentrations results in higher oxygen saturation at the same partial pressure of oxygen. Fetal hemoglobin cells are also more compliant than adult hemoglobin cells, making it easier for them to enter the capillaries of the microcirculation (14).

The fetal heart has an improved protection mechanism to prevent hypoxia-induced cell death compared to the adult heart. This resistance has been attributed to the enhanced capacity of the immature heart for glycolysis and protection against oxidative stress (15, 16). Increased adrenergic activity and elevated plasma concentration of catecholamines results in positive chronotropic and inotropic stimulation of the heart leading to an increase in cardiac output (17). There is also an altered distribution in cardiac output. Hypoxic stress in the ovine fetus increases umbilical venous return through the ductus venosus and the preferential streaming of this blood through the foramen ovale, facilitating the delivery of the most highly oxygenated blood to the upper body and thus to the heart and brain (18). Blood flow to the gastrointestinal, renal and peripheral vascular beds decreases (19).

Studies in both animal and human fetuses show an increase of oxygen extraction when oxygen delivery is diminished (20, 21). The fetus is also capable of switching metabolic rate depending on oxygen levels. In response to hypoxia, the fetus can reduce its oxygen demands or it can increase its metabolic demands to prevent hyperoxia (22). These adaptation mechanisms can maintain conditions until a 50% fall in placental blood flow occurs. Hereafter, a decrease in fetal movement is required to reduce oxygen consumption (23). Obstetricians consider this to be a risk factor for adverse pregnancy outcome.

Antenatal maladaptation to hypoxia

If fetal adaptation mechanisms are insufficient, chronic hypoxia leads to altered cardiac and pulmonary development. Lung development can be divided into the following five stages based on histological appearance: embryonic (1–7 wk gestation), pseudoglandular (5–17 wk gestation), canalicular (16–26 wk gestation), saccular (24–38 wk gestation), and alveolar stage (36 wk gestation onward) (24). By 17 weeks of gestation, all preacinar pulmonary and bronchial arteries are formed. Hereafter, there is an increase in lung capillaries. In response to hypoxia, pulmonary vascular remodeling occurs. This remodeling is characterized by the combination of hypertrophy and hyperplasia of the cells within each layer of the vessel wall. There is an increased smooth muscle proliferation and muscularization of the normally muscle-free peripheral arteries. The intima is infiltrated with fibroblasts, and the adventitia shows increased production of extracellular matrix with deposition of collagen and elastin (25). These changes result in thickening of the blood vessel wall, increased pulmonary vascular resistance (PVR), and reduced compliance. Vascular remodeling can occur in any stage of fetal lung development and also occurs in the postnatal period (26). Compared to adults, these vascular changes are greater and occur more rapidly in newborns (27). This results in more severe pulmonary hypertension and generally lower oxygen saturation levels. The intracellular mechanisms underlying vascular remodeling are complex and have recently been discussed in excellent reviews (26, 28). Unfortunately, vascular remodeling can only be observed by histopathological examination and cannot be predicted on antenatal ultrasound.

In normal cardiac development, at 8 weeks of gestation, the heart is developed to a completely looped 4-chamber organ (29). Hemodynamic characteristics, such as ventricular volume, stroke volume, and cardiac output, increase with gestational age, whereas ejection fraction decreases as gestation advances. The combined cardiovascular output in human fetuses estimated by the Doppler technique steadily rises from 50 ml/min at 18 weeks to 1200 ml/min (approximately 400 ml/ kg/min) at term. Normal fetal cardiovascular physiology is characterized by ventricular volumes that are larger on the right and ejection fractions that are greater for the left ventricle. This results in similar left and right ventricular stroke volume and cardiac output (30). In chronic hypoxia, fetal cardiac development is impaired in both structure and function (31). Animal studies have shown that chronic hypoxia in the first trimester caused ventricle septum defects, myocardial thinning, cardiomyocyte hypertrophy, ventricle dilation and detachment of the epicardium (32-34). This phenomenon leads to a lower heart rate, decreased cardiac output and lowered contractility (35, 36). Developmental changes to chronic hypoxia are not restricted to cardiovascular tissue.

Postnatal hemodynamics and maladaptation

The fetal circulation is characterized by a high pulmonary vascular resistance (PVR), and this resistance is accompanied by a constant state of pulmonary hypertension. Of the blood pumped out by the right ventricle, approximately 10% enters the lung via pulmonary arteries; the majority enters the aorta via the ductus arteriosus (37). As a result of the low vascular resistance of the placenta, the high vascular resistance of the fluid-filled fetal lungs, and the presence of the fetal channels, the fetal circulation functions as a parallel circuit with right-to-left shunting of relatively oxygenated blood through the foramen ovale and less-saturated blood across the ductus arteriosus. At birth, an impressive fall in PVR and an increase in systemic vascular resistance results in the transition from fetal to an adult circulation, including the closure of the foramen ovale and ductus arteriosus. Various mechanical factors and vasoactive agent signaling pathways contribute to the decrease of PVR. Of these factors, the endothelium-derived nitric oxide pathway (EDNO), prostacyclin (PGI_2) and endothelin-1 (ET-1) pathways seem to have the greatest clinical importance. A newborn infant prior to the 37th week of gestation is considered to be preterm. The use of antenatal corticosteroids and exogenous surfactant therapy made it possible to treat premature infants as young as 22 weeks of gestation. Cardiovascular physiology in preterm newborns is altered compared to adults, and hemodynamic values differ between the broad ranges of prematurity. Reference values for gestational age are shown in Table 1.

The preterm newborn has a limited ability to respond to changes in determinants of the cardiac output, due to fewer mitochondria and energy stores. Consequently, the preterm infant heart is less able to respond to stresses that occur in the postnatal period, such as increased peripheral vascular resistance with resultant increased afterload. Large ductus arteriosus and high mean airway pressure can further impede

Table 1. Reference values for heart rate and blood pressure in premature infants (38). Data are presented as means and 95% confidence intervals

Gestational age	Heart rate [beats/minute]	Mean blood pressure [mmHg]	Systolic blood pressure[mmHg]	Diastolic blood pressure[mmHg]
<24	160 (120-190)	27 (20-37)	35 (25-45)	23 (15-30)
24 - 28	157 (115- 185)	31 (23-40)	40 (30-50)	26 (17-34)
28 - 32	150 (110 -180)	36 (28-45)	45 (35-55)	30 (22-40)
32 - 37	136 (90 -175)	42 (33-50)	55 (45-65)	35 (28-43)
37 - 40	130 (70-170)	45 (36 -55)	60 (55-75)	40 (32-47)

systemic blood flow during a period when the preterm infant is particularly vulnerable to blood flow changes and hypoxia.

Chronic hypoxia has major influence on postnatal adaptation and vice versa. Most data in humans on this subject were gathered from comparison of high-altitude versus sea-level newborns. At birth, mean pulmonary artery pressure (PAP) is equal in newborns born at high altitude and at sea level with values of 60 mmHg. Within 72 hours, PAP decreases and remains at values of 12 mmHg through further life. At high altitude, there is a persistent high PAP with values of 55 mmHg after 72 hours of birth and 28 mmHg during adolescence and adulthood (39). As a result, there is persistent right ventricle hypertrophy (RVH) with a similar decreasing pattern as in PAP (40). These factors contribute to a persistent fetal circulation including a prolonged right-left shunt over the ductus arteriosus, a 30-40-fold higher incidence of persistent ductus arteriosus and persistence of an anatomically patent foramen ovale in 44% of children at the age of 6 months (41-43). Most striking is the estimation of a 100-fold increase of persistent pulmonary hypertension of the newborn (PPHN) born in hypoxic conditions (44).

Persistent pulmonary hypertension of the newborn (PPHN)

PPHN must be distinguished from pulmonary hypertension in children or adults. In children, congenital heart disease or idiopathic pulmonary hypertension accounts for the majority of cases (45), while in adults, Chronic Obstructive Pulmonary Disease (COPD), left heart disease and connective tissue disease are more frequent causes of pulmonary hypertension.

PPHN due to maladaptation is different than other forms of PPHN. PPHN generally can be divided into the following three groups: 1) those with abnormally constricted pulmonary vasculature due to lung parenchymal diseases, such as meconium aspiration syndrome, respiratory distress syndrome, or pneumonia; 2) those with hypoplastic vasculature, as observed in congenital diaphragmatic hernia; and 3) those with lungs of normal parenchyma but remodeled pulmonary vasculature (46). This last group suffers from what is known as idiopathic PPHN, but antenatal vascular remodeling almost certainly plays a major role in the etiology. Understanding the regulation of

the perinatal pulmonary circulation has helped the development of new treatments for PPHN. Inhaled NO has proven its value in newborns, decreasing the need for extracorporeal membrane oxygenation (ECMO) (47). Other potential treatment options currently being studied in neonates include inhibition of cGMP degradation by PDE5 with sildenafil, inhibition of cAMP degradation with milrinone, and inhibition of ET-1 with bosentan (48-50). However, as only small randomized controlled trials have been performed in newborns, their clinical value remains not fully known.

Consequences of maladaptation to hypoxia

Exposure to chronic hypoxia and persistent fetal circulation leads to increased mortality and morbidity in newborns and children (51, 52). Epidemiological studies have indicated that high-altitude pregnancies increase the risk of intrauterine growth restriction (IUGR) and low birth weight. Infants born small for gestational age (SGA) are at higher risk for prematurity, asphyxia, necrotizing enterocolitis and bronchopulmonary dysplasia and thus have increased morbidity and mortality rates. (53, 54). In adult life, SGA infants also have a more than 1.5-fold increased risk of cardiovascular disease compared to age- and gender-matched controls who were born appropriate for gestational age (AGA) (55).

Reaching a situation of normoxia can reverse the cellular and structural changes in the pulmonary vasculature. In pulmonary hypertensive rats, 6 weeks of normoxia resulted in reversal of vessel wall thickness and vascular smooth muscle cell infiltration in preacinar arteries (56). However, right ventricle systolic pressure shows incomplete reversal. Sartori et al. demonstrated that survivors of perinatal pulmonary hypertension had a significant greater increase in PAP at high altitude compared to healthy controls (57). These findings suggest that a transient perinatal insult to the pulmonary circulation leaves a persistent and potentially fatal imprint, which predisposes patients to a pathological response when activated in adult life.

Oxygen: treatment or trouble?

While technology helped to treat newborns as young as 22 weeks gestational age, the use of oxygen therapy has become more and more standard as the perception that dysoxia is prevalent and can be corrected by administering oxygen via respiration gained acceptance. As gestational age decreases, the incidence of maladaptation and specific neonatal disorders, such as PPHN, persistent ductus arteriosus and respiratory distress syndrome (RDS), is increasing (58). However, as knowledge of oxygen therapy has increased over time, it has become clear that administration of high fractions of oxygen also has serious side effects. Excessive oxygen administration results in increased mitochondrial production of ROS, as well as reactive nitrogen species (RNS), which cause membrane and DNA fragmentation (59). Under normal physiological conditions, O_2 and NO form hydrogen peroxide (H_2O_2), which is a stable cell-signaling molecule in vascular homeostasis (2). In the situation of increased ROS availability, however, ROS

and NO can form the highly reactive peroxynitrite ($ONOO^-$). This oxidant and others have the potential to produce vasoconstriction, cytotoxicity, and damage to surfactant proteins and lipids. The premature infant is especially susceptible to ROS- and RNS-induced damage for two major reasons. First, increases in antioxidant capacity occur in the latter part of gestation in preparation for the transition to extra-uterine life, and in early stages, this capacity is undeveloped. Adequate concentrations of antioxidants like catalase, glutathione peroxidase and superoxide dismutase may therefore be absent at birth (60). Second, the ability to increase synthesis of antioxidants in response to hyperoxia or other oxidant challenges is relatively impaired (3).

ROS is directly responsible for DNA damage and thus carcinogenesis (61). A higher target of oxygen saturation increases the incidence of severe retinopathy of prematurity (ROP) and bronchopulmonary dysplasia (BPD) in extreme preterm infants (62, 63). Resuscitation in the delivery room with 100% oxygen even increases mortality rates (64, 65). In later life, ROS is responsible for cell aging (66, 67), carcinogenesis and childhood leukemia (68, 69).

Conversely, higher targets of oxygen saturation reduce mortality in extreme preterm infants (62, 70). These data indicate that there is a clear balance between "drug action" and side-effects in oxygen therapy. In standard critical care, tissue oxygenation and oxygen availability at the microvascular level is not routinely measured. We hypothesize that gaining such insight into the oxygen transport pathways at the level of the microcirculation for the critically ill neonatal patient can result in an improved monitoring environment, leading to treatment strategies aimed at optimizing tissue oxygenation and resolving dysoxia. Such a strategy would result in a more rational use of oxygen therapy, thereby restricting its harmful side effects.

Monitoring of the newborn

In (preterm) newborns, hemodynamic monitoring remains complicated compared to adults; methods should be accurate along the entire spectrum of gestational age and birth-weight, useful in neonates with extra- and intra-cardiac shunting, validated against one of the 'gold standards' and most of all be practical and non-invasive (71). However, many measuring techniques are impractical, due to the lack of appropriately sized materials, and the risks of use outweigh the potential benefits.

The following three key components in the physiology of oxygen delivery to the tissues can be identified: uptake of oxygen in the lung, transport and delivery of oxygen from the lung to the tissues and oxygen uptake and utilization by the tissues (72). Recent innovations in hemodynamic monitoring helped neonatal and pediatric intensivists to visualize tissue perfusion and oxygenation in a non-invasive manner. These techniques have allowed evaluation of cardiac output, systemic vascular resistance, organ blood flow distribution and tissue oxygen delivery at the bedside. However, lack of validation and reference values complicates clinical use (71). Adaptation is not limited to oxygen delivery or tissue oxygen extraction. Techniques need to be combined

to allow monitoring of changes in neonatal adaptation. This approach has not yet been attempted in neonatal critical care. The identification of whether tissues suffer from dysoxia, i.e., the condition where oxygen availability is inadequate to meet the mitochondrial needs of the parenchymal cells to sustain oxidative phosphorylation, can be regarded as a "holy grail" in this respect.

Tissue oxygenation has been measured in newborns during the adaptation phase using near infrared spectroscopy (NIRS). NIRS is based on the transparency of biological tissue to light in the near infrared part of the spectrum (700–1,000 nm) and its subsequent absorption by oxygenated hemoglobin (O_2Hb) and deoxygenated hemoglobin (HHb) in the blood vessels that are within the near infrared light beam. Absorption changes in near infrared light can later be converted into concentration changes of O_2Hb and HHb. NIRS has mainly been used for cerebral oxygen saturation (78-80). However, small numbers and great inter-patient variability gave limited information concerning reference ranges during the first weeks of life.

Tissue perfusion has been measured in newborns during the adaptation phase mainly using orthogonal polarization spectral (OPS) imaging and its successor, sidestream dark field (SDF) imaging. SDF imaging uses a light guide, which is surrounded by green light (530 nm) emitting diodes (LED). The light penetrates the tissue, illuminates the microcirculation, and is absorbed by hemoglobin of the erythrocytes. The flowing red blood cells perfusing the microvessels are visualized. Quantitative assessments of microvascular variables are possible using specialized software off-line. Different variables can be measured. Generally these outcome parameters can be divided in quantity and quality of the microcirculation. Quantity is measured by the density of the microcirculation, i.e. the total length of the vessels divided by the measured surface are. The quality of the microcirculation takes in to account the flow of the blood running through the vessels. This is the proportion of perfused vessels (PPV) of microvascular flow index (MFI). Combining quality and quality will give the perfused vessel density (PVD). Finally, to assess the heterogeneity of the flow, the heterogeneity index (HI) was created.

A disadvantage of the OPS /SDF imaging techniques is that the resolution is limited due to the use of analogue video cameras and the fact that the technology does not allow instant on-line bedside quantification of images (73). These shortcomings may have been resolved by the recent introduction of a computer-controlled high resolution imaging sensor-based device based on incident dark field imaging (74), which holds promise in this respect (73). This thesis will critically review the new aspects of incident dark field imaging.

The PVD in the skin of premature infants decreases significantly over the first month of life. A correlation has been found with decreases in hemoglobin concentration (75). It has been supposed that higher PVD in the first week of postnatal life may be related to higher cardiac output in the first week. The microcirculation has been measured in newborns with severe respiratory failure directly after birth. ECMO treatment did not

improve the microcirculation after 24 hours. Interestingly, the use of inhaled nitric oxygen did have this effect, which highlights the role of nitric oxygen in adaptation (76, 77).

All of these imaging techniques however remain relatively new. Its clinical benefit to the patient has not been demonstrated yet. Moreover, basic elements as reproducibility and inter-observer variability are unknown. This potentially can cause bias in future studies.

Research questions

The aims of this thesis are:

- To establish reference ranges for cutaneous microcirculation in term and preterm infants.
- To evaluate the effect of clinical variables influencing the microcirculation
- To evaluate the strengths and weaknesses of Incident Darkfield Imaging (IDF) compared to its predecessors.
- To evaluate reproducibility of newly introduced hemodynamic diagnostic techniques.

In Part 1 of this thesis we present the results of microcirculatory research obtained with incident darkfield imaging. In **chapter 2** we demonstrate how to use incident darkfield imaging to obtain high quality images of the cutaneous microcirculation in preterm infants. The manuscript provides a step-by-step learning tutorial for new users with state of the art tips and tricks. The procedure is visualized and can be seen online. In **chapter 3** we compared incident darkfield imaging with its predecessor sidestream darkfield imaging. The comparison comprised two elements: 'standard' microcirculatory outcome parameters measured by the two camera's in the same infants and analysis of the quality of these obtained images. **Chapter 4** describes the results of a single center observational study of cutaneous microcirculation in preterm infants. This is the largest study ever performed in preterm infants on this subject. During the first month of life, the adaptation of the cutaneous microcirculation is measured on six different time points (day 1, day 3, day 5, day 7, day 14, day 28). This study creates reference values and identifies variables that influence the cutaneous microcirculation.

The effect of antenatal hypoxemia on the cutaneous microcirculation is described in **chapter 5**. The cutaneous microcirculation was measured on the first day of life in healthy, term born infants at high altitude (Puno, Peru, 3830 meter) and a cohort of sea-level born infants (Rotterdam, The Netherlands, 0 meter).

In Part 2 of this thesis we present the results of reproducibility studies of several new technical devices. In **chapter 6** the reproducibility of manual offline analysis of buccal and cutaneous microcirculation is studied. In **chapter 7** the reproducibility of the pleth variability index (PVI) is discussed, while **chapter 8** focuses on the reproducibility of laser doppler spectrometry measured by the oxygen to see (O2C).

In the general discussion (**chapter 9**) the overall findings of this thesis, general considerations, recommendations and future perspectives will be addressed. Finally, a summary is provided in **chapter 10**.

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PART I

MICROCIRCULATORY IMAGING USING INCIDENT DARK FIELD

CHAPTER 2

TRANSCUTANEOUS MICROCIRCULATORY IMAGING IN PRETERM NEONATES

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Online video: http://www.jove.com/video/53562/transcutaneous-microcirculatory-imaging-in-preterm-neonates

SHORT ABSTRACT

Microcirculatory imaging (MI) is used to monitor peripheral perfusion in critically ill or preterm neonates. This manuscript and video demonstrates the optimal approach for obtaining high-quality images.

LONG ABSTRACT

Microcirculatory imaging (MI) is a relatively new research tool mainly used in the intensive care setting. MI provides a clear view of the smallest capillaries, arterioles and venules. The magnifying effect visualizes the flow pattern of erythrocytes through these vessels.

It's non-invasive character makes it suitable to apply in (preterm) neonates, even in cardiorespiratory unstable patients. In adults and children, MI is mainly performed sublingually, but this is not possible in preterm infants as these cannot cooperate and the size of the probe is problematic. In preterm infants, MI is therefore performed transcutaneously. Their thin skin makes it possible to obtain high quality images of peripheral microcirculation.

In this manuscript we will demonstrate the method of transcutaneous MI in preterm infants. We will focus on the different techniques and provide tips to optimize image quality. The highlights on software settings, safety and offline analysis are also addressed.

INTRODUCTION

Hemodynamic diagnostics in critically ill preterm neonates has always been difficult. Most diagnostic tools used in adults cannot be applied in these tiny preterm infants; and then there is a problem of the sensitivity of the outcome parameters. But most of all, these infants are so vulnerable, that the risks of diagnostic procedures do not outweigh the benefits. As a result, in the field of neonatology, hemodynamics has been neglected and therefore there is a lack of knowledge on this topic.

An interesting option for handling these problems might be visualizing the microcirculation. The introduction of handheld microscopes in the late 1990s made it possible to visualize the microcirculation in a non-invasive manner. Three generations of devices have been introduced: Orthogonal Polarization Spectral (OPS) imaging[1], Sidestream Dark Field (SDF) imaging[2], and Incident Dark Field (IDF) imaging[3]. They all use more or less the same technique in which green light with a specific wavelength (548nm) stroboscopic illuminates the microcirculation. The green light is absorbed by oxy- and deoxyhemoglobin and mostly reflected by the surrounding tissue. This property of green light therefore creates visible contrast. The reflected light passes a magnification lens and is projected on a camera sensor. Hereby it is possible to visualize the flowing red blood cells at a depth of approximately one millimeter of mucosal tissue or directly at solid organs.

Over the past 15 years, the microcirculation has been mainly studied in adults, especially in patients with septic shock[4-6]. These observational studies found that persistent microcirculatory alterations were associated with organ failure and mortality. This observation cannot be extrapolated directly to (preterm) infants however, as in the adults the microcirculation was measured sublingually. High quality images of the sublingual microcirculation cannot be obtained in preterm infants because they are unable to cooperate. In term infants the buccal microcirculation has been the area of interest[7]. Fortunately, in preterm infants the thin skin allows transcutaneous microcirculatory imaging. This approach has been applied in neonatal studies focusing on blood transfusion[8], therapeutic hypothermia[9] and hypotension[10].

In this manuscript we present our protocol for transcutaneous microcirculatory imaging using Incident Dark Field imaging in preterm neonates. We will focus on different strategies to acquire the highest quality images. Technical details and differences between the SDF and IDF devices can be found elsewhere[11].

PROTOCOL

This protocol follows the guidelines of the local human research ethics committee.

1. Preparation

1.1) Schedule the microcirculatory measurement so that it does not coincide with other procedures such as blood sampling. In term neonates it is best performed after feeding. This prevents agitation and will ease the measurement.

1.2) Ensure that a nurse or a parent attends to support and comfort the neonate during the examination, using the principles of Newborn Individualized Developmental Care and Assessment Program[12]

Note: Although measurements can be performed by a single person, it is highly recommended to have a second person assist. One holds the camera and is focused on the neonate whilst the other operates the computer and software. In our experience, this results in higher quality images and a shorter duration of the procedure.

1.3) If the clinical condition of the neonate permits, place the neonate in supine position. Microcirculatory imaging can be performed in prone position, but this requires more skill and patience.

1.4) Make sure the body temperature of the preterm infant is within appropriate range (36,5 – 37,5 degrees Celsius)

2. Procedure

2.1) Install the device along the incubator. Make sure the incubator is at the right height.

2.2) Put the disposable cap on the camera.

2.3) Apply gel, oil or saline on the tip of the probe; this will help smoothen the contact between probe and skin.

2.4) Place the camera on the ventromedial side of the infants upper arm. To prevent focus-artefacts, make sure the probe is perpendicular to the skin. This may require repositioning of the infants arm.

Note: The ventromedial side of the upper arm is the primary location to measure the cutaneous microcirculation. This location has little lanugo hair and is therefore less prone to artefacts. It is most easily reached if the patient is positioned in supine position.

2.5) Gain time to minimize the total length of procedure by finding the optimal depth of focus (Figure 3) while searching the location with the fewest artefacts.

Note: Depth of focus depends primarily on postnatal age rather than gestational age. The average depth of focus in the first week of life is 0 - 80 μm (Figure 1). Hereafter, due to maturing of the skin, the focus depth rapidly increases with average values of 80-200 μm between 1-4 weeks of postnatal age (Figure 2). In term born neonates the average depth of focus is 80-160 μm at birth.

2.6) Stabilize the probe to avoid movement artefacts. To do so, rest the elbow on the incubator window and the wrist beside the neonate. Alternatively, position the probe alongside the neonate on a pillow.

2.7) Avoid pressure artefacts by letting the camera only have the slightest contact with the skin. Pressure artefacts can be recognized during image capturing if there is back-and-forth flow in vessels or if large vessels are non-perfused while there is

good flow in small vessels. Also if the flow pattern is identical throughout the whole screen, beware of pressure artefacts.

2.8) Record videos for a minimum duration of 5 seconds.

2.9) After a successful capture, move the camera to another spot on the upper arm.

Note: It is recommended to capture in total 5-10 videos at 3-5 different locations, as some artefacts are only recognized at offline analysis, which means that the video in question is not usable for analysis.

2.10) Gently remove the gel, oil or saline from the skin with a small gauze.

3. Offline analysis

3.1) Crop the video if there is a significant movement that impedes analysis. Go to the 'Tools' section and use the button 'Editor'. Select the frame interval eligible for analysis and click the 'Crop Video' button. Note: Videos are acceptable if movement is within ½ of the field view[13].

3.2) Select the cropped video and stabilize it. Go to the 'Tools' section and use the button 'Analysis'. Click the 'Stabilize' button. Note: All movies must be stabilized before automatic analysis can be performed.

3.3) Select the stabilized video. Go to the section 'Analysis' and click the 'Detect' Button. Make sure the options 'Capillaries' and 'Vessels' are highlighted.

3.4) After detection (Figure 4), click the 'CNA' or 'De Backer' button for a full microcirculatory report. This report includes the most used outcome parameters like the total vessel density (TVD), perfused vessel density (PVD) and proportion of perfused vessels (PPV).

Note: As an alternative, videos can be exported offline to be manually analyzed. This option can be found in the section 'Tools'. Select the option 'Export' and click the 'AVA export' button.

REPRESENTATIVE RESULTS

Figures 1 and 2 show representative still images of high-quality MI videos. These examples demonstrate the difference in skin thickness in the same infants between day 1 (Figure 1) and day 28 (Figure 2) of postnatal age.

On day 1, there is a bright illumination, adequate focus on the micro vessels and minimal presence of artefacts. On day 28 it is more difficult to find the right balance between focus on the micro vessels and artefacts due to the thicker skin. Note that stability, duration and pressure artefacts cannot be judged on these still images. This should be noticed during the acquiring of the images or before offline analysis.

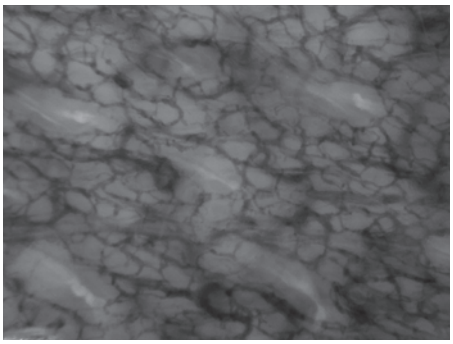

Figure 1. MI of preterm infant 24 weeks gestational age, day 1. Transcutaneous MI of an infant born at a gestational age of 24 weeks. The postnatal age of the infant is 1 day. The used depth of focus is 40 μm.

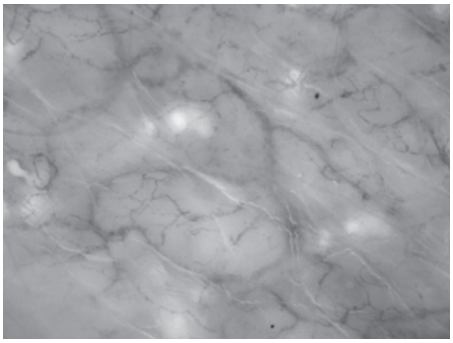

Figure 2. MI of preterm infant 24 weeks gestational age, day 28. Transcutaneous MI of an infant born at a gestational age of 24 weeks. The postnatal age of the infant is 28 days. The used depth of focus is 160 μm.

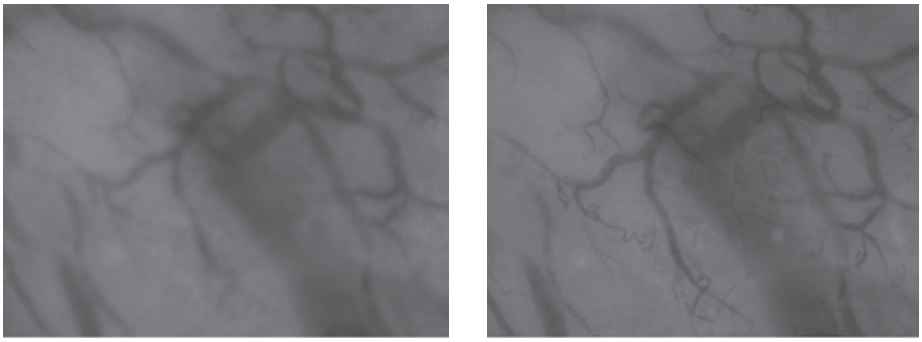

Figure 3. Differences is focus depth. These two images of the exact same area highlight the importance of adequate focus depth. Inadequate focus (left) results in loss of vessel visuability compared to adequate focus (right).

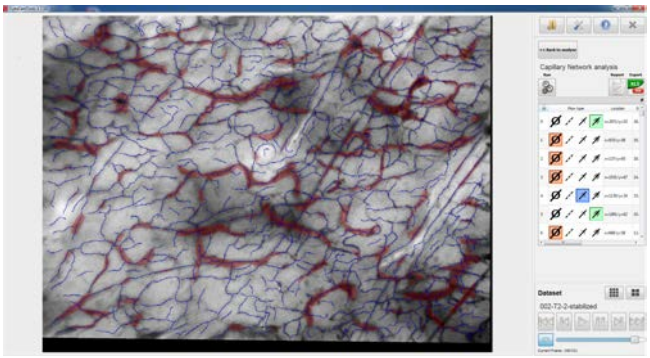

Figure 4. Results of automatic offline analysis. This figure shows the results of automatic offline analysis.

DISCUSSION

In this manuscript we describe and demonstrate the approach for transcutaneous microcirculatory imaging in preterm neonates. Visualizing this method will help researchers overcome two of the biggest challenges in research: reproducibility and the time and labor intensive nature of learning new techniques.

This technique can provide useful information of peripheral microcirculation in preterm infants in a non-invasive manner. Serial measurements can help clinicians evaluate the effects of therapeutic interventions. The microcirculation is the area where the final step of the oxygen transport chain occurs. Observational studies have demonstrated a relation between microcirculatory deterioration over time and the presence of bacterial infections[14]. As deterioration of the peripheral microcirculation is one of the first signs of sepsis in preterm infants, it is generally hypothesised that the peripheral microcirculation can help predict the development of sepsis.

The quality of the images mainly depends on the skills of the operator. Although the latest camera devices provide better technical specifications and hardware, this is still of secondary importance. Operators should be properly trained in microcirculatory imaging. They should be familiar with the recommendations of the round table conference held in Amsterdam in 2006[15] and with the quality criteria developed by Massey *et al*[13]. These six criteria (illumination, duration, focus, content, stability, pressure) are the foundation of high quality and trustworthy data. Inexperienced operators are likely to fail in recognizing frequently occurring pressure artefacts[16]. Note however that these quality standards and recommendations have been drafted for sublingual MI measurement in adults and cannot be directly extrapolated to cutaneous MI measurements in preterm infants. The quality criteria of duration and content are therefore harder to accomplish.

Also, it is highly recommended to perform the measurements with two operators and ask a nurse or parent to attend. The camera must be held steady, pressure artefacts must be avoided, the software must be operated and the infants well-being must be

considered. Especially if the infant moves a lot, this is too much for one operator. It is therefore better to have one person handle the camera and focus on the area of measurement and another person operate the software and check the infant's vital signs. Naturally, besides the technical aspects of MI, experience in handling (critically ill) preterm neonates is essential for operators.

Despite its non-invasive character, MI still carries a potential burden and risks. Limit the duration of measurements to a minimum to prevent that the incubator and thereby body temperature drops too much. Always apply a disposable protection cap on the camera before measurements. The function of this cap is twofold: It protects the skin from potential warming of the probe tip and it serves as an artificial barrier to prevent bacterial transmission. In addition, MI equipment should be regularly disinfected.

Also, apply just a little amount of gel or oil on the skin and gently remove it after the measurement. If misused, the combination of oil and therapeutic phototherapy can have devastating effects on the skin. Although mostly infants hardly react to the measurements, cardio-respiratory instability cannot be excluded. This is the reason why we recommend having the infant supported by a parent or nurse.

After data acquisition, offline analysis of the videos should be performed in a standardized manner. Commercial software is available to automatically analyze MI videos according to the guidelines[15]. Manual analysis comprises three steps. First, contrast enhancement can be applied in order to attain the optimal contrast. Second, videos should be stabilized. This step will bring out the importance of stability during the measurements. For the slightest drift can make a video unusable for analysis. Third, vessels must be drawn and flow must be categorized. Note that this final step carries a high risk of poor inter observer variability[17]. Manual offline analysis should therefore be performed by trained researchers only. There are two methods for reporting the outcome measures. The conventional method of capillary network analysis (CNA) reflects the total length of vessels divided by the measured surface area (mm/mm^2). Alternatively, the less time consuming De Backer Score (DBS) can be used. In this score, three equidistant horizontal and three equidistant vertical lines are drawn on the screen. Vessel density can be calculated as the number of vessels crossing the lines divided by the total length of the lines (n/mm).

There are drawbacks to the research field of microcirculation. Heterogeneity of the microcirculation makes is complicated to establish reference values. Therefore, most of the research are observational studies or comparisons between groups. The rather complex evaluation of microcirculatory videos and subjective human interference increases the possibility of poor reproducibility[17]. It is therefore important that the method of obtaining videos is standardized.

For the near future, technical advances will improve the research in cutaneous MI. An example is automated computer analysis of the video images, which will replace the relatively subjective human assessment of microvessels and thereby rule out inter observer variability. A good example to standardize this field of research is the use of wireless imaging sensors, which serially measure the microcirculation at the exact same location. This will make cutaneous MI less operator dependent.

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CHAPTER 3

CUTANEOUS MICROCIRCULATION IN PRETERM NEONATES: COMPARISON BETWEEN SIDESTREAM DARK FIELD (SDF) AND INCIDENT DARK FIELD (IDF) IMAGING

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ABSTRACT

Purpose

Incident dark field imaging (IDF) is a new generation handheld microscope for bedside visualization and quantification of microcirculatory alterations. IDF is the technical successor of sidestream dark field imaging (SDF), currently the most used device for microcirculatory measurements. In (pre)term neonates the reduced thickness of the skin allows non-invasive transcutaneous measurements. The goal of this study was to compare the existing device (SDF) and its technical successor (IDF) in preterm neonates. We hypothesized that IDF imaging produces higher quality images resulting in a higher vessel density.

Methods

After written informed consent was given by the parents, skin microcirculation was consecutively measured on the inner upper arm with de SDF and IDF device. Images were exported and analyzed offline using existing software (AVA 3.0). Vessel density and perfusion were calculated using the total vessel density (TVD) proportion of perfused vessels (PPV) and perfused vessel density (PVD). The microcirculation images quality score was used to evaluate the quality of the video images.

Results

In a heterogeneous group of twenty preterm neonates (median GA 27,6 weeks, range 24 – 33,4) IDF imaging visualized 19,9 % more vessels resulting in a significantly higher vessel density (TVD 16,9/mm vs. 14,1/mm, *p-value*= <0.001). The perfusion of vessels could be determined more accurately in the IDF images, resulting in a significant lower PPV (88,7% vs. 93,9%, *p-value*= 0.002). The IDF video images scored optimal in a higher percentage compared to the SDF video images.

Conclusions

IDF imaging of the cutaneous microcirculation in preterm neonates resulted in a higher vessel density and lower perfusion compared to the existing SDF device.

Keywords: Microcirculation, preterm neonates, Sidestream Darkfield, Incident Darkfield

INTRODUCTION

The invention of handheld microscopes made it possible to visualize the microcirculation of critically ill patients in a non-invasive manner. Since the introduction approximately 15 years ago, several successive devices have been introduced for this purpose. In general, devices consist of an illumination unit for illumination and a light guide with a magnification lens for image transfer to an imaging module such as such as a video camera. Illumination light is green light with a wavelength (548 nm) which ensures optimal absorption of oxyhemoglobin and deoxyhemoglobin thus making it possible to visualize the red blood cell. The surrounding tissue mostly reflects the light, therefore creating contrast. Hereby it is possible to visualize the flowing red blood cells in microcirculation through mucus membranes and directly on the surface of solid organs at a depth of approximately 1 millimeter.

In 1999, Groner et al. introduced Orthogonal Polarization Spectral (OPS) imaging [1].The first commercially available device using this OPS technique was called the Cytoscan® (Cytometrics, Philadelphia, USA). OPS imaging uses polarizers to block surface reflection on tissues. The second generation of handheld microscopes was based on sidestream dark field (SDF) imaging. The illumination is provided by surrounding a central light guide with concentrically placed light emitting diodes (LEDs). Hereby the lens is less disturbed by tissue surface reflections [2]. The device gives an analogue output which requires external analogue to digital conversion for software analysis. The MicroScan® was introduced in 2007 by MicroVision Medical (Amsterdam, The Netherlands) and is the most commonly used device for microcirculatory research. A similar device as the Microscan but with a digital output able to connect directly to a lap top computer was introduced by KK Technology called the CapiScope [3].

Videomicroscopy has mainly been used in the adult population and specifically in the intensive care setting. Persistent microcirculatory alterations in the sublingual area has been identified a predictor of adverse outcome [4,5]. In the pediatric and neonatal population the microcirculation has been studied on a small scale. The small size of patients and lack of cooperation makes it impossible to measure the microcirculation sublingually. In preterm neonates the reduced thickness of the skin allows transcutaneous microcirculatory imaging. In observational studies using OPS imaging, the cutaneous microcirculation was described during transition [6], during erythrocyte transfusion [7] and in term newborns suffering from asphyxia treated with whole body cooling [8]. These studies provided an important insight in the physiology of the microcirculation in newborns. Other studies measured the buccal microcirculation in patients with septic shock [9] or hypoxemic respiratory failure [10,11]. In line with studies in adults, the microcirculation proved to be predictive for mortality in pediatric patients with sepsis [9].

Recently a third generation handheld microscope has been introduced called the CytoCam® (Braedius Medical, Huizen, The Netherlands). This device is based

on incident dark field (IDF) imaging [12]. SDF optically isolates the incoming light from the reflected whilst IDF according to Sherman illuminates the field in a non-homogeneous fashion according to darkfield. Technical improvements, such as digital signal, lower weight of de device and higher optical resolution, have been implemented to overcome persisting limitations of the earlier devices. The technical differences between the SDF and IDF technique are visualized in Figure 1 and summarized in Table 1. Our group compared the new IDF technique (CytoCam) with the existing SDF technique (MicroScan) in preterm infants. We hypothesized that the technical improvements would result in higher quality images.

Table 1. Technical overview of the SDF and IDF devices

		SDF	IDF
Dimensions	Length (mm)	206	190
	Diameter (mm)	64	28
	Weight (gram)	347	110
Sensor	Pixel size (µm)	6.25 x 6.25	1.4 x 1.4
	Number of Megapixel	0.43	14.6
	Pulse time (ms)	16	2
Optics	Resolution (lines per mm)	220	320
	Magnification	5	4
	Field of view (mm^2)	0.84	1.79
	Focusrange (µm)	0-400	0-400

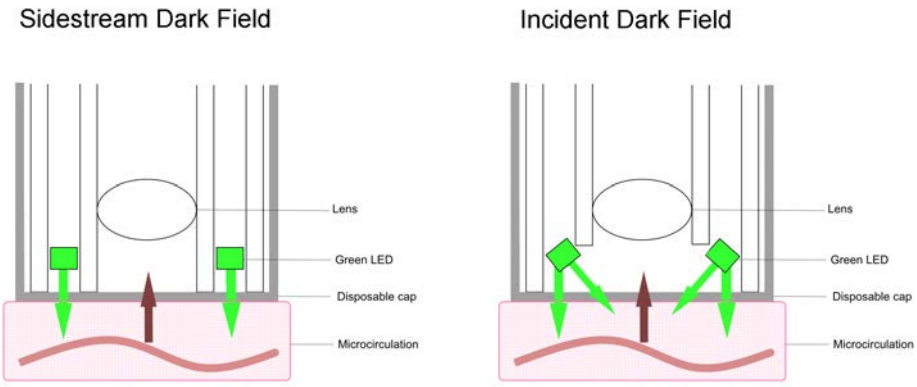

Figure 1. The conceptual differences between SDF and IDF imaging.

METHODS

2.1 Study design and setting

This prospective observational study included patients admitted to the neonatal intensive care unit (NICU) of the Erasmus MC – Sophia, a level III university children's hospital between November and December 2013. The local medical ethical review board approved the study. Parental written informed consent was obtained prior to the study start.

2.2 Patients

In order to obtain a heterogeneous group, the inclusion criteria in terms of gestational age and postnatal age was wide. All hemodynamically stable patients born preterm (GA < 37 weeks) were eligible. An arbitrary cut-off point for postnatal age was set at six weeks.

2.3 Microcirculatory Imaging

The microcirculation was consecutively measured by Sidestream Dark Field imaging (MicroScan) and Incident Dark Field imaging (CytoCam) at three sites of the upper inner arm. The microcirculation was measured according to the round table guidelines as published by De Backer et al.[13]. All videos were obtained by the same operator. Randomized video sequences were analyzed offline using dedicated software (Automated Vascular Analysis 3.0, Academic Medical Centre, Amsterdam, the Netherlands). Images obtained from the CytoCam were exported in the same video resolution (720 x 580) and surface area as the SDF images (0.94mm x 0.75mm). The observer was well-trained and experienced with offline analysis. On all videos, post-process contrast enhancement was applied. Thereafter videos were blinded and anonymized so that the observer was not aware of the used technique. An example of obtained microcirculatory videos is shown in Figure 2.

Total vessel density (TVD), perfused vessel density (PVD), proportion of perfused vessels (PPV), were calculated for small (Ø ≤ 10 μm) and non-small vessels (Ø 11–100 μm) according to the guidelines[13]. For determining the microvascular flow index (MFI) and heterogeneity index (HI), each video sequence was divided in four equally sized quadrants. Per quadrant the predominant type of flow was scored. MFI represented the mean score of the predominant type of flow, and HI represented the difference between the highest quadrant and the lowest quadrant score that is then divided by the mean score of all quadrants for one measurement. For all other scores, the average of the three video sequences per measurement was taken. To quantify the quality of the videos, the microcirculation imaging quality score was applied [14]. This score is based on six common image capture and analysis problem areas: illumination, duration, focus, content, stability and pressure. Each category is scored as optimal (0 points), suboptimal but acceptable (1 point) or unacceptable (10 points).

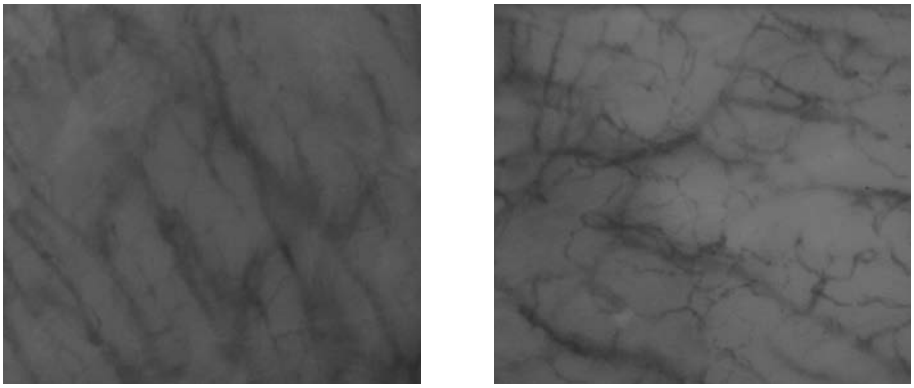

Figure 2. Frames of SDF (left) and IDF (right) microcirculatory videos.

2.4 Statistical analysis

Normal distribution of the population could not be assumed. Hereby the groups were compared using the Wilcoxon signed rank test for paired continuous data. For comparison of categorical data the Chi-squared test was used. Statistics were calculated using IBM SPSS 21. A two sided p-value < 0.05 was considered statistically significant. A Bland-Altman plot was created with the mean bias and 95% limits of agreement.

RESULTS

During the study period, a total of twenty patients were enrolled resulting in 60 video images per device. Table 2 shows the demographic data of the included patients. All patients were hemodynamically stable. Five patients (25%) were mechanically ventilated. Rectal temperature was between 36,5 and 37,5 degrees Celsius in all patients. Video images were obtained at a median postnatal age of 5 days (range 0 – 41 days).

Table 2. Demographic data of the population (n=20)

Male Gender (%)	50
Gestational age (weeks)	27.6 (24 – 33.4)
Gestational age (weeks)	27.6 (24 – 33.4)
Birth weight in grams	1117 (470 – 2650)
Apgar score at 1 min	6 (1-9)
Apgar score at 5 min	8 (3-10)
Caesarean Section (%)	45
Antenatal corticosteroids (%)	75

Data are presented as median (range) or percentage

The IDF technique visualized significantly more vessels which resulted in a higher total vessel density (mean 16.9/mm vs. 14.1/mm $p < 0.001$). This was mainly because more small vessels were visualized. The proportion of perfused vessels was lower in the IDF group versus the SDF group. This was seen in both small as non-small vessels. The differences in total vessel density and proportion of perfused vessels also resulted in a significant higher perfused vessel density for small and total vessels (Table 3).

Table 3. Mean microcirculatory parameters total vessel density (TVD), perfused vessel density (PVD), proportion of perfused vessels (PPV), microvascular flow index (MFI) and heterogeneity index (HI) for small and non-small vessels measured by IDF and SDF

	IDF (SD)	SDF (SD)	Change	*p*-value*
TVD small (n/mm)	14.8 (2.1)	12.5 (1.8)	+18.4%	.001
TVD non-small (n/mm)	2.1 (1.1)	1.6 (1.1)	+31.3%	.070
TVD total (n/mm)	16.9 (2.0)	14.1 (1.3)	+19.9%	.000
PPV small (%)	88.0 (9.4)	93.6 (7.6)	-5.6%	.003
PPV non-small (%)	95.1 (8.4)	98.9 (2.2)	-3.8%	.023
PPV total (%)	88.7 (9.1)	93.9 (7.4)	-5.2%	.002
PVD small (n/mm)	13.0 (2.1)	11.6 (1.9)	+12.1%	.033
PVD non-small (n/mm)	2.0 (1.1)	1.6 (1.1)	+25.0%	.103
PVD total (n/mm)	15.0 (2.2)	13.2 (1.7)	+13.6%	.001
MFI small (au)	2.78 (0.3)	2.75 (0.3)	+1.1%	.621
MFI non-small (au)	3.00 (-)	3.00 (-)	-	-
HI small (au)	0.22 (0.19)	0.24 (0.18)	-8.3%	.650
HI non-small (au)	0.00 (-)	0.00 (-)	-	-

* Wilcoxon signed rank test for paired continuous data
au = arbitrary units

Figure 3 shows Bland-Altman plots of the TVD, PPV and PVD. It demonstrates that the SDF structurally measures a lower TVD and PVD and a higher PPV (mean bias TVD -2.8 95% limits of agreement 1.2 to -6.9; mean bias PPV 5.3, 95% limits of agreement 18.4 tot -7.9; mean bias PVD -1.8, 95% limits of agreement 2.1 tot -5.7)

Table 4 shows the microcirculation imaging quality score. In the majority of the video clips, the duration was below three seconds and therefore scored 10 points. However, the stability of the video clips was optimal (0 points). This was for both the IDF as the SDF device. In the categories focus and pressure, the majority of the IDF videos were optimal (52/60) while the SDF video clips scored suboptimal (1 point) in a much higher rate (37/60). This difference was highly significant with a p-value below .001. The percentage of artefacts in the category content was equal.

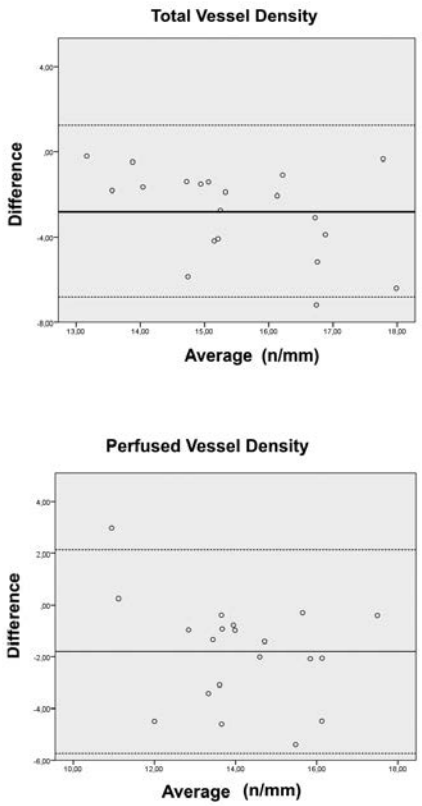

Figure 3. Bland-Altman for TVD, PPV and PVD comparing SDF with IDF.

Table 4. The microcirculation image quality score for IDF (n=60) and SDF (n=60) video images

	Device	Score 0	Score 1	Score 10	p-value*
Illumination	IDF	56	4	0	.032
	SDF	48	12	0	
Duration	IDF	5	5	50	.238
	SDF	6	11	43	
Focus	IDF	52	8	0	<.001
	SDF	20	37	3	
Content	IDF	44	16	0	.838
	SDF	43	17	0	
Stability	IDF	59	3	0	.648
	SDF	58	2	0	
Pressure	IDF	59	1	0	<.001
	SDF	44	16	0	

*Chi-squared test for categorical data

DISCUSSION

This study demonstrates that the IDF device visualized approximately 20% more vessels and therefore provides a higher total vessel density than the SDF device. The increased vessel density visualized with the IDF device can be explained by the improved imaging technology. Compared with the SDF device, the IDF device signal is fully digital and contains a high resolution sensor. A shortened pulse time (2ms vs 16 ms) creates more contrast and contours of the moving red blood cells. The ergonomic improvements are probably even more important for the higher quality of the images. The low weight of the device (120 gram vs. 350 gram) minimizes pressure artefact problems. The focus control is integrated in the computer software. This adjustment makes it easier to determine the accurate focus and avoids disturbance of camera handling during recording. These improvements are likely to cause the differences in microcirculation images quality scores (Table 4). Especially in the category focus, the IDF device produce higher quality video images. This also applies to the category of pressure. The difference in proportion of perfused vessels is therefore likely to be caused by the quality of the images. Due to the suboptimal focus mechanism in the SDF device, it sometimes may be difficult to accurately judge the flow pattern and the presence of pressure artefacts. In a healthy patient the flow is most likely to be undisturbed. In case of doubt, the flow in specific vessels may therefore more likely to be judged positive.

To our best knowledge, this is the first paper reporting a comparison between two microcirculation imaging techniques in preterm infants. Second, it is the first paper presenting the microcirculation quality scoring system for cutaneous microcirculatory images. The scoring system was based on the experience of investigators of the sublingual microcirculation in an adult population. We fully support this effort of quantifying and improving the research field of the microcirculation. However, in the field of pediatric and neonatal intensive care, most of the patients are non-sedated and non-cooperative and therefore the sublingual area is not feasible for microcirculatory research. The buccal and cutaneous microcirculation are good alternatives but the lack of patient cooperation still makes it a challenge to obtain microcirculatory images that fully meet the criteria of the microcirculation image quality score, especially in the category 'duration'. Obviously, duration and stability of the video images go hand-in-hand. The majority of our video images were perfectly stable but below three seconds of length (10 points) and according to the scorings system would be unacceptable for analysis. In our view, the shorter length of the video images is inherent in performing microcirculatory research in neonates and infants. The other five categories of the scorings system is fully applicable to buccal or cutaneous video images.

It must be said that the compared videos are not measured on the exact same spot as the skin of preterm infants is too vulnerable for marking. The comparison of the two devices is therefore not based on completely identical video clips. Also, in the majority of the cases only three movies where used for analysis. Since three movies is reliable

for analysis[13], we did not want to burden the preterm neonates further, as incubator- and thereby body temperature can decrease to increased measurement time. We do however think the results of this study reflect the differences between the devices as the measured vessel density was structurally higher in the IDF device compared to the SDF device.

CONCLUSION

The IDF imaging device represents the third generation hand held microscope visualizing the microcirculation. Technical and ergonomic improvements, in comparison with its predecessor SDF imaging, provides higher quality images of cutaneous microcirculation in preterm infants. This resulted in a higher total vessel density count and a more accurate judgment of the red blood cell flow and proportion of perfused vessels, making it a promising technique for the use of microcirculatory observations in (preterm) newborns.

Conflict of interest

Professor Ince has developed SDF imaging and is listed as inventor on related patents commercialized by MicroVision Medical (MVM) under a license from the Academic Medical Center (AMC). He has been a consultant for MVM in the past, but has not been involved with this company for more than five years now, except that he still holds shares. Braedius Medical, a company owned by a relative of Professor Ince, has developed and designed a hand held microscope called CytoCam-IDF imaging. Dr Ince has no financial relation with Braedius Medical of any sort, i.e., never owned shares, or received consultancy or speaker fees from Braedius Medical.

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CHAPTER 4

ADAPTATION OF THE CUTANEOUS MICROCIRCULATION IN PRETERM NEONATES

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ABSTRACT

Objective

Transition from fetal to neonatal circulation is characterized by multiple hemodynamic changes. The role of the microcirculation in this process is underexposed. Visualizing the cutaneous microcirculation can help us understand peripheral perfusion in a non-invasive manner.

Methods

Cutaneous microcirculation of term and preterm infants born below 32 weeks of gestational age was measured in the first month of life of using Incident Dark Field (IDF) imaging. Linear mixed modeling was used to identify clinical variables which influence the cutaneous microcirculation.

Results

Sixty preterm and 33 term infants were included. Total vessel density (TVD) of preterm infants significantly decreased in the first month of life (31.7mm/mm^2 day 1 vs 27.9mm/mm^2 day 28) but remained significantly higher compared to TVD of term infants on day 1 (25.8 mm/mm^2). Besides postnatal age, no clinical variables were associated with total vessel density. Infants born small for gestational age had significantly higher TVD values directly after birth than those born appropriate for gestational age (35.4 mm/mm^2 vs 31.6 mm/mm^2; p=0.015).

Conclusions

Total vessel density decreases in the first month after birth and is higher in preterm infants compared to those born term. Differences in antenatal oxygen exposure might explain the adaptation of the microcirculation.

Keywords: Preterm, Neonates, Cutaneous, Incident Dark Field, Adaptation

INTRODUCTION

The process of postnatal adaptation in preterm neonates directly after birth is characterized by multiple cardio-pulmonary changes. Within the first week of life, the fetal circulation transposes into the neonatal circulation. This process starts immediately after birth as the removal of the placenta and endocrine hormones lead to increases in systemic vascular resistance[23]. The microcirculation plays a key role in maintenance of systemic vascular resistance, thermoregulation and supply of gasses and nutrients on a cellular level.

The introduction of handheld microscopes 15 years ago allowed researchers to explore buccal and cutaneous microcirculation in (preterm) infants[13]. The cutaneous microcirculation can be divided in two components; an upper horizontal network in the papillary dermis and a lower horizontal plexus at the dermal subcutaneous interface[38]. The upper layer is 1-2 mm in depth and can be visualized with videomicroscopy. It is composed of arterioles, capillaries and post capillary venules[4] and the majority of the vessels have a diameter between 10 and 35 μm[3]. The vascular structure of the skin is similar between term and preterm infants. Vessel diameters do not differ and, contrary to adults, capillary loops are not present until the postnatal age of 4 months[25]. There are important differences between the skin of term infants and preterm infants. In preterm infants the underdeveloped stratum corneum results in a reduced barrier function. Barrier maturation of the skin is fulfilled in 2-4 weeks for very preterm infants and 4-6 weeks for extremely preterm infants after birth[16].

Observational studies in critically ill children demonstrated that the microcirculation can be prognostic for survival[5,29]. In preterm infants, the effects of multiple interventions and clinical variables on the microcirculation have been studied such as erythrocytes transfusion[12], patent ductus arteriosus[14] and hypotension[28]. All studies used orthogonal polarization spectral (OPS) or sidestream dark field (SDF) imaging. The microcirculation of preterm infants during postnatal transition has been studied in term [30] and preterm infants previously[17]. Only univariable analyses were performed and the role of the microcirculation during transition remains not fully understood. Also, a new generation of handheld microscope was recently introduced using incident dark field (IDF) technology[15]. A comparison study between IDF and SDF technology in preterm infants demonstrated that IDF technology visualizes 20 percent more vessels[34]. Against that background, and the fact that modern video microscopes visualize more vessels, we conducted this study. The aim is to create microcirculatory reference values for healthy preterm neonates and identify the influence of clinical variables on the cutaneous microcirculation using multivariate statistics.

MATERIALS AND METHODS

This prospective observational cohort study included patients admitted to the neonatal intensive care unit (NICU) of the Erasmus MC – Sophia, a level III university children's

hospital between February 2014 and June 2015. The local medical ethical review board approved the study (NL40946.078.12). Parents gave written informed consent prior to the study start.

Patients were eligible for inclusion if the gestational age (GA) was between 24 and 31 6/7 weeks and postnatal age was less than 24 hours. Patients were excluded in case of denied parental informed consent, any known congenital cardiac or hematological disorder and any lethal congenital condition.

A control group of healthy term infants was included as well. These infants were born from apparently healthy mothers not suffering from antenatal or postnatal pregnancy complications. GA was between 37 and 41 6/7 weeks.

Imaging

Microcirculatory data were obtained at six time points: within 24 hours after birth (T1), day 3 (T2), day 5 (T3), day 7 (T4), day 14 (T5) and day 28 (T6). The cohort of term infants was only measured on T1, as these healthy newborns and their mothers were discharged soon after birth. During imaging, body temperature of the infants was kept between 36.5 and 37.5 degrees Celsius. Images were obtained using IDF technology (CytoCam, Braedius, The Netherlands). All videos were obtained using two operators and conform guidelines of transcutaneous microcirculatory imaging in preterm infants[33]. A minimum of 8 videos were captured. This allowed critical evaluation of the video quality using the microcirculation imaging quality score[20], and videos that did not meet this criteria were discarded. According to the guidelines, three to five videos were eventually used for analysis [10]. Vessel density (small vessels = Ø <10μm, medium vessels = Ø 10-20μm, large vessels = Ø 20 -100μm) was analyzed using automated analysis software (CCTools, Braedius, The Netherlands). Automated analysis for vessel density was chosen over manual analysis as manual analysis of cutaneous microcirculation has a poor reproducibility[32]. Microvascular flow index (MFI) was analyzed manually by one experienced researcher (HvE). Each video sequence was divided in four equally sized quadrants. Per quadrant the predominant type of flow was scored continuous (3), sluggish (2), intermittent (1), or absent (0). MFI represented the mean score of the predominant type of flow.

Demographic and clinical data

The following clinical characteristics were collected: gender, gestational age, birth weight, mode of delivery, Apgar scores at 1 and 5 minutes, arterial cord blood gas analysis, antenatal corticosteroid administration (complete course defined as two doses given 24 hour apart administered 48 hour prior to delivery), maternal pre-eclampsia (PE) or HELLP syndrome (defined as the combination of hemolysis (LDH > 550 U/l) elevated liver enzymes (ASAT or ALAT >41 U/l) and thrombocytopenia (<100 x 10^9/l), hemodynamically significant patent ductus arteriosus (defined as PDA confirmed by ultrasound and in need for medical or surgical treatment).

The following clinical parameters were recorded during microcirculatory imaging in preterm infants: arterial oxygen saturation, fraction of inspired oxygen (FiO_2), heart rate, mean arterial blood pressure (MAP) cerebral oxygen saturation ($rScO_2$) and cerebral fractional tissue oxygen extraction (cFTOE) and rectal temperature. If measured, blood gas analyses, hematocrit, hemoglobin, thrombocytes and leukocyte count were recorded.

In order to create reference ranges preterm infants were classified as healthy or non-healthy. Reference ranges were based on analysis of healthy patients only. Patients were considered non-healthy in case of infection (defined as clinical symptoms in combination with c-reactive protein levels >20mg/l and/or positive blood culture test), necrotizing enterocolitis (NEC) stadium Bell 2 or above, persistent pulmonary hypertension of the neonate (PPHN) defined as the need for inhaled nitric oxygen (iNO), circulatory failure that required blood pressure supportive medicine or the presence of a pneumothorax. These conditions were likely to influence macro- and microcirculatory parameters based on current literature.

Statistics

A priori sample size calculation was virtually impossible as there is very little known about cutaneous microcirculation in preterm infants, especially not on subgroups. One study measured microcirculation in preterm infants after birth [17], but this was performed with a SDF technology which visualized 20% less vessels compared to IDF technology [34]. Therefore as much patients as possible were included for a predefined period of time (January 2014 – March 2015).

Data are presented as mean (standard deviation) for normally distributed variables, and as median (range) for continuous variables that were not normally distributed. Normality of the distribution of the data was assessed using the Shapiro-Wilk test. The following subgroups were defined: patients considered not healthy, patients born small for gestational age (SGA) defined as birthweight below the 10^{th} percentile and patients born to mothers suffering from pre-eclampsia.

A linear mixed model was used to identify the associations between clinical parameters and the outcome parameter total vessel density (TVD) over time. The independent variables in the linear mixed model included gender, GA (weeks), birthweight z-score (based on Cole et. al [6]) , SGA (defined as birth weight below the 10^{th} percentile), body temperature (°C), PDA, maternal PE, hematocrit (L/L), antenatal corticosteroid administration, postnatal age (days, coded as a categorical variable), health status (healthy or not healthy) and mean arterial blood pressure (mmHg). A random intercept and a random slope of postnatal age (coded as a continuous variable) were included in the linear mixed models to account for the within-subject correlations. Univariable linear mixed models with only postnatal age (coded as a categorical variable) as independent variable were used to compare TVD and MFI between time points. Multiple imputation by chained equations was used to impute missing values (45%) of the independent

variable hematocrit and 200 imputed data sets were generated and the linear mixed model was estimated for each imputed data set. The results were pooled using Rubin's rules for combining results of imputed data sets. Comparison of variables that were not normally distributed was performed using the Mann-Whitney test for 2 groups (i.e. small for gestational age yes/no, maternal pre-eclampsia yes/no and healthy yes/no).

All statistical tests were two-sided and used a significance level of p-value ≤0.05. The statistical analyses were performed using SPSS version 21.

RESULTS

A total of 60 preterm infants and 33 healthy term infants were included Demographic data are shown in Table 1. The number of preterm participants decreased over time due to transfer to high care facilities in other hospitals and death. In one case, parents withdrew their consent (Figure 1). The development of heart rate, arterial oxygen saturation, cerebral oxygen saturation and mean arterial blood pressure of the healthy preterm infants is shown in Figure 2.

Median (5^{th} and 95^{th} percentile) microcirculatory values of healthy preterm and term infants are shown in Table 2. These values can be interpreted as reference values. TVD decreased over time (Figure 3). The decrease was seen in small, medium and large vessels. Using univariable mixed model, TVD was significantly lower from day 5 onwards compared to day 1. (31.6 mm/mm^2 vs 30.5 mm/mm^2, p=0.017). Preterm infants had a significantly higher total vessel density on day 1 compared to term infants (31.7 mm/mm^2 vs 25.8 mm/mm^2, p<0.001). Even though in preterm infants total vessel density decreased over time, values on day 28 remained higher compared to term infants directly after birth (27.9 mm/mm2 vs 25.8 mm/mm2, p=0.029).

Table 1. Demographic data of the preterm (n=60) and term (n=33) population

	Preterm	**Term**
Male gender	25 (42%)	19 (58%)
Female gender	35 (58%)	14 (42%)
Gestational age (weeks)	28 0/7 (24 0/7 – 31 1/7)	39 5/7 (370/7 – 41 3/7)
Birth weight (grams)	1110 (400 – 1770)	3353 (2475 – 4450)
Caesarean Section	36 (60%)	20 (60.6%)
Apgar score at 1'	6 (1-10)	9.0 (2 -9)
Apgar score at 5'	8 (4-10)	10 (7-10)
Umbilical cord arterial pH	7.32 (7.06 – 7.45)	7.31 (7.01 – 7.40)
Antenatal corticosteroids	57 (95%)	-
Small for gestational age	6 (10%)	-
Maternal PE/HELLP	8 (13%)	-

Data are presented as median (range) or number (percentage)

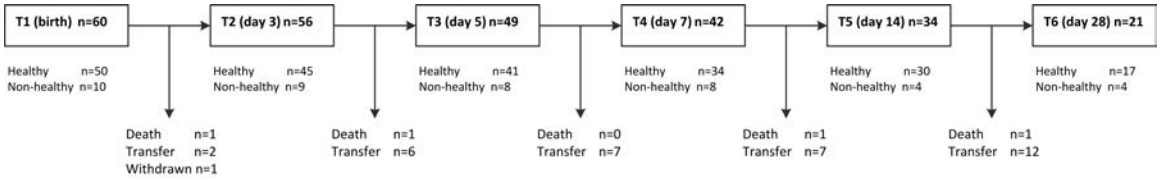

Figure 1. Flowchart of preterm patients participating in the study per time moment.

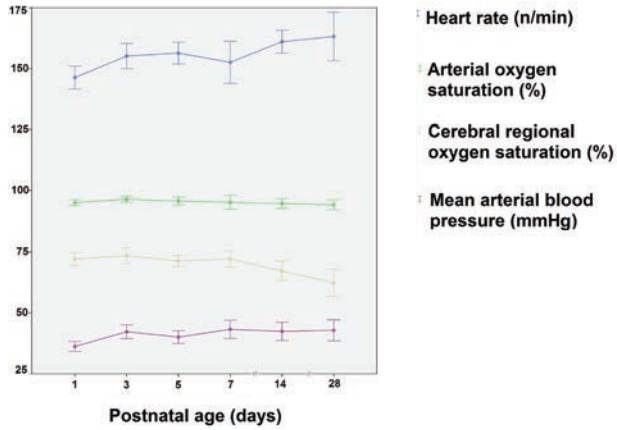

Figure 2. development of heart rate, arterial oxygen saturation, cerebral oxygen saturation and mean arterial blood pressure of the healthy preterm infants. Error bars showing 95% CI.

Table 2. Median (5th and 95th percentile) microcirculatory values of healthy preterm and term infants

	Term	Preterm					
	Day 1 (n=33)	**Day 1 (n= 50)**	**Day 3 (n= 45)**	**Day 5 (n=41)**	**Day 7 (n=34)**	**Day 14 (n=30)**	**Day 28 (n=17)**
TVD (mm/mm²)	25.8 (21.6-31.8)	31.7 (27.0-35.6)	31.7 (27.1-35.0)	30.5 (25.5-35.9)	30.3 (24.9-34.1)	28.9 (23.9-33.5)	27.9 (22.4-30.6)
TVD small (mm/mm²)	1.7 (0.9-4.2)	2.7 (1.2-4.7)	2.7 (1.5-3.6)	2.5 (1.3-3.4)	2.2 (1.3-4.3)	2.0 (1.1-3.2)	1.7 (0.7-3.8)
TVD medium mm/mm²)	13.6 (8.5-18.9)	18.4 (13.0-23.7)	19.6 (15.5-24.4)	19.2 (13.7-23.5)	19.9 (14.1-24.0)	17.6 (12.5-21.3)	17.3 (13.2-19.5)
TVD large (mm/mm²)	9.9 (6.2-13.3)	10.6 (6.8-14.6)	9.4 (5.8-13.0)	9.1 (5.0-11.7)	8.4 (5.7-12.4)	9.5 (5.7-12.4)	8.9 (6.3-12.9)
MFI small (au)	2.83 (2.13-3.00)	2.56 (1.52-3.00)	2.65 (1.86-3.00)	2.75 (2.03-3.00)	2.92 (2.42-3.00)	2.92 (2.45-3.00)	3.00 (2.83-3.00)
MFI non-small (au)	3.00 (2.26-3.00)	2.81 (1.99-3.00)	2.88 (2.00-3.00)	3.00 (2.56-3.00)	3.00 (2.81-3.00)	3.00 (2.88-3.00)	3.00 (2.5-3.00)
HI small (au)	0.35 (0.00-0.59)	0.40 (0.00-1.06)	0.37 (0.00-1.00)	0.36 (0.00-0.99)	0.34 (0.00-0.83)	0.34 (0.00-0.41)	0.00 (0.00-0.35)
HI non-small (au)	0.00 (0.00-0.35)	0.41 (0.00-0.86)	0.35 (0.00-0.75)	0.00 (0.00-0.44)	0.00 (0.00-0.35)	0.00 (0.00-0.38)	0.00 (0.00-0.34)

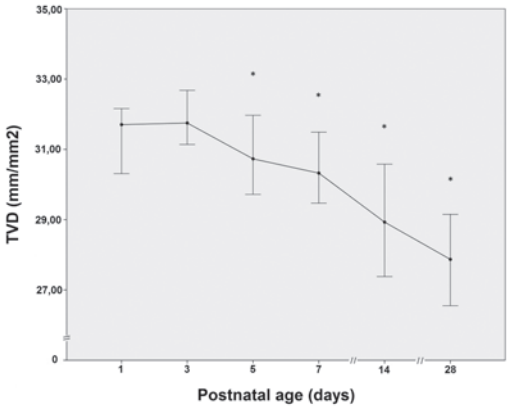

Figure 3. Total Vessel Density of healthy preterm infants over time.
*: Significant compared to day 1; Error bars showing 95% CI.

Table 3 shows the estimated coefficients of the independent variables in the linear mixed model for TVD. Beside the significant effect of postnatal age, no clinical variable had significant influence on total vessel density, although the effect of hematocrit on TVD (0.5mm/mm2 lower TVD per 0.1 l/l decrease in hematocrit) was borderline significant (p=0.080).

Infants born small for gestational age (n=6) had significantly higher TVD values directly after birth compared to appropriate for gestational age infants (35.4 mm/mm^2 vs 31.6 mm/mm^2; p=0.015) but this difference was not significant in subsequent time points.

Neonates born to mothers suffering from PE or HELLP syndrome (n=8) had similar TVD scores, however the distribution of vessels was altered (significantly higher TVD medium and lower TVD large). MFI values were remarkably low at birth (MFI small 2.56) and day 3 (MFI small 2.65), but normalized over time. Overall comparison of healthy and not-healthy neonates showed no statistically significant differences in vessel density and MFI scores. Comparison per time point showed a significant difference in MFI non-small values on day 1 (median MFI healthy [n=50] 2.81, not healthy [n=10] 2.44; p=0.046). Comparison of other time points showed no significant differences.

DISCUSSION

In this study we report the relationship between total vessel density and clinical variables, showing that the decrease of total vessel density in preterm infants is explained by postnatal age and not by clinical parameters such as gestational age, birth weight, blood pressure or hematocrit. These results were unexpected as many studies have shown correlations between microcirculatory parameters and clinical variables such as hematocrit [13], hemoglobin [17] and birthweight [8] . However, correlation does not imply causation. The linear mixed model used in this study implies that there is only an effect of postnatal age on total vessel density. It could be that the decrease in

Table 3. Estimates of fixed effects in the linear mixed model for total vessel density (TVD)

Variable	Estimate	95% CI	p-value
Male sex	-0.54	-1.52 – 0.44	0.282
Gestational age (weeks)	-0.13	-0.38 – 0.12	0.297
Birthweight z-score	-0.11	-0.89 – 0.67	0.786
Small for gestational age	0.57	-1.71 – 2.85	0.625
Body Temperature (°C)	-0.09	-0.35 – 0.17	0.489
Patent ductus arteriosus	0.01	-0.92 – 0.94	0.983
Maternal pre-eclampsia	-0.19	-1.57 – 1.19	0.788
Hematocrit	5.35	-0.64 – 11.35	0.080
Corticosteroid			
Full	-0.03	-2.30 – 2.24	0.978
Partial	0.61	-1.75 – 2.98	0.611
No	REFERENCE	-	-
Timepoint			
Day 1	REFERENCE		
Day 2	0.25	-0.64 – 1.14	0.583
Day 3	-0.42	-1.52 – 0.69	0.459
Day 4	-0.79	-1.97 – 0.39	0.187
Day 5	-1.74	-3.13 – -0.34	0.014
Day 6	-3.35	-5.03 – -1.67	<0.001
Healthy	0.11	-0.91 – 1.13	0.830
Mean arterial blood pressure	-0.01	-0.06 -0.04	0.638

vessel density is explained by a unknown variable which we did not include in this study. However, besides the variables that were used in the linear mixed model (Table 3) other variables such as Apgar score, heart rate, arterial oxygen saturation (SaO_2), fraction of inspired oxygen (FiO_2), delivery mode, ventilation mode, thrombocyte count, leukocyte count and pH did not had a significant effect and were eliminated from the model.

The higher total vessel density found in preterm infants compared to term infants is perhaps one of the earliest signs of hemodynamic differences between these groups of neonates. It could be that a compensation mechanism is triggered, in response to upcoming preterm delivery, to increase oxygen and nutrient delivery to the fetus. Another explanation might be that vessel density also decreases during intra uterine life. Term born infants might have a similar vessel density, but the delivery at term age and therefore a later time point of measuring, resulted in a lower vessel density.

Our findings are in line with research investigating microcirculatory differences between these groups [8]. Studies have shown a decrease in vessel density after birth in both preterm [17] as term [30] newborns. In adolescence, vessel density of former

preterm infants was higher compared to a healthy control group [2,18], implying that the microcirculatory differences might be structural until at least adolescence. In adult life, preterm birth is associated with cardiovascular disease and a higher systolic and diastolic blood pressure [24]. The differences found in this study might be one aspect of the complex process of influences in fetal and neonatal life leading to life-long persistent changes in physiology.

One study has previously studied the cutaneous microcirculation of preterm infants in the first month of life [17]. Using OPS imaging, Kroth et al. found a decrease in vessel density in 25 preterm infants. A comparison between results is difficult, as in this study an alternative outcome parameter (functional small vessel density in cm/cm^2) was used. The 12% decrease in vessel density found by Kroth et al. is however similar to the decrease in vessel density between day 1 and day 28 in this study.

The decrease in vessel density in the first month after birth can be explained by the different pre- and postnatal environmental conditions. Extrapolations from animal studies suggest that human fetal PaO_2 levels are between 2.5 and 3.5 kPa [22] while postnatal PaO_2 levels in preterm infants range from 4.0 to 9.0 kPa [26]. The hypoxic conditions in utero leads to hypoxia-induced angiogenesis [31]. After birth, the higher PaO_2 levels result in a decrease of vascular endothelial growth factor (VEGF) and thereby a decrease in angiogenesis. The hypothesis that oxygen exposure influences the adaptation of the microcirculation is supported in four ways: First, studies found a higher VEGF concentrations in newborns suffering from intrauterine growth restriction, a condition were the fetus is exposed to a greater hypoxic condition[27,36]. Secondly, and in line with our results, when exposed to normo- and hyperbaric hyperoxia (FiO_2 100%), vessel density decreased in healthy volunteers and in a recent animal study [7,21]. Furthermore, subgroup analysis in small for gestational age newborns in our own study showed significantly higher TVD values directly after birth. Lastly, term newborns born at high-altitude, have a higher TVD than newborns born at sea-level (Gassmann et. al, unpublished data). The antenatal environment therefore seems to be a key factor in the adaptation of the microcirculation in newborn infants. Another explanation of the decrease in vessel density is the maturation of the skin. The development of the stratum corneum leads to an increase of skin-artefacts and thereby a drop in video quality. The decrease in vessel density is not only seen in automated computer analysis, but also in manual analyzed videos [17], ruling out a technical explanation.

An interesting finding was the relatively low values of MFI in infants considered to be healthy on day 1, 3 and 5 after birth. A large number of healthy infants had a MFI value below 2.6, which is considered to be abnormal in adults [11,35]. This threshold however, is based on sublingual measurements and the lingual artery arises directly from the external carotid artery. Therefore, sublingual measurements must be considered as more central perfusion compared to the peripheral perfusion measured on the skin. MFI values showed no correlation with blood pressure ($r=0.192$) and might represent the normal process of hemodynamic adaptation. Also the higher

percentage of poorly deformable erythrocytes in preterm infants compared to adults could be accountable for the lower MFI values [19].

The comparison between healthy and non-healthy infants revealed no differences in vessel density and only a significant difference in MFI on day 1. This could be explained by time point of the measurement. Previous studies have shown that severity of microcirculatory alterations is related to the onset of disease, with more severe alterations at early stages and less severe alterations in persistent sepsis [9]. If a measurement was performed well after the onset of sepsis or the start of blood pressure supportive medication, this could have led to more favorable outcome parameters for the non-healthy patient. Observational studies in term and preterm infants found a 10% decrease in vessel density in 5 days prior to infection [1,37]. Analysis from our own data cannot confirm this observation.

LIMITATIONS

There were some limitations of our study. First, the number of participants in the subgroups was low which undermines the statistical power of these analysis. Second, for technical reasons skin temperature was not recorded while this could be a possible confounding variable. Rectal temperature was chosen primarily for temperature monitoring instead of skin temperature.

Also, the definitions used for patients being 'non-healthy' were broad. Beside infection, which is a well- known variable for microcirculatory alterations, also NEC, low blood pressure, pneumothorax and PPHN were identified as critical diseases potentially disturbing the microcirculation. Although all these conditions are severe and potentially life-threatening, the diversity in conditions could have caused the failure to show sustained differences between healthy and non-healthy infants.

Finally, the frequently used outcome parameters proportion of perfused vessels (PPV) and perfused vessel density (PVD) are not given in this manuscript as it is our opinion that the output of the automated analysis of these parameters are currently not fully accurate.

PERSPECTIVES

In preterm infants the vessel density decreases in the first month after birth. There only is a significant effect of postnatal age on total vessel density. No association was found between total vessel density and clinical parameters such as gestational age, birthweight, blood pressure or hematocrit. Patients small for gestational age had a significantly higher total vessel density directly after birth. These finding suggest that there is antenatal and postnatal adaptation of the microcirculation, presumably in response to oxygen exposure. The cutaneous microcirculation is a non-invasive manner to study the peripheral perfusion in preterm infants. Reference values for healthy preterm infants provide a basis for future studies focusing on transcutaneous microcirculation and clinical interventions.

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CHAPTER 5

PREGNANCY AT HIGH ALTITUDE IN THE ANDES LEADS TO INCREASED TOTAL VESSEL DENSITY IN HEALTHY NEWBORNS

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ABSTRACT

The developing human foetus is able to cope with the physiological reduction in oxygen supply occurring *in utero*. However, it is not known if microvascularisation of the foetus is augmented when pregnancy occurs at high altitude. Fifty-three healthy term newborns in Puno, Peru (3,840m) were compared to sea-level controls. Pre- and post-ductal arterial oxygen saturation (SpO_2) was determined. Cerebral and calf muscle regional tissue oxygenation were measured using near infrared spectroscopy (NIRS). Skin microcirculation was non-invasively measured using Incident Dark Field imaging.

Pre- and post-ductal SpO_2 in Peruvian babies was 88.1% and 88.4% respectively, which was 10.4% and 9.7% lower than in newborns at sea level ($p<0.001$). Cerebral and regional oxygen saturation were significantly lower in the Peruvian newborns (cerebral 71.0 % vs. 74.9%; regional 68.5% vs. 76.0%, $p<0.001$). Transcutaneously measured total vessel density (TVD) in the Peruvian newborns was 14% higher than that in the newborns born at sea level (29.7 vs. 26.0 mm/mm^2; $p\leq 0.001$). This study demonstrates that microvascular vessel density in neonates born to mothers living at high altitude is higher than that in neonates born at sea level. Evidence is provided that the observed elevation in TVD is independent of the neonates' ethnicity.

INTRODUCTION

It is estimated that in the South American Andes over 30 million people - most of them belonging to the Quechua or Aymara population - permanently live above 2,500m (8,200ft), defined as high altitude [1,2]. At high altitude, the environmental conditions are extreme, including dramatic temperature changes and low atmospheric pressure leading to hypobaric hypoxia. The consequences of this are often exacerbated by low socio-economic status and negatively impact upon the health of infants [3]. Of note, people living at high altitude do not only show genetic adaptation but also plasticity in development in response to hypoxia [4,5]. Despite the harsh conditions at the high altitudes of the Andes, most foetuses develop well and are delivered at term [6]. For that matter, it must be understood that the intrauterine environment already represents an extreme surrounding at sea level that is exacerbated in pregnancies at high altitude. In general, proper *in utero* development requires adequate oxygen delivery to the foetus, which is achieved by increased maternal ventilation rate and thus increased blood oxygen saturation (SpO_2) level [7,8]. Under conditions of chronic hypoxia, however, the utero-placental blood flow is lower and, consequently, oxygen uptake by the foetus is reduced. This process can even be exacerbated by the presence of maternal preeclampsia [9]. When pregnancy occurs at 3,100m, however, the placenta increases antioxidant capacity [10] while the foetus is able to adapt to maternal and placental hypoxemia by increasing nitric oxide production *in utero* and after birth. This adaptive response might be necessary to sustain placental blood flow but may also lead to improvement of microcirculatory blood flow [11].

It was shown, decades ago, that babies born to indigenous Quechua or Aymara women have a higher birth weight than non-Andean neonates both born at high altitude [12]. A more recent study revealed that elevated uterine artery blood flow and thus increased oxygen delivery protect Andeans from foetal growth retardation when pregnancy occurs at high altitude [13]. Perinatal Doppler and ultrasound studies in Andean foetuses performed at 3,600m showed reduced umbilical blood flow, compensated for, however, by the foetuses' elevated neonatal haemoglobin concentration and increased oxygen extraction capability [6]. As a result, foetal oxygen delivery and oxygen consumption at high altitude do not differ from values measured at low altitude [6], supporting the notion that the foetus copes with the extreme *in utero* situation by increasing systemic blood flow and thus oxygen delivery. Note that the present study does not include the Tibetan population which is known to maintain better neonatal oxygenation than Andeans (reviewed in [14]).

Apart from vasodilation, an obvious strategy to increase blood and thus oxygen supply to the tissue is to enhance microvascular density. Microcirculation studies in critically ill neonates [15] found a low microvascular density to be a predictor for mortality in sepsis [16]. However, no studies have reported on the effect of antenatal hypobaric hypoxia on foetal microcirculatory development. Thus, in the present prospective observational study the aim was to obtain microcirculatory profiles of term babies born at high altitude and compare these with the profiles of babies born at sea level. We postulated that the microvascularisation of the neonate born to mothers at high

altitude is elevated and that this phenomenon reflects a general adaptive mechanism and thus is independent of the ethnicity.

MATERIALS AND METHODS

Subjects

This prospective observational study was performed in August 2014 at the paediatric department of the Hospital EsSalud III in Puno (Peru) located at 3,840m above sea level. The Peruvian microcirculatory measurements were compared to those performed at sea level in the maternity ward of the Erasmus MC - Sophia Children's Hospital in Rotterdam, the Netherlands (altitude: 0m) where measurements were performed by the same operator using identical instrumentation. Before any measurements were taken, all parents gave their written informed consent. The study protocol was approved by the Ethics Committee of the Universidad Peruana Cayetano Heredia (UPCH 180-17-14; 62794) as well as by the local Ethics Authorities represented by the Red Asistencial Puno EsSalud and the Erasmus MC Rotterdam Ethics Committee (NL48445.078.14). The measurements were carried out in accordance with the approved guidelines. Eligible for participation were healthy, singleton newborns of women either residing at high altitude (Puno and surroundings) or at sea level (Rotterdam and surroundings) at least during pregnancy, delivered either vaginally or by caesarean section, with Apgar scores of 8 or higher and not older than 30 hours at the time of measurement. Newborns were considered healthy if born at term to apparently healthy mothers not suffering from obvious pregnancy complications (no ante- or postnatal abnormalities). Maternal data on smoking was not collected. Babies delivered by caesarean section at high altitude (n=19), but not those at low altitude, were placed in an incubator (33°C) with oxygen-enriched air. Exclusion criteria included gestational age below 37 or above 42 weeks, any known congenital, hematologic or cardiorespiratory disorder and refusal of written parental informed consent.

Ethnicity was assigned by analysing the babies' parental surnames, a method that was validated by analysing ancestry informative genetic markers [17,18]. In brief, babies born to Andean parents acquire both parental surnames that are not changed upon marriage. Accordingly, this custom yields four parental surnames for every child. By the method taking into account this tradition, further refined by Lorna Moore and her team [13,19], a baby was considered indigenous if she or he had three or four Andean parental surnames. If three or four parental surnames were of Hispanic origin, the baby was considered as being of low-altitude population origin (Hispanic). Babies with two Andean and two Hispanic surnames were considered of 'mixed origin'. Classification was not possible in all other cases.

Data collection

Clinical data of 53 healthy term-born neonates born at high altitude were retrieved from the medical files of the Hospital III Puno EsSalud and clinical data of 33 healthy

term-born neonates born at sea level from the medical files of the Erasmus MC - Sophia Children's Hospital. Data included gender, gestational age, birth weight, mode of delivery, and rectal temperature. Additional data - only available in Peruvian newborns - included heart rate, respiratory rate, haematocrit, haemoglobin concentration as well as thrombocyte and leukocyte count. For classification of ethnicity (see above), the surnames of the babies, the mothers and (if known) of the fathers were obtained.

Full microcirculatory profiles were obtained by the following measurements performed simultaneously: pre- and post-ductal arterial oxygen saturation (SpO_2), regional and cerebral tissue oxygen (rSO_2 and $crSO_2$) and total vessel density (TVD) using transcutaneous microcirculatory imaging. All newborns were asleep, or awake and calm during measurements. While full microcirculatory profiles were obtained in Puno, in 33 newborns from Rotterdam only the transcutaneous microcirculation profiles were obtained.

Measurement methods

Pre- and post-ductal arterial oxygen saturation (SpO_2) levels were measured on the right and left wrist using two Masimo Radical 7 pulse oximeters (Masimo Corp., Irvine, CA, USA).

Regional tissue oxygen saturation was measured by near infrared spectroscopy (NIRS) using the INVOS® device (Somanetics Corp., Troy, Michigan). This device uses near-infrared light at wavelengths of 730 and 810nm to measure oxygenated and deoxygenated haemoglobin. Tissue oxygen saturation, defined as the percentage of oxygenated haemoglobin/total haemoglobin, was measured on the forehead to determine the cerebral oxygen saturation ($crSO_2$) and on the skeletal calf muscle to determine the regional oxygen saturation (rSO_2). Fractional tissue oxygen extraction (FTOE) was calculated as (pre-ductal arterial saturation -cerebral saturation) / pre-ductal arterial saturation [(SO_2 - $crSO_2$)/ SO_2] for cerebral (crFTOE) and with the rSO_2 for the skeletal calf muscle measurements (rFTOE). Pulse oximetry and NIRS measurements of Peruvian newborns were compared to published reference values [20-23].

Skin microcirculation was measured on the upper inner arm using incident dark field (IDF) technology (Braedius, Huizen, the Netherlands). This device (CytoCam®) is a handheld microscope with an illumination unit (green light, 450nm) that allows optimal absorption of deoxy- and oxyhaemoglobin thereby permitting visualization of the erythrocytes [24] (Figure 2, upper left). The transcutaneous approach was chosen because sublingual measurement in newborns is not possible and a newborn's skin is thin enough to allow this [25]. Identical instrumentation was used in Puno and Rotterdam and the measurements were performed by one and the same technical study operator present at both sites. A minimum of three video clips were recorded and those that did not met the quality criteria according to Massey *et al.* [26] were excluded from further analysis. Total vessel density was automatically analysed using CCTools (Version 1.7.12, brightness 500, sensibility level 95%). A distinction was made into

small vessels, medium and large vessels: Ø ≤10, 10-20 and 20-100 μm, respectively. The automated analysis standardizes the process of analysis and thereby excludes inter-observer variability [27]. Following standard guidelines, a minimum of three video clips per newborn was used for automated analysis [28].

The microvascular flow index (MFI) and the heterogeneity index (HI) semi-quantitatively describe the velocity of microcirculatory perfusion [28]. Each video image was divided in four equally sized quadrants. Each quadrant was scored manually by one experienced operator according to the predominant type of flow (continuous: 3, sluggish [e.g. continuous but very slow]: 2, intermittent: 1, or absent: 0). The MFI is represented by the mean score of the type of flow, and HI by the difference between the highest quadrant and the lowest quadrant score divided by the mean score of all quadrants for one measurement. The MFI and HI for small (Ø ≤10 μm) and non-small vessels (Ø 10 - 100 μm) were determined. This method shows good intra-rater variability and is described in more detail elsewhere [29].

Statistical analysis

Continuous data are presented as median and range for non-normally distributed variables and as mean and standard deviation (SD) for normally distributed parameters. Non-continuous variables are presented as percentages of total and 95% confidence intervals (CI) of proportions.

Normally distributed continuous data were compared using an unpaired t-test. Pre- and post-ductal arterial saturation and cerebral saturation were compared with the aforementioned international reference values using a one-sample t-test. Median values were compared using a one sample Wilcoxon signed rank test. One way-ANOVA was used to compare means between more than two groups. Multivariable linear regression analyses adjusting for possible confounding variables were performed using SPSS version 21(IBM Co., Armonk, NY, USA). The crude association was adjusted for country (Peru/Netherlands), sex, gestational age, birth weight z-score, Apgar score (5 minutes), mode of delivery, pregnancy (primigravida/multigravida) and rectal temperature. Collinearity analysis to explore correlation between all covariates using a correlation matrix was performed. A cut-off value of 0.7 was used for the exclusion of variables in the model. Residual plots were constructed to check for normality of the distribution of the residuals.

RESULTS

Comparison of demographic data is shown in Table 1. Gender distribution was approximately even, gestational age and birth weight were comparable between Peru and Rotterdam. About one third of the Peruvian newborns were delivered by caesarean section, versus circa 60% in Rotterdam. In Puno, 18 babies had 3 or 4 Andean surnames and were classified as "indigenous", 6 had an even mixture of Andean and Hispanic surnames and were classified as "mixed", while 19 carried three or four Hispanic surnames and were

considered as "Hispanic". The remaining 10 babies could not be classified by surnames. The birth weight of indigenous, mixed and Hispanic newborns was 3,374 (SD 315), 3,325 (SD 414) and 3,196 (SD 220) grams, respectively. Comparison of birth weight between these groups, adjusted for sex and gestational age, showed no significant difference (the comparison between indigenous vs. Hispanic resulting in p=0.100). Nevertheless, this trend of higher birthweight in indigenous newborns was in accordance to recent studies [30,31] reporting that high altitude generally decreases birth weight but that birth weight of neonates of Andean descent was higher than that of neonates of non-Andean origin.

Additional clinical data of the 53 healthy Peruvian newborns (3,840m above sea level) were the following: mean heart rate 145 (SD 13) n/min, mean respiratory rate 53 (SD 5) n/min, mean haematocrit 0.57 (SD 0.06), mean haemoglobin 19.0 (SD 1.9) g/dL, mean thrombocytes count 247 (SD 53 x 10^{-9}) dL and mean leukocytes count 18.6 (SD 4.1 x 10^{-9} dL).

Table 1. Clinical parameters of the newborns at high and low altitude

	High altitude: Puno (n=53)	**Low altitude: Rotterdam (n=33)**
Male gender (%, CI)	49.1 (27.1 – 51.0)	57.6 (40.8 – 72.8)
Caesarian section (%, CI)	35.9 (24.3 – 50.3)	60.6 (43.7 – 75.3)
Gestational age (weeks+days)[#]	39+0 (37+0 – 40+0)	39+5 (37+0 – 41+3)
Birth weight (grams)[#]	3310 (2590 – 4180)	3353 (2475 – 4450)
Rectal temperature (°C) [*]	36.8 (0.3)	36.9 (0.3)

[#] Median (range)
[*] Mean (Standard deviation)

Mean pre- and post-ductal saturation in Peruvian newborns was 88.1% (SD 4.1%) and 88.4% (SD 4.6%) respectively (Figure 1). These values were significantly lower (p<0.001) than reference values [20] obtained from a total of 13,714 term newborns at sea-level, that is 98.5 and 98.7%, respectively. The relative difference between pre- and post-ductal saturation in high and low altitude born babies thus was 10.4% and 9.7%, respectively. The results of cerebral and regional NIRS measurements at high altitude are also shown in Figure 1. These data were compared to published reference values of term infants (cerebral n=339 and regional n=72), born at sea level and measured with the same NIRS device [21-23]. Tissue oxygen saturation was significantly lower (cerebral 71.0% vs. 74.9%; calf muscle 68.5% vs. 76.0%, p<0.001). Lower arterial and tissue saturation was not associated, however, with different tissue oxygen extraction (crFTOE 0.19 vs. 0.19, p=0.610; rFTOE 0.22 vs. 0.24, p=0.199).

Regarding cutaneous microcirculation data, in only two cases (one from Peru and one from Rotterdam) microcirculation data could not be analysed due to low

quality video imaging and thus both were excluded from further analysis. Regarding the remaining cases, the mean total vessel density (TVD) in the Peruvian babies born was 14% higher than that in the Rotterdam babies (Figure 2, upper right). Automated morphometric analysis revealed that both small and medium sized vessels (but not large ones) were significantly longer in the Peruvian newborns (Figure 2, lower part). To determine whether ancestry has an impact on increased microvascularisation in newborns at high altitude, TVD was calculated for the three ethnicity subgroups mentioned above: indigenous, mixed and Hispanic (n = 18, 6 and 19, respectively). No statistical differences in TVD were found between any two groups tested.

Lastly, multivariable linear regression analysis adjusted for possible confounding variables between countries showed no collinearity between the independent variables used in the model and normal distribution of the residuals. Table 2 shows the corresponding crude and adjusted differences for microcirculatory parameters; after adjustment the difference between the Peruvian and Rotterdam groups remained significant. Moreover, both the MFI and HI were not altered in either group.

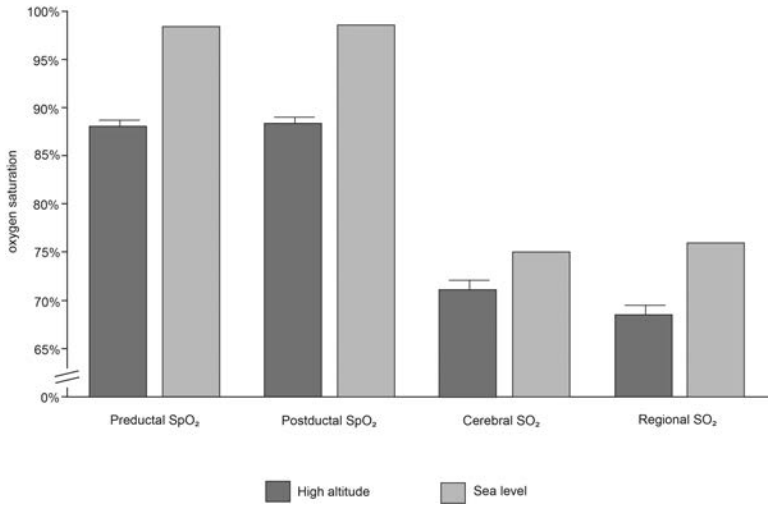

Figure 1. Pre- and post-ductal arterial saturation (SpO_2) as well as cerebral and skeletal calf muscle (regional) oxygen saturation (SO_2) measured at high altitude are compared to sea level reference values.

DISCUSSION

Reduced oxygenation of the placenta is linked to severe complications including intra-uterine growth retardation and preeclampsia [9,32,33]. Of note, despite reductions in systemic oxygen supply, such as occurs at high altitude, the foetus is able to cope with this extreme but still physiologic hypoxic condition. While many studies have addressed the hypoxic placenta's vascular remodelling and metabolic changes (reviewed in [32,34]), data on the

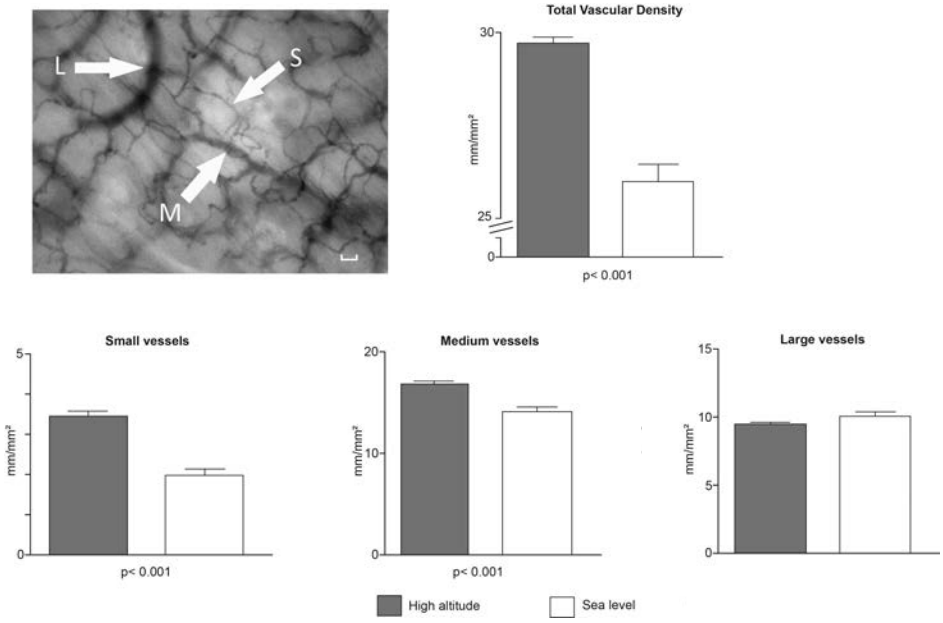

Figure 2. Imaging and morphometric analysis of vessel density of the skin from newborns at high and low altitude. The image in the upper left shows a representative single shot of the video images obtained by a CytoCam®. Small, Medium and Large vessels with Ø of <10, 10-20 and 20-100 µm, respectively, are labelled. The bar represents 25µm. Error bars: SEM.

Table 2. Crude and adjusted difference between Puno and Rotterdam for microcirculatory parameters

	Difference between high altitude and sea-level (95% CI)			
Variable	**Unadjusted difference (95% CI)**	**p-value**	**Adjusted* difference (95% CI)**	**p-value**
Total Vessel Density (mm/mm²)	3.67 (2.68 – 4.66)	<0.001	3.57 (2.37 – 4.77)	<0.001
TVD small (mm/mm²)	1.46 (1.02 – 1.91)	<0.001	1.14 (0.64 – 1.64)	<0.001
TVD medium (mm/mm²)	2.79 (1.91 – 3.66)	<0.001	3.08 (2.00 – 4.16)	<0.001
TVD large (mm/mm²)	-0.58 (-1.26 – 0.10)	0.129	-0.64 (-1.49 – 0.20)	0.132
MFI small (au)	-0.02 (-0.14 – 0.09)	0.688	-0.08 (-0.21 – 0.06)	0.261
MFI non-small (au)	0.03 (-0.04 – 0.09)	0.381	0.02 (-0.06 – 0.09)	0.646
HI small (au)	0.001 (-0.08 – 0.09)	0.854	0.02 (-0.08 – 0.13)	0.640
HI non-small (au)	0.03 (-0.04 – 0.09)	0.367	0.04 (-0.04 – 0.11)	0.368

mature foetus's adaptation to a hypoxic environment are scarce. The present study is the first, to our knowledge, to examine microvascular density in healthy term neonates born to mothers that were living at high altitude during pregnancy (3,840m). Our major finding was that their TVD was approximately 14% higher than in neonates born at sea level, pointing

towards a possible adaptive foetal strategy to cope with reduced oxygenation. In addition, we provide evidence that the increase in TVD was independent of the babies' ethnicity.

The microcirculation is defined as vessels equal to or smaller than 100μm in diameter that form the capillary network [35]. The above-mentioned difference in TVD was still significant when the crude data was adjusted for the following predefined, potentially confounding variables: country, gender, gestational age, birth weight, Apgar score (5min), mode of delivery, primigravida/multigravida, and rectal temperature. Increased vascularization was observed in small (Ø ≤10μm) and medium (Ø 10-20μm) vessels but not in larger ones. This implies that vessel density is only increased at the level of gas exchange (i.e. capillaries and small arterioles). In a study of healthy adults with no high altitude ancestry [36] a 10.9% increase in TVD was found in subjects first measured at sea level and thereafter at high altitude (5300m). Also, in preterm infants born small for gestational age, most often caused by more extreme hypoxic conditions, TVD was significantly higher soon after birth (van Elteren *et al.*, unpublished observations).

Considering that blood flow in the umbilical vein is reduced at high altitude [6] and that vascularization seems to be independent of ethnicity, it is plausible to speculate that enhanced microvascularisation is a general adaptive mechanism that might be induced by the PHD2-driven stabilization of the a-subunits of the hypoxia-inducible factors 1 and 2 (HIF-1 and HIF-2) (reviewed in [37,38]). In turn these heterodimeric regulatory transcription factors up regulate oxygen-dependent genes including those that trigger angiogenesis such as the vascular endothelial growth factor (VEGF) [32,39]. In contrast to the Andean population, evolution has selected a blunted erythropoietic response for Tibetans as an adaptive strategy to high altitude: a missense mutation in the EGLN1 gene encoding PHD2 results in lower O_2 affinity and increased HIFa degradation [40]. Thus, it would be of interest to determine TVD in healthy babies born to Tibetan mothers at high altitude. Apart from such mutations also epigenetic modifications may support adaptation to exposure to exogenous factors such as hypoxia, which can be inherited to next generations. As such, Julian *et al.* recently provided convincing evidence that unique DNA methylation patterns occur in genes known to influence vascular development and integrity in offspring of hypertensive pregnancies [41].

While the babies' heart rate at high altitude (mean 145, SD 13 n/min) did not deviate from published data, levels of haematocrit (0.57 vs. 0.49-0.50) and haemoglobin concentration (19.0 vs. 16.8 -17.1 g/dl) values in our Peruvian population were higher than those reported in a study performed at 3,600m [6]. We cannot explain this difference as the hospital in which our study was conducted was located only about 300m higher. Nevertheless, in the present study the flow-related parameters MFI and HI did not differ between the high-altitude and sea level groups despite a physiological higher haematocrit level in the high-altitude group. However, haematocrit values measured in arterial or venous blood differ greatly from haematocrit at a microcirculatory level. Known as tube haematocrit, it is significantly lower and highly variable in the presence of a constant systemic haematocrit [42]. Systemic haematocrit is therefore not correlated to viscosity and

blood flow at a microcirculatory level. Moreover, it should be noted that MFI values are often lower in disease states, especially in individuals suffering from septic shock [43].

Previously, a study on NIRS measurements in 24 children reported a significant decrease in cerebral tissue oxygen saturation on ascent from 1610m to 3109m (78% to 67%, p<0.001) [44]. In another study, reporting NIRS measurement in 17 children during emergency helicopter transport, NIRS decreased from 69.2% to 66.3% in patients transported to altitudes higher than 5000ft (1524m) above sea level [45]. Although these two studies measured the response to acute hypoxia, these observations are in line with our results showing that exposure to high altitude significantly lowers cerebral tissue oxygenation.

LIMITATIONS

Due to unforeseen administrative delays in Peru, measurements could not be performed in the local sea level control group, mainly represented by a Hispanic population. Therefore, measurements at high altitude were compared to sea level values either found in the literature (pulse oximeter and NIRS data) or by own data obtained from our Rotterdam cohort (determination of TVD). Although a control group of children born at sea level in Peru is also not completely comparable, the use of a Dutch control group might have introduced extra, unknown confounding factors. The number of participants in the referred studies exceeded the number of participants in our control group, thereby serving as a reliable comparison group unless ethnicity plays an important role. This issue was analysed and despite the fact that all four parental surnames of the neonates were not always obtained, it was possible to classify a significant number as indigenous (n=18) or Hispanic (n=19). Although classification of ethnicity by surname is not as precise as genetic analysis, this strategy first described and validated in 1989 [17], has been successfully applied recently [19,46]. Considering that elevated TVD was observed in all neonates independent of their ethnicity, we propose that comparison of our data obtained in neonates born at high altitude to sea level neonates from the literature is sound.

The automated computer IDF technology used for microcirculatory analysis has, just like its predecessor methods (sidestream darkfield imaging and orthogonal polarization spectral imaging), only been validated against its predecessor. However, given that the same method was used in both the Peruvian and the Rotterdam group, under supervision of the same experienced operator, any limitation of the software should be equally reflected in both groups. Thus, the data provided are comparable within this study but cannot be extrapolated to other studies.

To conclude, in this study, microvascular vessel density measured using IDF imaging was higher in babies born at high altitude than in babies born at sea level. Surname-inferred ancestry suggests that the observed TVD elevation is independent of the neonates' ethnicity. Neonatologists are often confronted with hypoxemia, and our observations open new doors for the diagnosis and treatment of the hypoxemic critically ill newborn.

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PART II

REPRODUCIBILITY OF MODERN DIAGNOSTIC TECHNIQUES USED IN CRITICAL CARE

CHAPTER 6

REPRODUCIBILITY OF MICROVASCULAR VESSEL DENSITY ANALYSIS IN SIDESTREAM DARK FIELD DERIVED IMAGES OF HEALTHY TERM NEWBORNS

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ABSTRACT

Objective

Microcirculatory visualization has already been used to investigate buccal and cutaneous microcirculatory alterations in neonates. Still, the reproducibility of these microvascular measurements has never been studied in (premature) neonates. This study aimed to determine reproducibility of the microvascular vessel density in cutaneous and buccal Sidestream Dark Field (SDF) clips in one-day old term newborns.

Methods

Buccal and cutaneous microcirculation was measured using SDF Imaging. Vessel density was independently assessed by two investigators. Reproducibility was assessed from the intra-class correlation coefficient (ICC) and Bland-Altman analysis.

Results

Reproducibility of vessel density assessment in the buccal area was good, with ICC's for total and perfused vessel density of 0.93 (0.88-0.97) and 0.93 (95%-CI 0.85-0.97), respectively, and a near zero bias and acceptable limits of agreement in the Bland-Altman analysis. Reproducibility of assessment of the cutaneous microcirculation was poor with ICCs for total and perfused vessel density of 0.31(0-0.70) and 0.37(0-0.74), respectively, and large biases (3.09 and 2.53) in the Bland-Altman analysis.

Conclusions

Evaluation of buccal microvascular vessel density in SDF derived images in term newborns is reproducible in contrary to the cutaneous vessel density.

Keywords: Reproducibility, Neonates, Sidestream dark field, Buccal, Cutaneous

INTRODUCTION

Orthogonal Polarization Spectral (OPS) imaging and its technically successor Sidestream Dark Field (SDF) imaging are non-invasive methods for directly visualizing a patient's microcirculation at the bedside. Critically ill patients may show altered microcirculation even when clinical parameters representing the systemic blood flow are in normal range. [17] The degree of microcirculatory distress may serve as an indicator of disease severity and has proven to be a predictor for poor outcome of adults and children. [3,5,17,21] The non-invasive character of the SDF imaging allows investigation of the microcirculation in any body surface with a thin cover of epithelium, even in case of severe critical illness.

SDF illuminates the tissue of interest using light at a wave length that is at the isobestic point of both oxy- and deoxy-hemoglobin (530-548 nm). The scattered light is reflected by the background and absorbed by hemoglobin regardless of the oxygenation status. The images are created by projecting the reflected light. [9,10] SDF images are analyzed offline total vessel density (TVD) and perfused vessel density (PVD). These analyses are semi-quantitative and thus subject to inter-observer variability.

In adults microcirculatory measurements are routinely performed in the sublingual area. In children -and particularly neonates- this is not feasible, however, and measurements are performed in the buccal mucosa. [3,20,21] Nevertheless, size-constraints do not allow for buccal measurements in preterm neonates; hence, transcutaneous measurements are performed. [7,11,13,25]

Microcirculatory visualization has already been used to investigate microcirculatory alterations in neonates. In term neonates with respiratory failure, it demonstrated PVD of the buccal microcirculation after inhaled nitric oxide and extracorporeal membrane oxygenation. [22,23] A study by Weidlich et al. investigating the cutaneous microcirculation found a decrease in PVD of preterm infants one day prior to infection. [25] Using SDF imaging Hiedl et al. found a decreased PVD in the cutaneous microcirculation of premature neonates with a significant persistent ductus arteriosus. [11] Still, the reproducibility of these cutaneous microvascular measurements has never been studied in (premature) neonates. It is also unknown if microcirculatory clips of both areas are comparable to one another or show any correlation.

The aims of this observational study were (i) to evaluate the reproducibility of the semi-quantitative analysis of buccal and cutaneous microcirculatory measurements in one-day old term newborns and (ii) to investigate if the vessel density analysis of the buccal and the cutaneous SDF clips show any correlation.

MATERIALS AND METHODS

This single-center prospective observational study was performed between April and May 2013 at the Obstetrics and Gynecology department of Erasmus MC – Sophia, a level III university children's hospital. Approval for this study was granted by the medical

ethical review board of this hospital. All healthy term newborns younger than 24 hours were eligible for participation in the study. Exclusion criteria were gestational age below 36 weeks or above 42 weeks, age over 24 hours, any known congenital, hematologic or cardiorespiratory disorder, the absence of written parental informed consent and failure to obtain high quality microcirculatory measurements. All neonates were born to mothers with a maternal indication or maternal request for hospital delivery.

Data collection

The buccal and cutaneous microcirculations were visualized using SDF imaging (Microscan™; Microvision Medical, Amsterdam, the Netherlands) according to the guidelines for optimal image acquisition. [6] All video sequences were acquired by the same researcher (EB) and recorded on DV-tape (Sony DSR-20P digital video recorder), and clips (avi-format) were digitized and stored. Blinded, randomized video sequences were analyzed offline using dedicated software (Automated Vascular Analysis 3.0, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands). Both the horizontal and the vertical pitch were calibrated using the SDF calibration files. In all participants, three measurements were obtained in both the buccal and the cutaneous sites, in this specific order. Before measuring the buccal microcirculation saliva was removed using gauze and the lens of the device was covered with a disposable sterile cap. To avoid pressure artefacts, the standard operating procedure as published by Trzeciak et al. was adhered. [24] Next, the cutaneous microcirculation was measured on the upper inner arm. This site contains little lanugo and is less prone to movement artifacts caused by breathing. [8] A drop of oil ensured better contact of the probe with the surface.

Quality of all clips was assessed, and those that did not meet the quality criteria were not included in the analysis. [6] For every clip, the TVD was estimated according to guidelines by dividing the amount of blood vessel crossings with three equidistant horizontal and vertical lines drawn on the screen with the total length of the lines. Blood vessels were considered perfused in case the flow was either hyperdynamic, continuous, or sluggish [6].

The clips were analyzed independently by two investigators (authors VvdB and HvE) with the software program AVA 3.0 (MicroVision Medical, Amsterdam, the Netherlands). They had been instructed by another author (EB), who is an experienced user of the SDF technique and the AVA software. First, a training phase was commenced during which VvdB and HvE independently analyzed 13 microcirculatory video clips that were obtained during another study. These results were discussed in detail. The training phase served to familiarize the two investigators with the methodology and the pitfalls of the analysis procedure. Thereafter, all clips obtained from the healthy newborns were analyzed and the reproducibility of the microcirculatory vessel density assessment was determined. Small vessels and non-small vessels were counted separately and the TVD and PVD were calculated. The cut-off value for the small vessels was 10 μm in diameter. [3,21,23] During analyses, the investigators were blinded for the order of the images.

The following demographic data were retrieved from the medical files: Gender, gestational age, birth weight, Apgar scores, rectal temperature and mode of delivery.

Statistical analysis

For each patient the mean TVD and PVD of three images per site were calculated. However, if clips were discarded due to poor image quality, the mean was calculated over the remaining clips. The inter-observer variability was determined by calculating the differences from the means of both authors and presenting these in a Bland Altman plot with 95% limits of agreement. [14] Also two-way mixed intra-class correlation coefficients (ICCs) for inter-observer reliability were calculated and are presented with 95% confidence intervals (CI). [19] This coefficient can vary between 0-1 and reliability is considered very good when ICC >0.81; good when between 0.61-0.80; fair to moderate when between 0.21-0.60; and poor when below 0.20. [1] Paired T-test and Pearson correlations were performed for both investigators individually to assess any correlation between vessel density analyses of the buccal and cutaneous SDF clips. Results were considered significant when $p<0.05$. Demographic data are presented with mean (SD) for normally distributed variables and median (range) for skewed distributed variables. Statistical analysis was performed using SPSS 21 (IBM Corp., Armonk, New York) and GraphPad Prism 5 (GraphPad Software Inc., La Jolla, California).

RESULTS

A total of 28 healthy term newborns were included. Eighty-four buccal and 84 cutaneous clips were recorded of which 14 buccal clips and 19 cutaneous clips were excluded because they did not meet the quality criteria. Three newborns were excluded for analyses of cutaneous inter-observer variability as all three cutaneous clips did not meet quality criteria. Examples of the buccal and cutaneous microcirculation are shown in Figure 1. Demographic data and the means of the results of the assessment of the vessel density from both authors are presented in Table 1 and Table 2, respectively.

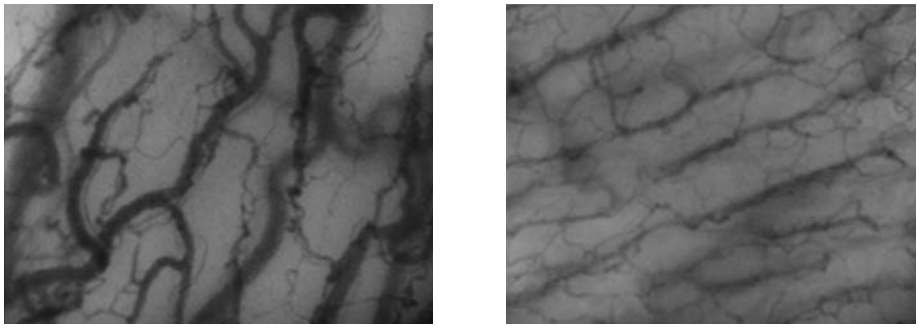

Figure 1. Stills from a buccal (left) and cutaneous (right) SDF-derived image.

Table 1. Baseline demographics

	Healthy term neonates (n=28)
Male sex [a]	13 (46.4)
Gestational age (weeks) [b]	39+3 (36+5 – 41+5)
Birth weight (grams) [b]	3483 (3070-3693)
Caesarian section as mode of delivery [a]	12 (38.7)
Temperature (°C) [b]	37.1 (37.0-37.3)
Apgar 1 minute [b]	9 (6 – 9)
Apgar 5 minute [b]	10 (8 – 10)

[a] number of subjects (%)
[b] median (range)

Table 2. Mean scores of buccal and cutaneous assessment of vessel density

	Buccal (n=28)		**Cutaneous (n=25)**	
	HvE	**VvdB**	**HvE**	**VvdB**
Crossing small [a]	41.9(14.1)	40.4(12.8)	76.3(8.2)	51.0(6.9)
Crossing nonsmall [a]	29.7(5.5)	31.6(6.1)	7.6(3.9)	16.6(4.3)
TVD [a]	13.7(2.4)	13.8(2.8)	15.7(1.6)	12.7(1.2)
PVD [a]	13.6(2.4)	13.6(2.7)	15.0(1.5)	12.4(1.2)

a = mean (SD)

Inter-observer variability

Table 3 presents the ICCs for interobserver variability in both the buccal and cutaneous microcirculatory parameters. The buccal ICC for total PVD and TVD were 0.93 (0.85-0.97) and 0.93 (0.88-0.97), respectively (excellent variability); the cutaneous ICC were 0.37 (0-0.74) and 0.31 (0-0.70), respectively (moderate variability).

Bland-Altman plots were created presenting the differences of the mean between the analyses of the buccal and cutaneous vessel density of both authors. An overview of biases and 95% limits of agreement is presented in Table 4. To visualize the relationship between the mean and the differences, the plots of the buccal and the cutaneous TVD and PVD are shown in Figure 2.

Relationship between the buccal and cutaneous microcirculation

For both investigators the paired t-test and Spearman correlation showed no significant relationship between the analysis of the buccal and the cutaneous SDF clips. Bland-Altman analyses and ICC calculations were not performed due to the lack of any correlation.

Table 3. Intra-class correlation coefficient for inter-observer variability

	Buccal (n=28) ICC (95%CI)	**Cutaneous(n=25)** ICC (95%CI)
Crossing small[a]	0.95(0.88-0.97)	0.13(0-0.44)
Crossing nonsmall[a]	0.70(0.36-0.86)	0.27(0-0.64)
TVD[a]	0.93(0.88-0.97)	0.31(0-0.70)
PVD [a]	0.93(0.85-0.97)	0.37(0-0.74)

a = mean (SD)

Table 4. Overview of Bland-Altman plots

	Bias (95% limits of agreement)	
	Buccal (n=28)	**Cutaneous (n=25)**
Crossing small[a]	1.5(-10.4 – 13.4)	25.3(-10.0 – 40.7)
Crossing nonsmall[a]	-2.0(-12.6 – 8.8)	-8.9(-16.81 – -1.1)
TVD[a]	-0.094(-2.8 – 2.6)	3.1(0.6 – 5.6)
PVD [a]	-0.005(-2.6 – 2.6)	2.5(0.2 – 4.9)

a = mean (SD)

DISCUSSION

This is the first study to evaluate the reproducibility of the analysis of microcirculatory data obtained in one-day-old neonates. Reproducibility was high for the buccal measurements, but moderate to poor for the transcutaneous measurements. Also, there was no significant relationship between the analysis of the buccal and the cutaneous SDF clips.

SDF images are non-invasively obtained, which makes it a promising technique to learn more about microcirculatory alterations in (pre-) term infants during their adaptation phase after birth or as a predictor of infection/ sepsis. Furthermore it helps understand microcirculatory changes after therapeutic interventions to improve cardiovascular performance. An essential requirement, however, is reproducible quantification of the recorded images. Our results validate the use of the TVD and PVD as a method of assessment of SDF clips in the buccal area in one-day old term infants.

Quality of the SDF microcirculatory clips of the skin was lower than that of the buccal clips, which might be the main reason for the difference in reproducibility of the buccal and cutaneous TVD and PVD. Even though the skin of the term infant is still developing and made up of a disorderly capillary network in which the capillaries are more horizontal orientated and more superficial than in the adult skin, [16] it is not thin enough for good-quality SDF imaging results. However, applying extra pressure

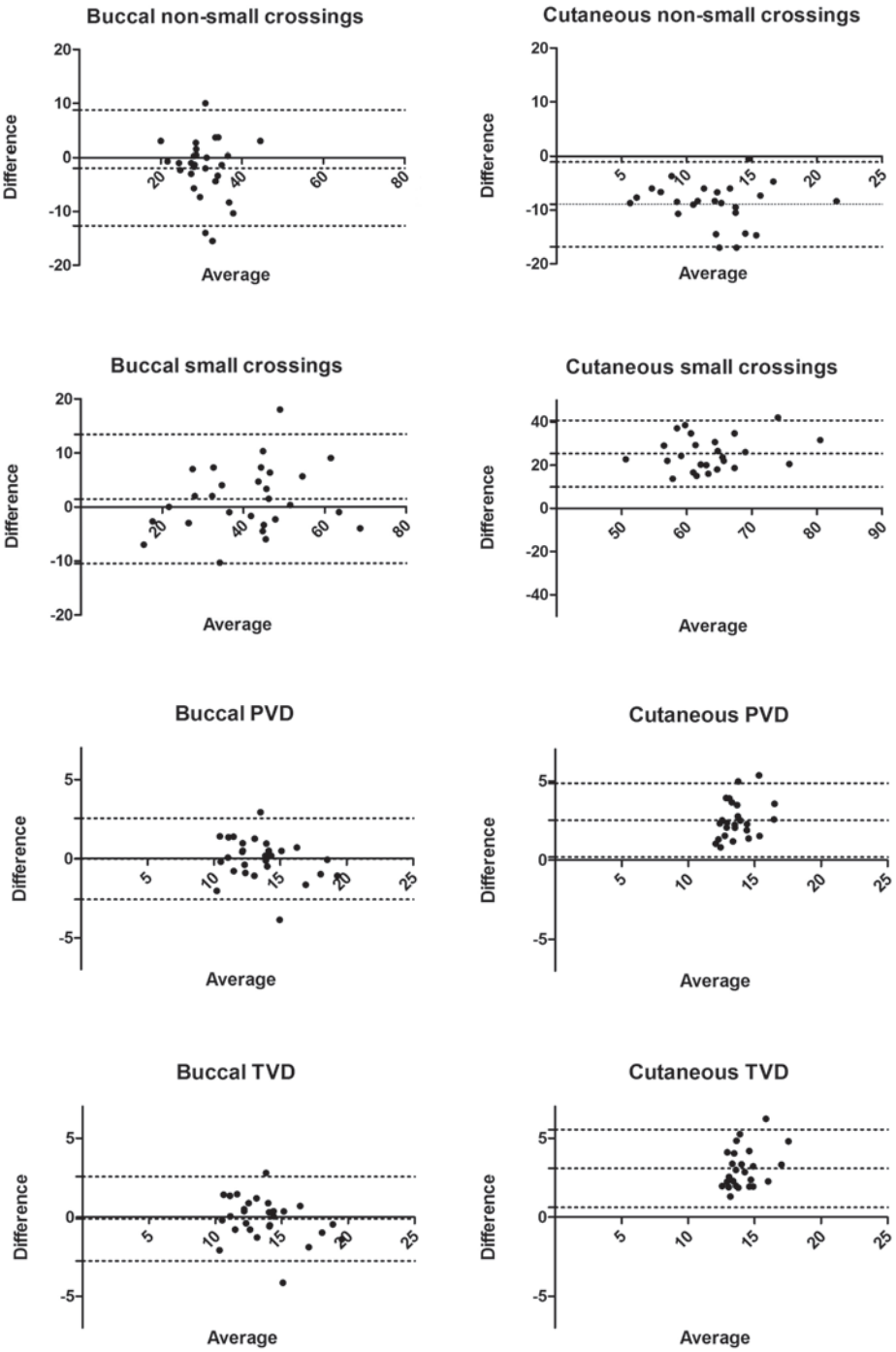

Figure 2. Bland-Altman plots of non-small crossings, small crossings, PVD and TVD of buccal and cutaneous microcirculation.

in order to look past the superficial skin cells would cause pressure artifacts and would also negatively affect the quality. In low quality clips, crossings and perfusion become more arbitrary and distinctions between vessels structures are harder to make. The lower quality of the cutaneous clips, with more extensive movement artifacts, could also be due to agitation in the newborns induced by the preceding buccal measurements. In our study interpretation of the cutaneous clips clearly differed between the two investigators: scores of one investigator (HvE) were consistently higher than those the other (VvdB). Such a difference in interpretation was not seen in the analysis of the buccal clips.

To our knowledge this is the first study reporting on interobserver reproducibility for the TVD and PVD in SDF clips using Bland-Altman plots and ICC in this patient group. Previous studies in healthy and (critically) ill adult patients have already established good inter-observer variability in the sublingual area. [4,5,12] A study by Buijs et al. reported ICCs ranging from 0.57 to 0.87 for microcirculatory assessment of buccal SDF clips in pediatric patients after cardiac arrest. However, when interobserver agreement is presented as ICC only, all information of the relation between the variability and the mean is lost which makes this method inferior to a Bland-Altman plot. No studies have reported on the inter-observer variability of the analysis of cutaneous SDF clips. Our study showed excellent ICCs and near zero bias for the analysis of the buccal measurement. For both the TVD and the PVD the difference between the two raters was less than one in 64.3% of all measurements. In contrast, the cutaneous measurements had poor ICC and a large bias and more scattering of the differences.

Even though the results of analysis of cutaneous SDF clips in healthy term infants using TVD and PVD are poorly reproducible, measuring the microcirculation of the skin might still be feasible in preterm infants. For this specific group of patients buccal measurements are impossible due to the size of the probe and lack of cooperation. Several studies have already been performed in this group using OPS and SDF and have reported the microcirculatory status by presenting the cutaneous TVD and PVD. [7,8,11,13,25] It can be argued that because of the even less far developed and thinner skin of the premature infants, the quality of the clips and thus the reproducibility of the analysis would drastically improve. However, until now the inter-observer variability has never established in this specific patient group.

The limitations of the OPS and SDF imaging devices, which can be regarded as first and second generation devices, respectively, include the use of relatively low resolution analogue camera technology and lenses of limited optical properties. These limitations can possibly explain the limited image quality found in the current study in the neonatal cutaneous microcirculation. Recently a new third generation device was introduced based on Incident Dark Field (IDF) imaging [18] incorporating improved optical lenses coupled to and a high resolution computer controlled imaging sensor [2]. In view of the expected future sophistication of automatic image analysis this new

generation hand held microscopes may perhaps overcome the limitation of SDF images of the cutaneous capillary network of the newborn we identified in the present study.

Our results showed no correlation in vessel density analysis of buccal and cutaneous SDF clips in healthy term newborns. This should be taken into account when interpreting neonatal microcirculatory studies. However since we only did a single microcirculatory measurement in every healthy newborn, we cannot conclude that no dynamic properties are shared between the two microcirculatory beds.

LIMITATIONS

Several limitations of this study should be addressed. Most importantly, the feasibility of SDF imaging is highest in capacitated/cooperative and/or non-awake/sedated subjects. [15] We included healthy neonates who could not be instructed and who were awake. As a result, the average length of the clips was 3 seconds (90 frames). We believe that the shorter frame length of the video's comes with microcirculatory research in non-cooperative (pre)term neonates. In the balance between frame length and stability, we choose for stability. Therefore all are videoclips are perfectly stable, but reduced in length compared to adult standards. However, 3 seconds does allow the investigator to accurately analyze the video clip for vessel density and perfusion of the vessels. Secondly, the clips were not analyzed for microvascular flow index (MFI) because this score is specifically designed for critically ill patients and not for healthy subjects. Future validation studies should include a heterogeneous patient cohort also including critically ill patients. Thirdly, of a total of 70 analyses of the buccal clips, seven needed to be extensively discussed before consensus on analysis method was reached. These clips were included in the study and thus create a bias. Nevertheless, the overall agreement in analysis of the buccal clips is of such a high level that even after the exclusion of these clips the ICCs for TVD and PVD are still 0.92 and 0.93 respectively. Finally, De Backer et al. recommended to regularly review the clips with several researchers in order to prevent drift in analyses. [6] In our study, we have only reviewed a total of 13 clips to create a consensus, after which first all buccal clips were analyzed randomly and then all cutaneous clips. However it is unlikely that the difference in the reproducibility of the buccal and cutaneous TVD and PVD are caused by a drift in analyses. A drift in analysis method would create subtle differences worsening over time while our results showed an abrupt difference in reproducibility between the buccal and cutaneous TVD and PVD. Following our own experiences, we recommend starting researchers to first discuss the microcirculatory analysis extensively with an experienced researcher. Hereafter, a minimum of 15 training video clips should be analyzed. During this training, there should be at least two moments of comparison with the experienced researcher.

In general, there can also be inter observer variability in the technique of acquiring the video clips. This issue does not apply to our study since all the video clips were

acquired by the same researcher. Although this issue seems inferior to the inter observer variability of the offline analysis, it is recommended that acquiring images should be performed by experienced researchers only.

CONCLUSION

For the evaluation of the vessel density of SDF derived images in term newborns younger than 24 hours, the semi-quantitative analysis using the TVD and PVD have high inter-observer reproducibility in the buccal area. The reproducibility of the assessment of the cutaneous microcirculation in this specific patient group turned out to be poor. There is no correlation between vessel density analysis of the buccal and the cutaneous SDF clips.

PERSPECTIVES

There has been growing interest in the buccal and cutaneous microcirculation as a potential biomarker in pediatric and neonatal critical care. The reproducibility of the buccal microcirculation was excellent, however the reproducibility of the cutaneous microcirculation turned out to be poor. Improved video quality and standardization of the offline analysis are urgently needed to overcome this issue.

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CHAPTER 7

PLETH VARIABILITY INDEX IN PRETERM INFANTS: IS IT FEASIBLE?

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ABSTRACT

Aim

To assess the reproducibility of the Pleth Variability Index (PVI), developed for non-invasive monitoring of peripheral perfusion, in preterm neonates below 32 weeks of gestational age.

Methods

Three PVI measurements were consecutively performed in stable, comfortable preterm neonates in the first 48 hours of life. On each occasion, pulse oximeter sensors were attached to two different limbs for five minutes. Reproducibility was assessed with the intra-class correlation coefficient (ICC) and Bland-Altman analysis.

Results

A total of 25 preterm neonates were included. Inter-limb comparison showed fair to moderate ICC's with 95%-confidence intervals (95%-CI). Left hand – right hand ICC= 0.504, 95%-CI (0.114 – 0.759); right foot – right hand 0.470 (0.71 – 0.740); right foot – left foot ICC=0.310 (-0.089 – 0.623). Intra-limb comparison showed fair to moderate ICC for right foot – right foot ICC=0.386 (-0.021 – 0.684); and good ICC for right hand – right hand 0.731 (0.455 – 0.879). Bland-Altman plots showed moderate reproducibility of measurements between different limbs and of the same limb in consecutive time periods, with large biases and a wide limits of agreement.

Conclusions

The findings from this study indicate that PVI measurement is poorly reproducible when measured on different limbs and on the same limbs in stable and comfortable preterm neonates.

Keywords: Microcirculation, Premature neonates, Pleth variability index, Reproducibility.

Keynotes:

- Pleth variability index (PVI) is a non-invasive parameter of peripheral perfusion and might be reliable to predict fluid responsiveness.
- PVI is poorly reproducible in stable and comfortable preterm neonates.
- PVI seems not to be feasible in preterm infants.

INTRODUCTION

The principal goal of fluid administration is to increase cardiac output without the accumulation of fluid, which would cause tissue edema. For this reason, a predictive index of fluid responsiveness would be useful. Some studies have demonstrated that the Pleth Variability Index (PVI) is reliable to predict fluid responsiveness in the operating room and the ICU (1-5). The PVI is a parameter based on the changes in the perfusion index (PI) during a complete respiratory cycle (6). It can be measured continuously by most Masimo pulse oximeters at the bedside and is calculated based on the difference between the lowest and highest PI (PVI = ((PI_{max} - PI_{min}) / PI_{max}) x 100%) (4).The PI is calculated by indexing the infrared pulsatile signal (AC) from the blood flow in the arterioles against the non-pulsatile signal (DC) absorbed by skin, other tissues, and non-pulsatile blood, and it expressed as a percentage (PI = (AC / DC) x 100%) (4).

Others, however, have shown that PVI is not suitable to predict fluid responsiveness in critically ill, hemodynamic unstable adult patients receiving norepinephrine (7). Due to physiological differences between newborns, children and adults – such as in chest, lung and arterial compliance (8, 9) – the predictive ability of PVI cannot be extrapolated directly from adults to infants or (preterm) neonates. Studies evaluating the effectiveness of PVI to predict fluid responsiveness in children reported contradictory results. Three studies in mechanical ventilated children found a significant difference in PVI between responders and non-responders based on an increase in stroke volume index (SVI) (10-12). Yet another study in mechanical ventilated children showed no predictive relation between PVI and fluid responsiveness (13). All above-mentioned studies investigated the usefulness of PVI to predict fluid responsiveness in children beyond neonatal age. One pilot study showed that PVI might be an useful indicator of volume-response hypotension in newborn infants during surgery (14).

Since preterm infants regularly are administered fluids as treatment for hypotension, PVI is of interest for monitoring their fluid management in the neonatal intensive care unit (NICU) setting. Accumulation of fluid in tissue would cause tissue edema, in particular in the lungs, and would influence closure of the ductus arteriosus and necessitate prolonged mechanical ventilation (15). If PVI is found to be able to predict fluid responsiveness, it could be a valuable non-invasive device in this population. To determine its usefulness in the NICU setting, it is important to prove that the PVI is reproducible in patients who are not sedated or ventilated, because previous studies were performed in mechanically ventilated and sedated patients.

Therefore the aim of this study was to assess the reproducibility of the PVI measurement on the same limb and on two different limbs in preterm infants younger than 32 weeks of gestational age (GA).

METHODS

The study protocol was approved by the medical ethical review board of the Erasmus Medical Center and written informed consent was given by all parents. Newborns between 26 and 32 weeks of gestational age, younger than 48 hours after birth and admitted to the NICU of the Erasmus MC - Sophia Children's hospital were eligible for this study. Patients with any known cardiac or chromosomal defect were excluded from this study.

Study procedures

To calculate the PVI, two Masimo Radical 7 pulse oximeters (Masimo Corp., Irvine, CA, USA) with NeoPt Softtouch sensors were connected to a laptop. Three five-minute measurements were performed. On each occasion, two different limbs were each fitted with a pulse oximeter sensor. The first measurement compared right wrist vs. left wrist. The second measurement compared right wrist vs. right foot. The third measurement compared right foot vs. left foot. If a sensor could not be attached to the wrist because a peripheral intravenous or arterial catheter was in place, it was attached to the palm of the hand. For all study subjects, sensors were placed by the same operator. Recording of data started when a clear PVI signal was obtained within five minutes after the sensors were placed. PVI values were recorded with a frequency of 1 per second for a total of five minutes, resulting in 300 data points per measurement. Since "0" is not an actual PVI value but a representation of a poor signal or calculation of PVI, all data points with a value of "0" were discarded. The mean of the PVI values per measurement was calculated over the remaining values.

Pulse oximeter derived variables such as heart rate, oxygen saturation and PI were synchronously recorded. Since PVI is influenced by behavioral status, all measurements were conducted while the neonates were quiet and comfortable. Furthermore, they were left undisturbed during the measurements. Changes in behavioral or circulatory status were reported (16).

The following baseline characteristics were retrieved: gestational age (GA), birth weight, sex, mode of delivery, type of ventilation, age at start of measurement, and Apgar scores.

Statistical analysis

Differences in means of the PVI inter and intra limb were assessed with paired t-tests. Reproducibility of the mean PVI per sensor side was assessed with two-way mixed intra-class correlation coefficients (ICC) (17). This coefficient can vary between 0-1.0, with 1.0 reflecting perfect agreement and 0 no agreement between the measurements. Reproducibility is considered very good at an ICC > 0.80; good at 0.61-0.80; fair to moderate at 0.20-0.60; and poor if below 0.20 (18). For further analysis of the reproducibility Bland-Altman plots with bias and 95% limits of agreement were created by calculating the differences between the means of measurements (19).

To asses if the average PVI of 300 PVI values per measurement influences the reproducibility of the PVI, Bland-Altman analyses of single PVI values for one measurement per patient (left foot – right foot) were made.

Continuous data are presented as median and range for non-normally distributed variables and as mean and standard deviation (SD) for normally distributed parameters. Non-continuous variables are presented as number of events and percentages of total.

Statistical analysis was performed using SPSS21 (IBM Corp., Armonk, New York) and Prism 5 (GraphPad Software Inc., La Jolla, California).

RESULTS

A total of 25 preterm newborns were included between March 2014 and August 2014. Their background characteristics are presented in Table 1. Two infants were intubated and received synchronized intermitted mandatory ventilation (SIMV) during data collection; 21 infants required nasal continuous positive airway pressure (CPAP) or nasal intermittent positive pressure ventilation (NIPPV); and two infants received respiratory support from a nasal cannula without positive end expiratory pressure (PEEP).

Table 1. Background characteristics of the 25 included preterm neonates

Male sex [a]	9 (36%)
Gestation Age (weeks) [b]	30 0/7 (27 2/7 – 31 5/7)
Birth weight (grams) [b]	1175 (430 – 1910)
Apgar score 1 minute [b]	6 (2 – 9)
Apgar score 5 minutes [b]	8 (1 – 10)
Twins [a]	10 (40%)
Caesarian section [a]	17 (68%)
Age at start measurement (hours) [c]	32 (10)
Type of ventilation	
SIMV [a]	2 (8%)
CPAP or NIPPV [a]	21 (84%)
Nasal cannula without PEEP [a]	2 (8%)

[a] number of subjects (%)
[b] median (range)
[c] mean (SD)

Observations

In total 72 measurements were performed in 25 children. For 21 neonates all three measurements were successful. Three measurements could not be performed due to an arterial line or peripheral catheter at the intended site of the sensor. During six individual measurements the neonate was described as restless and during 66 measurements the neonates were quiet or a sleep. The manufacturer of the Masimo sensors describes

that PVI will be calculated after two minutes if a clear plethysmographic waveform is displayed. However, in the majority of cases it took over five minutes to calculate PVI at the start of the measurement. During twelve measurements, (in nine neonates; 36% of all patients), PVI was not calculated for a brief period, i.e. a mean of 85 seconds, with a maximum of 163 seconds. During these periods no changes in behavioral status were seen and PI was presented for the whole period.

Reproducibility

Eighteen hundred PVI data points were recorded for every patient. For each sensor side the average PVI during 300 seconds was calculated. In total five comparisons were made to determine reproducibility of the PVI. First, three inter-limb comparisons: left hand (LH) – right hand (RH), right hand (RH) – right foot (RF) and right foot (RF) - left foot (LF). Second, to determine a difference over time on the same limb, two intra-limb comparisons were made: right hand (RH) – right hand (RH) and right foot (RF) – right foot (RF). Paired t-tests showed significant differences for two of the comparisons, i.e. inter-limb RF - RH (RF=19.3, RH=27.7, $p < 0.001$) and intra-limb RH – RH (RH1= 22.9, RH2=27.7, p=0.002).

Inter-limb comparison showed fair to moderate ICC for left hand – right hand, right foot – right hand and right foot – left foot. Intra-limb comparison showed fair to moderate ICC for right foot – right and good ICC for right hand – right hand 0.731. Table 2 gives an overview of the paired t test p value and ICC per comparison.

Table 2. Paired T tests and Intra class correlation coefficient inter limb and intra limb measurements

Measurement	Paired t-test	ICC (95% confidence interval of the difference)
Inter-limb		
LH – RH (n =22)	p =0.234	0.504 (0.114 – 0.759)
RF – RH (n =22)	p =0.000	0.470 (0.710 – 0.740)
RF – LF (n =25)	p =0.515	0.310 (-0.089 – 0.623)
Intra-limb		
RF – RF (n =23)	p =0.222	0.386 (-0.021 – 0.684)
RH – RH (n =22)	p = 0.002	0.731 (0.455 – 0.879)

To visualize the reproducibility of PVI and to assess bias between the measurements, Bland-Altman plots and 95% limits of agreement were created. Figure 1 shows Bland-Altman plots of inter-limb measurements; Figure 2 shows Bland-Altman plots of intra-limb measurements.

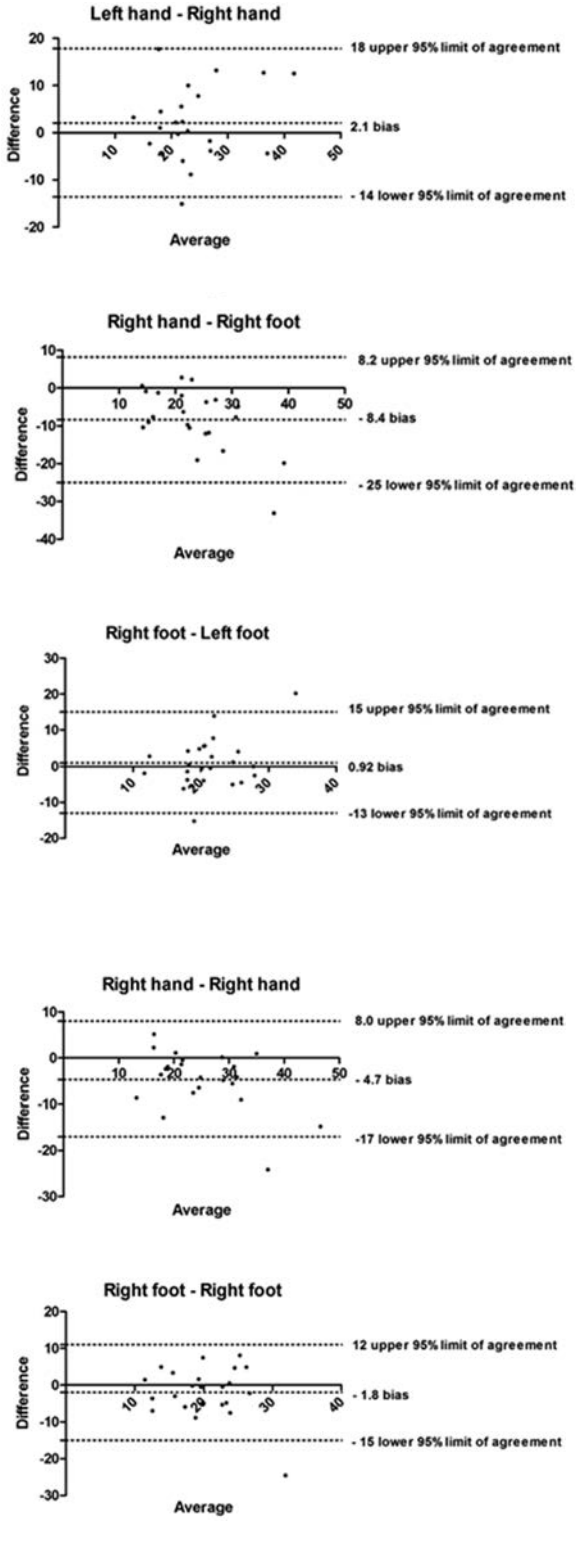

Figure 1. Bland-Altman plots showing the agreement of inter-limb measurments.

Figure 2. Bland-Altman plots showing the agreement of intra-limb measurements.

For one type of measurement (LF – RF), Bland-Altman plots for all 25 patients were created to asses if there was a difference with the average PVI value and to asses if there is a bias by calculating the average PVI of the 300 values per measurement (Table 3). Only six Bland-Altman plots of the feet had a bias lower than 1.5 and almost all measurements had a wide spread of 95% limits of agreement.

Table 3. Agreement of PVI-measurement comparing left foot – right foot in individual patients; results of Bland-Altman analysis

Patient no.	N (amount of seconds with PVI result)	Bias (PVI value)	95% limits of agreement (PVI value)
1	266	-5.1	-12 – 1.5
2	257	7.7	4.7 – 11
3	258	4.9	-2.2 – 12
4	266	-20	-29 – -11
5	260	-5.4	-8.9 – -1.9
6	253	2.8	-1.7 – 7.3
7	189	2.8	-0.19 – 5.7
8	282	0.43	-4.8 – 5.7
9	264	4.3	-3.8 – 12
10	255	-0.02	-3.1 – 3.1
11	249	-2.7	-19 - 13
12	184	14	14 – 14
13	271	-15	-31 – 0.29
14	261	-4.8	-14 – 4.8
15	249	-3.8	-20 – 12
16	258	1.3	-4.1 – 6.8
17	246	-5.9	-13 – 1.1
18	256	-2.9	-7.1 – 3.2
19	259	5.8	-0.27 – 12
20	260	-4.4	-8.1 – -0.77
21	264	-0.40	-3.2 – 2.4
22	254	-0.35	-4.0 – 3.3
23	257	5.4	-1.1 – 12
24	258	-1.1	-4.4 – 2.2
25	243	-3.7	-5.7 – 1.7

DISCUSSION

To the best of our knowledge, this is the first reproducibility study of PVI in preterm neonates. The reproducibility of PVI was found to be fair to moderate during the first 48 hours of life of these circulatory stable preterm neonates between 26 and 32 weeks of GA. For two comparisons (RF – RH and RH – RH) a paired t-test showed significant differences between measurements, and therefore t-test only shows that these measurements are not reproducible. For the other three comparisons, an ICC between 0.31-0.50 (fair to moderate) was found with wide 95% confidence intervals. Similarly, the Bland-Altman analysis showed a poor reproducibility of the PVI with large biases and wide limits of agreement. The Bland-Altman analysis of single PVI values of one measurement did not show better results.

PVI reproducibility in neonates has not been studied before, although three studies of PVI measurements in neonates have been published (14, 16, 20). Latini et al. established reference values for 242 spontaneously breathing term neonates; the median PVI value was 20 (19-20) (16). Vidal et al. included 56 newborns below 29 weeks of gestational age and established a median PVI value of 22 (18-27) (20). Bagci et al showed in a pilot study that PVI might be an useful indicator of volume-response hypotension in newborn infants during surgery (14). It is unfortunate that two studies did not indicate whether the reported PVI values are a mean of several data points or just a value on one point (16, 20). The mean PVI values per measurement of our study were in the same range as found by both studies (16, 20). However, in the study of Vidal et al., PVI was measured on either foot, whereas we did not find reproducibility of PVI on the foot.

Besides the fact that PVI was not found to be reproducible some other observations made during the study period are worth mentioning. Even though other parameters were stable and behavioral status did not change, our results are consistent with a wide range of PVI values measured in 300 seconds. Studies in adults noted that PVI could predict fluid responsiveness in mechanically ventilated hemodynamic stable patients in the operating theatre (3, 4, 6). Since we found such wide range of values in 300 PVI values per measurement per patient, it seems that PVI is not useful as an indicator for fluid responsiveness in preterm neonates.

Another observation was that in the majority of the cases it took more than five minutes before PVI was calculated by the Masimo pulse oximeter, and in nine (36%) neonates the PVI was not calculated for a mean of 85 seconds, with a maximum of 163 seconds. Since all other parameters (heart rate, oxygen saturation and PI) showed uninterrupted data, and a normal plethysmographic waveform was displayed, we cannot explain this observation. Latini et al. showed that PVI was significantly influenced by the behavioral status (16). We hypothesized that a change in behavioral status could cause the loss of PVI, but behavioral status did not change during the short periods when PVI was not calculated.

Kinoshita et al. evaluated the reproducibility of PI in thirty preterm infants younger than 32 weeks of gestational age. They concluded that PI was highly reproducible (ICC 0.982) on the same limb and between limbs (21). Since the calculation of the PVI is primarily based on the PI we expected that PVI would be reproducible in preterm neonates. To compare our results with those found by Kinoshita et al., and to understand why we failed to show good reproducibility in PVI measurement, we performed post-hoc analysis of the PI as well. Similar to Kinoshita et al., reproducibility of PI on the same limb was found to be good (RH – RH ICC 0.759 (95% CI 0.504 -0.893) and RF – RF ICC 0.836 0.651 – 0.927) and Bland-Altman plots showed very small biases and 95% limits of agreement. Taking the formula into account (PVI = $((PI_{max} - PI_{min}) / PI_{max})$ x 100%), we cannot explain why PVI in this study is not reproducible on the same limb even when PI is reproducible.

Several limitations of this study should be addressed. The sample size of this study was relatively small with a total of 25 patients. However, both the poor ICCs and the wide spread in the Bland-Altman plots suggest that larger samples will not necessarily demonstrate better reproducibility. A second limitation is that we could not measure at the exact same place and time. However, this is the reflection of clinical practice and since behavioral and circulatory states of the patient (heart rate and oxygen saturation) did not change in five minutes, the hypothesis was that the PVI would be similar during these measurements. A final limitation is that PVI values are compared as a mean of 300 values. When the average of 300 values is calculated a lot of information will be lost. Still, Bland-Altman plots of single PVI values of one measurement LH – RH per patient showed that the majority of the measurements had a bias > 1.5 and wide spread of 95% limits of agreement and therefore were not reproducible as well.

In conclusion, literature showed that PVI could be a valuable noninvasive parameter to predict fluid responsiveness for premature neonates. This study demonstrates that measurement of the PVI is not reproducible in stable and comfortable preterm neonates, and is thus not feasible in this population.

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CHAPTER 8

REPRODUCIBILITYOF LASER DOPPLER SPECTROSCOPY MEASURED BY O2C IN HEALTHY TERM NEONATES

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(To be submitted)

ABSTRACT

Background

The O2C (LEA, Germany) is a device that non-invasively measures a variety of hemodynamic microcirculatory parameters. The aim of this study has been to investigate its reproducibility both within the same as well as between limbs in healthy term neonates within their first 72 hours of life.

Methods

Subjects were collected from the maternity ward of the Erasmus MC. Sensors of the O2C were placed onthe skin of upper arm and calve.The device was allowed to measure four microcirculatory parameters (venous oxygen saturation (SvO_2), relative hemoglobin (rHb), blood velocity, and blood flow). Inter-, and intra-limb comparisons were made using paired t-tests and intra class correlation coefficients (ICC's).

Results

A number of 26 participants were included into the study. Paired t-tests indicated significant differences between the several inter-limb variables. These values were generally supported by their low respective ICC's. Moreover, great differences in normal range values were observed between participants.

Discussion

Intra-limb reproducibility was generally good compared to inter-limb comparisons. These differences may be explained by several anatomical and physiological factors. The results indicate that the O2C device would be more practical when used within the same patient at the same site.

INTRODUCTION

Despite technological advances, hematological and hemodynamic parameters remain difficult to assess in newborn infants. Most diagnostic tools used in adult patients are too large, too painful, or too invasive for neonates. Moreover, techniques involving the collection of blood cause distress to both baby and parents, and are a leading cause for iatrogenic anemia in small children[1]. It is for reasons like these that pediatricians and other healthcare workers in the field of neonatology are often forced to rely on less optimal and insensitive techniques. Changes in heart rate, blood pressure, urine output, lactate and pH are commonly used instruments to assess circulatory state, but may not be adequately reliable[2,3]. With the right instruments of measurement, however, the hematological system may function as an important predictor for complications, as a screening tool, or to determine the most effective treatment in the newborn infant[4,5].

Several techniques have emerged that aim at analyzing a variety of hemodynamic parameters in a non-invasive manner. Especially the examination of the microcirculation has been substantially facilitated[6,7]. Laser Doppler flowmetry is a commonly used technique in the field of neonatology that has contributed to this improvement. It relies on the use of a near-infrared laser ray, which is emitted and scattered in tissue. A Doppler shift of the reflected light is detected by the probe followed by a calculation of the velocity and the number of passing erythrocytes. This allows for the microcirculatory venous blood flow to be determined[8]. Another example of a similar technique can be found in white light spectroscopy. In this procedure, light at a wavelength of 450-850 nm is emitted. In strongly perfused tissue, a great amount of hemoglobin will be present. Since hemoglobin is the strongest absorbing agent of light, the machine is able to calculate its relative presence by the amount of reflected light. Moreover, since oxygenated hemoglobin will have a specific reflectance peak, the percentage of oxyhemoglobin can additionally be determined[9]. Recently, the Oxygen 2 See device (O2C, LEA Medizintechnik, Giessen, Germany) was manufactured, which combines the two aforementioned techniques, and allows for the continuous, non-invasive measurement of these variables in the microcirculation. Due to the fact that 80% of the erythrocytes are located in the venous capillaries, an area-specific microcirculatory analysis can be made[10]. Examples of the device's application include the management of burn wounds[11], diabetes[12,13], and free flap monitoring[14-17].

Because of its non-invasive nature, the O2C may significantly contribute to the monitoring of (pre-term) neonates. Microcirculatory changes may, for instance, predict sepsis[18,19] and negative effects associated with a patent ductus arteriosus[20]. Furthermore, due to the standard probe separation (3 mm.), it is possible to only investigate the superficial skin layers, thereby analyzing a homogenous area. The O2C was proven to be both valid[21] and reliable for this purpose in adults, with an average of 5% intra-subject variability[22]. Differences between adults and neonates however, create the necessity to investigate whether the device's output is reproducible. For example, neonates are non-cooperative and move spontaneously. This could potentially

create artifacts in the measurements. The primary aim of this study was therefore to determine the reproducibility of the O2C device between, and within the same limbs of healthy term neonates.

METHODS AND MATERIALS

The study protocol was approved by the medical ethical review board of the Erasmus MC - Sophia Children's Hospital in Rotterdam. Written informed consent was given by all parents before the start of the measurements. This study was carried out over a period of 3 months, and subjects were collected from the maternity ward of the Erasmus MC. Subjects were between 37 and 42 weeks of gestational age, and all were considered healthy. Patients with any known cardiac, hematological or chromosomal defects were excluded from participation. Measurements were performed during the first 72 hours of life.

Procedures

Measurements were performed with the O2C device. Four variables were derived by the machine: venous oxygen saturation (SvO_2), relative quantity of hemoglobin (rHb), velocity of erythrocytes, and flow of blood. All variables were presented by the device in Arbitrary Units (AU), except for oxygen saturation (presented as percentage of oxygenated hemoglobin of total hemoglobin). All subjects were preferably asleep and fed before the procedure so that they would be quiet and comfortable. Two identical sensors were attached on the skin with double-sided adhesive tape. In case of excessive movement of the subject, a Tegaderm (skin protective transparent plaster) was used to fix the cord of the sensor without the application of any pressure on the sensor itself. One operator conducted two consecutive measurements. During the first measurement (T1), sensor one was attached to the infant's right upper arm (RA1), and sensor two to the left upper arm (LA1). During the second measurement, sensor one remained in the same place (RA2), while sensor two was moved to the calf of the right leg (RL2). The recording of data was initiated after the device had stabilized within the first minute. 180 data points were collected during three minutes of measurement. Unrepresentative data points as a result of poor signal quality were excluded; a mean was calculated from the remaining scores. Movement artifacts were avoided; any change in behavioral status that could influence measurements was reported. The following baseline characteristics were retrieved from the newborn: date of birth, time of birth, birth weight, sex, GA (Gestational Age), rectal temperature at the time of measurement, Apgar score, mode of delivery, cord blood pH and base excess.

Statistical Analysis

Differences in means of all parameters between limbs were compared using paired t-tests. If background statistics appeared to be normally distributed, they were presented using the mean and standard deviation. Median and range were used for non-normally

distributed values. Reproducibility between measurements was measured by a two way mixed intra-class correlation coefficient (ICC)[23]. Values for this coefficient could vary between 0-1, with 1.0 reflecting perfect agreement and 0 no agreement at all. Reliability is considered very good at an ICC > 0.80; good at 0.61-0.80; fair to moderate at 0.20-0.60; and poor if below 0.20[24]. Statistical analysis was performed using SPSS21 (IBM Corp., Armonk, New York). To visualize the reproducibility of the measurements, Bland-Altman plots with deduced biases and 95% limits of agreement were created by calculating the differences of the means between measurements[25].

RESULTS

A total of 26 neonates participated in this study over a period of three months. Clinical characteristics of all participants are presented in Table 1 in the appendix. Gestational age and the male/female ratio are in line with expected values for healthy term neonates. In two out of 26 patients, data collection was incomplete due to difficulties with the device. An average was taken of the remaining data points. In as much as 18 children (69%) behavioral status at some point during the measurements was described as 'restless' or 'crying'.

Table 1. Background statistics of participants

	n=26
Male gender[a]	12 (46%)
Gestational age[b]	39 2/7 (37 2/7 - 41 2/7)
Birth weight[c]	3370 (540)
Apgar score 1 minute[b]	9 (3-9)
Apgar score 5 minutes[b]	10 (5-10)
Apgar score 10 minutes[b]	10 (6-10)
Cord blood pH[c]	7,4 (0,6)
Base excess[c]	-3,4 (3,6)
Twins[a]	4 (15%)
Ceasarian section[a]	13 (50%)

[a] part presented as percentage of total
[b] median and range
[c] average and standard deviation

Three comparisons were made with the obtained data. The sensor on the right arm performed two measurements at different times and compared these (RA_1-RA_2). The data from the right arm was also compared to both the left arm during the first timeslot (RA_1-LA), and right leg during the second (RA_2-RL). Medians and ranges for all parameters at all sites can be found in Table 2. paired t-tests were carried out to

assess correlations between measurements, and more specifically between variables. A significance level of α=0,05 was chosen as statistically significant. Second, the ICC coefficient was determined in order to establish correlations between measurements. This resulted in twelve comparisons, the results of which can be found in Table 3.

Two comparisons appeared to be significantly different (rHb , RA_1 - LA , p=0.024 and velocity , RA_2 – RL , p=0.034). These outcomes are supported by their ICC's, 0.322 and -0.201 respectively, both indicating moderate to poor reliability. Other poor ICC values were found in two inter-limb comparisons (SvO_2 , RA_2 – RL , ICC=0.026 and rHb , RA_2 – RL , ICC=0.146). Some variables display a discrepancy between the outcomes of the t-tests and their respective ICC's, demonstrating the significance of using multiple ways to establish reproducibility. Figure 1 displays the Bland-Altman plot for the RA^2-RL SvO_2 measurement. The ICC value for this comparison was considered poor (0.026),. As

Table 2. Median and range of variables at two timeslots and between sites

		Flow (Au)	SvO_2 (%)	Velocity (Au)	rHb (Au)
T1	Right arm	65.0 (32.2 – 191.8)	73.0 (53.0 – 95.6)	21.3 (17.6 – 33.0)	98.5 (77.2 – 110.4)
	Left arm	87.1 (37.8 – 166.3)	79.8 (46.7 – 96.6)	21.8 (17.3 – 27.3)	102.4 (81.2 – 116.3)
T2	Right arm	70.8 (31.5 – 200.0)	73.7 (49.8 – 97.0)	22.2 (16.3 – 34.0)	95.5 (78.4 – 110.3)
	Right leg	92.0 (21.3 – 145.1)	66.5 (50.9 – 94.5)	19.2 (16.5 – 32.2)	100.3 (57.1 – 111.1)

Table 3. Paired t-test and intra class correlation results

	Variable	Paired t-test	ICC (95% confidence interval)
Right arm (T1)	Flow	.915	.675 (.367 - .486)
vs.	SvO_2	.527	.460 (.093 - .716)
Right arm (T2)	Velocity	.961	.846 (.676 - .931)
	rHb	.480	.584 (.262 - .789)
Right arm (T1)	Flow	.176	.433 (.061 - .704)
vs.	SvO_2	.090	.395 (.032 – 673)
Left arm (T1)	Velocity	.723	.260 (-.166 - .598)
	rHb	.024	.322 (-.028 - .614)
Right arm (T2)	Flow	.650	.211 (-.196 - .552)
vs	SvO_2	.396	.026 (-.371 -.412)
Right leg (T2)	Velocity	.034	-.201 (-.492 - .164)
	rHb	.880	.146 (-.266 - .505)

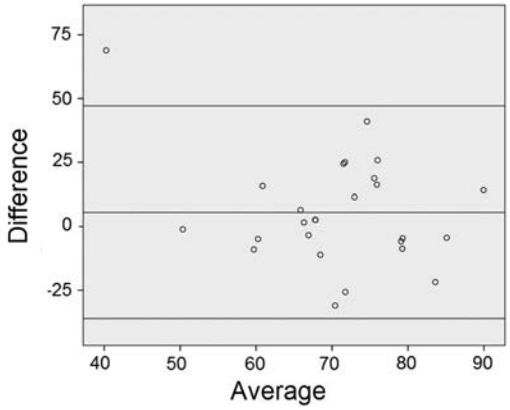

Figure 1. Bland-Altman plot indicating the scatter of participants of the RA^2-RL SvO_2 comparison.

the mean of the two measurements gets larger, the difference between the values does the same. This indicates that there is a consistent bias, thus reflecting the low ICC values.

DISCUSSION

Reproducibility should play an important role in all scientific research. A recent study that investigated the repeatability of 100 psychological experiments, however, indicated that in only 36% results were comparable[26]. This provides a strong illustration for the importance of thorough reproducibility research. To the best of our knowledge, this is the first reproducibility study of laser doppler spectroscopy in term newborns. Other fields in which research groups have investigated the O2C in pediatric medicine include testicular surgery[9,27,28], craniosynostosis surgery[29], and patients suffering from alveolar cleft gingiva[30]. Children in these studies were however older and more cooperative than our population of term newborns. Furthermore, measurements were mostly taken from organs other than the skin.

The obtained data sets point towards several conclusions. Firstly, intra-limb measurements display a high level of repeatability. Especially the Doppler flowmetry variables flow and velocity indicate highly reproducible values (P=0,915 , ICC=0,675 and P=961 , ICC=0,846 respectively). This means that repeated measurements within the same patient at the same site can be considered trustworthy. In contrast, lower reproducibility scores were obtained for most variables between limbs. According to the definitions established under *Methods,* RA_1-LA comparisons should be considered moderately reproducible, and RA_2-RL comparisons poor to moderate.

One study by Fredly *et al.*[31], has studied the differences between sites of laser Doppler perfusion- and diffuse reflectance spectroscopy measurements among healthy-term newborns. In this study, the chest region was compared to the left hand. The measured

variables in this experiment are comparable to those of the O2C. Laser Doppler measurements, which were expressed as blood flux, indicated significantly higher values in the chest ($P<0.001$). According to the authors, higher temperatures in this region could explain such a discrepancy. Due to our fixed superficial probe separation, we believe not to have experienced any heat artifacts as these only come from deeper regions. The spectroscopic measurements, defined as microvascular erythrocyte oxygen saturation, were also higher in the chest region in comparison to the hand ($P<0.05$). This could mean that microvascular architecture differs between the two sites of measurement. The results of this study are in line with the obtained data; there appear to be significant differences between sites of measurement. As laser Doppler perfusion measurements are presented in arbitrary units, a comparison between the two studies is difficult to establish. Moreover, even though a comparison was made between two different regions, we expect there to be other, physiologically explainable differences between our sites of measurement and those chosen by Fredly *et al.* During the transition from the fetal to neonatal circulation, the ductus arteriosus, and the foramen ovale only close several hours to days after birth[32]. This anatomical difference between newborn babies and older infants could result in a discrepant data set. A left-to-right shunt may have contributed to higher oxygen values in the right arm in comparison to the right leg or left arm[33]. This explanation is in line with the data sets we obtained. Inter-limb comparisons indicated significant differences whereas most intra-limb analyses did not.

No previous studies have reported reference values for term neonates of microcirculatory variables measured by O2C. Compared to reference values in adults, provided by the manufacturer, there are large differences (Table 4). This can be explained by the aforementioned anatomical and physiological differences between adults and neonates. A second element that may have caused these different values is excessive or sudden movement. As can be physiologically explained, small movements may already increase the flow of blood to muscles, or result in lower hemoglobin values. Movement may therefore have played a role in the wide ranges of normal values that we obtained. It is for these reasons that we were not able to establish any normal range values for neonates.

These observations in combination with the obtained correlations indicate that the O2C is most useful when it is used within the same patient at the same site. It can therefore be argued that it would be more useful as a means to repeatedly monitor the microcirculation instead of being used as a population-wide screening method for possible complications.

Table 4. Adult normal range values vs. extreme neonatal values

	Adults	**Neonates**
rHb	35-90 AU	57-116 AU
SvO_2	20-50%	50-97%
Blood Flow	10-50 AU	21-200 AU

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CHAPTER 9

GENERAL DISCUSSION

GENERAL DISCUSSION

The common theme of this thesis is research with advanced non-invasive monitoring systems used to evaluate the microcirculatory hemodynamic state of (preterm) neonates. Deprivation of the cardiovascular system is associated with increased morbidity and mortality (1-3). These studies underline the need for hemodynamic monitoring in preterm neonates. Various questions regarding the cardiovascular state however remain unanswered, despite numerous attempts to objectify and standardize outcome parameters (4). The most prominent examples are the quest to define circulatory insufficiency and significance and treatment of patent ductus arteriosus. The fact that these questions remained unanswered over the last decades, demonstrates the great need for innovation. With advances in (biomedical) technology, the ability of hemodynamic monitoring systems to collect and analyze the complex physiometric data provides a foundation for advances in diagnosis and management of neonatal cardiovascular compromise. Preterm infants are arguably the most vulnerable population in health care. This has led to the following:

1. Diagnostic tools are oversized to apply on preterm infants
2. Diagnostics are often to invasive to apply on preterm infants

As a result, hemodynamics is a neglected topic in neonatal critical care. The development of non-invasive diagnostic tools is therefore of particular interest for the field of neonatology. However, it must be emphasized that there are concerns on accuracy, reliability, feasibility, and the need for validation across different subpopulations. The design and interpretation of validation studies, including the interpretation of statistical methods can be difficult. Newly introduced devices are accompanied with one validation study, often performed in healthy, cooperative adults. Basis clinical limitations are often not addressed in these initial validation studies, because it's aims are to assess a measurement performance under study conditions in patients allowing optimal signal recording (5). These measurements do not reflect measurements from the clinical setting and therefore cannot be extrapolated to the daily clinical practice. The differences between healthy cooperative adults and vulnerable preterm infants are so big that validation studies in this specific population are most certainly needed.

Imaging of the microcirculation

The microcirculation reflects the area were the blood vessels have a diameter less than 100 micrometer. Despite these small dimensions, the microcirculation comprises 7% of the body's circulating volume(6). On this cellular level, important physiological processes take place. It is the terminus station of blood flow where individual cells are provided with gasses, body defense cells, nutrients and drugs by diffusion and active transport. Blood flow considered arterial merges into venous blood and waste products are disposed. The strong vasoconstrictive abilities of arterioles contributes to

the maintenance of systemic blood pressure. Lastly, the microcirculation plays a role in temperature regulation(7). Capillaries of the nail fold have been studied for decades, but the introduction of hand-held microscopes made it possible for researchers to visualize the microcirculation of any mucosal tissue(8). Over time, several video microscopes have been developed (9). They all, more or less, use the same principle. Light of a specific wavelength (540 nm) is emitted on the skin. These wavelength is at the isobestic point of oxyhemoglobin and deoxyhemoglobin and therefore absorbed by erythrocytes an reflected by the surrounding tissue (10). The microscopic feature of the camera produces images of the smallest blood vessels. (Figure 1). The element of moving erythrocytes give these movies an impressive character at first sight.

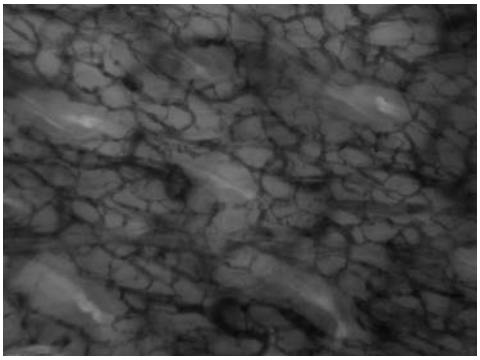

Figure 1. Cutaneous microcirculation of a preterm infant born 24 weeks of gestational age.

Outcome parameters

What can someone actually deduce from these images? In general, the quantity and the quality of the microcirculation can be described. Quantity refers to the density of vessels. The total length of all vessels divided by the measured area gives the Total Vessel Density (TVD) in mm/mm^2. However, equally important is the quality of the microcirculation. And by quality we mean the blood flow running through these vessels. The Proportion of Perfused Vessels (PPV) is the percentage of the vessels which are well perfused. Another parameter, Perfused Vessel Density (PVD) combines the quality and quality and is defined as the (TVD x PPV). In the literature this parameters is often referred as the Functional Capillary Density (FCD in mm/mm^2).

A more detailed outcome parameter of flow is the Microvascular Flow Index (MFI). This score is based on determination of the predominant type of flow in four quadrants. Flow is characterized as absent (0), intermittent (1), sluggish (2), or normal (3). The values of the four quadrants are averaged. The main advantage of this score is that it is relatively easy to measure. It also takes into account the fact that flow can be continuous but slow (sluggish).

Finally, the Heterogeneity Index (HI) gives information on the diversity of flow and is calculated as the highest site flow velocity minus the lowest site flow velocity, divided by the mean flow velocity of all sites per measurement.

This gives the researcher already five outcome parameters, which raises the questions "What is the most important parameter?". However, to complicate things even further, a distinction is made between the diameter of the blood vessels. In preterm infants small (diameter <10 μm), medium (diameter between 10-20μm) and large (diameter >20μm) vessels are distinguished for TVD, PPV and PVD. This results in almost 20 outcome parameters.

A statistical issue occurs in the presence of this variety of outcome parameters. The alpha value is set usually at 0.05 or less. This means that there is a less than five percent chance of rejecting the null hypothesis by chance alone. With this standard alpha level, about 1 in 20 results should come back significant when there really is no effect. Thus, by having so many outcome parameters "p-value fishing" can be a relevant problem in this research field. Proper methodology dictates that the experimenter choose which variables are being compared beforehand and to run post-hoc corrections on any further comparisons. Also, especially in studies with large number of participants, even a slight difference in means will become significant even though the effect size is close to nothing. This is why it's important to primarily look at the effect size and judge the p-value as an afterthought.

Image quality

Unquestionably, the quality of the video images is the most important factor for proper and trustworthy analysis. A round table conference held in 2007 was the first attempt to standardize image acquisition and give researchers sufficient guidance on quality. Five key points were given:

1. Three to five video clips should be obtained per measurement, each on different sites, given the intrinsic variability of the microcirculation
2. Avoid pressure artefacts. Excess pressure may collapse the microcirculation, especially larger vessels.
3. Eliminate all that is obscuring the image –e.g. secretions, lanugo.
4. Provide adequate focus and contrast.
5. Avoid movement artefacts; videos must be stable for at least 20 seconds.

Especially pressure artefacts are a big problem in the research field. Pressure artefacts can be recognized in two ways. First, the flow in large vessels should be evaluated. If this is much slower than in smaller vessels a pressure artefact is most likely the case. Second, the general flow in all vessels should be monitored. If the flow pattern in all these vessels is similar (decrease in flow at the exact same time), a pressure artefact must be suspected. Furthermore, from a physiological point of view, blood flow at a microcirculatory level is continuous and not pulsatile like the microcirculation.

Interrupted blood flow is therefore a strong sign of pressure artefact. Sallisami et al. demonstrated that, even after an extensive training period, 36% of the obtained movies contained pressure artefacts (11). If experienced users have a pressure artefact rate that high, imagine the video quality of inexperienced users.

The abovementioned key points for image quality leaves some room for interpretation. The introduction of the Microcirculation Image Quality Score (Table 1) however ensures there is less debate on what is right and wrong (12). Videos are judged on 6 different categories: illumination, duration, focus, content, stability and pressure (Figure 2). Per category the video can be good (preferably), acceptable, or unacceptable. If a video scores 'unacceptable' in one category, it cannot be used for data analysis. This score has been used to demonstrate the superiority of IDF technology over SDF technology (13, 14), but more important, it can (and should) be used by reviewers as a quality standard for manuscripts.

In order to obtain good and high quality images, operators should be experienced. This means familiar with the device, technical aspects and software but also familiar with the patients' setting and disease. In chapter 2, a detailed standard operation procedure (SOP), is described for transcutaneous microcirculatory imaging in preterm infants. Even more speaking is the visualization of the SOP which can be found online. http://www.jove.com/video/53562/transcutaneous-microcirculatory-imaging-in-preterm-neonates.

Although this SOP is for microcirculatory imaging in preterm infants, basics are given which can be applied on sublingual microcirculatory imaging too. Three things are extremely important for researchers:

- Awareness that it is not easy to obtain good images!
- Always perform measurements with two operators
- Always capture more than the required 3-5 videos

If operators stick to these recommendations, the foundation for good quality imaging is laid. Other recommendations in chapter 2 primarily focus on pressure and movement artefacts. Pressure artefacts in is less of a problem in microcirculatory imaging in preterm infants. The operator can actually see the area of measurement, contrary to sublingual measurements were the camera probe is usually hidden behind the tongue. However, movement artefacts are more difficult to avoid, as the patient is usually not sedated an non-cooperative. It can be a challenge to obtain a stable movie for more than five seconds.

Automated versus manual analysis

Another discussion is the one of manual vs automated analysis. Historically, video clips were analyzed manually. The most used program is provided with the SDF device and is called AVA 3.0 (Automated Vascular Analysis). The term 'automated' is somewhat misleading as the automated analysis are far from accurate. Operators still have to draw vessels manually and therefore the analysis with AVA 3.0 are considered manual

Table 1. Microcirculatory Image Quality Score (Adopted from Massey et al. Journal of Critical Care. Dec 2013)

	Good (0)	Acceptable (1)	Unacceptable (10)
Illumination: brightness and contrast of the video	Even illumination across the entire field of view. Contrast sufficient to see small vessels against a background of tissue	The video borders on being too dark or bright to distinguish vessels from tissue but the vessels are still identifiable	The video is oversaturated/too bright or too dark to make out analyzable features. Insufficient contrast to resolve flow rate
Duration: number of frames in the video clip and how it represents the actual pathology	Analyzable video segment is ≥5 s long (>150 frames)	Analyzable video segment is 3-5 s (between 90 and 150 frames)	Analyzable video segment is <3 s (<90 frames)
Focus: image sharpness in region of interest	Good focus for all vessels (small and large) in the entire field of view. Plasma gaps and red blood cells are visible.	<1/2 field of view is out of focus or edges of the vessels are slightly out of focus.	Video is completely out of focus such that no small vessels can be seen.
Content: determination of the types of vessels and/or presence of occluding artifacts in the image. If the video presents overall pathology	Video is free of occlusions. Good distribution of large and small vessels. Less than 30% of the vessels are looped upon themselves.	Video may have a few artifacts. Acceptable distribution of large and small vessels. About 30% to 50% of the vessels are looped.	Most of the field of view has occluding artifacts such as saliva or bubbles. More than 50% of the vessels are looped upon themselves.
Stability: frame motion that can be adequately stabilized without motion blur	Movement is within 1/4 of the field of view. No motion blur	Movement is within 1/2 of the field of view. No motion blur	Movement is greater than 1/2 of the field of view and/or motion blur in frame.
Pressure: iatrogenic mechanical pressure causing misrepresentation of flow	Flow is constant throughout the entire movie. No obvious signs of artificially sluggish or stopped flow. Good flow in the largest vessels	Signs of pressure (localized sluggish flow in a specific large vessels), but flow appears to be unimpeded based on good flow in most large vessels	Obvious pressure artifacts associated with probe movement, and/or flow that starts and stops, reversal of flow. Poor or changing flow in larger venules

analysis. This is a very time-consuming process. It takes about 30 minutes, for an experienced operator, to analyze one video clip. Per measurement, the average of 3-5 video clips is calculated, meaning that the analysis of 1 patient takes over 1.5 hour of work. This is one of the main reasons why the technique of videomicroscopy still is not incorporated in clinical care.

The introduction of automated analysis software was promising. IDF technology was supported with the software program CC-Tools and SDF technology upgraded AVA to the automated AVA 4.0 version. The automated analysis successfully overcame the problem of time-consuming manual analysis An even bigger advantage is the elimination of 'human factor'. Inter-observer variability of the cutaneous microcirculation is shown to be poor (15). Studies of the sublingual microcirculation claim good reproducibility of vessel density, PPV and MFI (16, 17). One have to take in mind that these studies are 'single-centre'. Each centre can claim good reproducibility of their own data, but it is doubtful if different centres have identical interpretation of definitions. For example, there can be a thin line between sluggish flow and normal flow. This problem, together with the enormous amount of outcome parameters, make studies hard to compare. Automated computer analysis will therefore be beneficial and improve standardization in the research field.

However, soon after its introduction questions arose on the accuracy and reliability of the automated analysis. To this day, technical challenges interfere proper flow measurements. Therefore, flow-derived parameters (PPV, PVD, MFI) are untrustworthy and cannot be used for research. This applies to CCtools and AVA 4.0. Hopefully these issues can be solved in the near future. If flow could be determined and presented in μm/sec, the semi-quantative measurement MFI could be set aside and a clear cutoff point can be determined for good and bad flow. This could narrow the gap towards clinical use.

Also, consensus should be reached on specific technical settings like sensibility, minimal vessel length, maximum capillary diameter. These settings could differ between adults and infants.

Despite these major drawbacks, IDF technology is claimed to be 'validated' (18). There are a lot of different kind of (definitions of) validation but in this case validation should mean: "Is the given outcome of the analysis (vessel density) in agreement with the true measurement". The SDF technology was however 'validated' in the same manner: The technique was compared to its predecessor (10) and inter-observer variability (16, 17, 19) was briefly noted in some studies. These studies made the techniques fully acceptable for clinical research.

Microcirculation in (preterm) infants: review of literature

Few research groups have performed studies of the microcirculation in newborns and infants using videomicrocscopy. Besides our own research group, the major contributors are Genzel-Boroviczény (Munich, Germany), Ergenekom (Ankara, Turkey) and Tibboel (Rotterdam, The Netherlands). Fredly (Oslo, Norway) used an similar videomicroscope

called Computer Assisted Video Microscopy (CAVM). An overview of all studies in children and newborns using OPS, SDF or IDF technology is given in Table 2. This table is adopted from Buijs et al. and completed with additional studies.

Table 2. Overview of microcirculatory studies performed in (preterm) infants and infants

First Author	N	Age Group	Device	Type of disease	Conclusions
Genzel-Boroviczeny, 2004	13	Preterm	OPS	Anemia	BTx improves the MC for at least 24h in anemic preterm infants
Kroth, 2008	25	Preterm	OPS	-	The MC is higher in 1-week-old preterms than in 4-week-old preterms
Weidlich, 2009	25	Preterm	OPS	Proven infection	The MC decreased in infants with proven infections from d5 to d1 before starting antibiotics
Top, 2009	14	Term	OPS	Respiratory failure	Before VA, the MC is lower in VA patients than in non-ventilated controls, but it does not differ from ventilated controls. After VA, the MC is increased in VA patients whereas it is not in ventilated control patients
Den Uil, 2009	3	Children	SDF	Congenital heart disease	The MC of alveoli can be visualized
Hiedl, 2010	25	Preterm	SDF	Significant PDA	The MC of PDA+ patients was lower than the MC of PDA- patients The MC differences disappear after closing PDA+ The MC is better in the left than in the right arm, irrespective of treatment and PDA type
D'Souza, 2011	44	Preterm	OPS	Low Birth Weight	The MC is increased in LBW infants compared to normal weight infants
Ergenekon, 2011	15	Term	SDF	Polycythemia	The MC is improved after partial exchange transfusion
Top, 2011	18	Term	OPS	Septic shock	The MC does not differ at ICU d1 and increases thereafter in the survivors, but not the non-survivors MC impairment predicts mortality more accurately than the PRISM-II
Top, 2011	8	Term	OPS	Respiratory failure	The MC increases after iNO whereas macrocirculatory and ventilator parameters are unaltered
Top, 2011	45	Term	OPS	-	The MC is higher in neonates aged 0d to 7d compared to all older children
Milstein 2012	11	Children	SDF	Alveolar cleft gingiva	The MC is impaired in patients with an alveolar cleft

Table 2. (continued)

First Author	N	Age Group	Device	Type of disease	Conclusions
Paize, 2012	20	Children	SDF	Menigococcal disease	The MC is impaired in MCD patients and resolves as MCD regresses. MC impairment at admission predicted the length of ventilation
Top, 2012	21	Term	OPS	Respiratory failure	The MC is maintained, but not improved immediately after starting VA EMCO.
Antonios, 2012	22	Term	OPS	Maternal hypertension	While the MC is increased in preterm neonates born from hypertensive mothers, it is decreased in term neonates born from hypertensive mothers when compared to neonates born from normotensive mothers.
Schwepcke, 2013	21	Preterm	SDF	Hypotension	The MC is increased early after birth in hypotensive preterms compared to normotensive preterms.
Caixeta, 2013	2	Children	SDF	Dengue shock	The MC is severely impaired during dengue shock
Alba-Alejandre, 2013	16	Term	OPS	Infection	The MC is impaired in neonates with infection that does not cause shock
Ergenekon, 2013	7	Term	SDF	Perinatal asphyxia	The MC is decreased during therapeutic hypothermia
Raghuraman, 2013	26	Term	OPS	-	The MC is higher in twin infants than in singleton infants
Tytgat, 2013	12	Term	SDF	Pyloric stenosis	Pneumoperitoneum impairs the MC
Buijs, 2014	28	Term	SDF	CDH	Whereas HF and/or MABP rise after dopa ± E or NE, the MC fails to improve Abnormal MC is associated with need for E or NE and with need for VA
Buijs, 2014	48	Children	SDF	Respiratory failure	The MC is impaired prior to both VA and VV and it requires 24h of VA or VV support to improve MC. The MC evolution does not differ between VA and VV There is no relation between MC impairment and mortality
Buijs, 2014	20	Children	SDF	Post-cardiac arrest	The MC is impaired during TH and increases thereafter to a level comparable to normothermic, healthy controls. At TH start, MC impairment is associated with poor outcome
Lee, 2014	54	Children	SDF	Prematurity	FVD was significantly higher in former preterm children compared to controls at baseline

Table 2. (continued)

First Author	N	Age Group	Device	Type of disease	Conclusions
Nussbaum, 2014	28	Children	SDF	Diabetes type 1	The TVD of the sublingual vasculature and the MFI were found to be similar between children with diabetes and controls
Van den Berg, 2014	28	Term	SDF	-	Buccal MC measurements in term newborns are highly reproducible in contrast to cutaneous MC measurements.
Nussbaum, 2015	49	Term	SDF	Cardiac surgery	Reduction of PVD and MFI after surgery, normalization after 24 hours.
Van Elteren, 2015	20	Preterm	Both	-	IDF visualized 19.9 % more vessels resulting in a significantly higher vessel density
Fredly, 2016	28	Term	CAVM	Perinatal asphyxia	FCD was higher both during and following TH
Schinagl, 2016	37	Children	SDF	Anemia	Erythrocyte transfusion can improve, but also temporarily reduce the MC
Van Elteren	60	Preterm	IDF	-	The MC decreases in the first month of life. Only postnatal age influences the MC.

Focusing on studies in preterm infants, we see that these are exclusively performed by the group of Genzel-Boroviczény. In 2002 the first paper was published, introducing the OPS camera as a new method for visualization of the microcirculation in preterm infants (20). In 9 term and 28 preterm infants (gestational age 24-33 weeks, weight 550-2070 grams) the cutaneous microcirculation was measured from birth till day 5. No change was seen in this time period and no statistical differences were seen between preterm and term infants. A significant negative correlation is claimed between hematocrit and red blood cell velocity. However, this correlation must be considered 'weak/low' as the ρ value was -0.37 (21).

In 2004 a study was published on microcirculatory changes after an erythrocyte transfusion (22). No differences were seen macrocirculatory variables like heart rate and blood pressure, but a whopping 45% increase in functional capillary density was seen after transfusion (before FCD 142cm/cm^2, after 206 cm/cm^2). No correlation was found between hemoglobin and FCD. This paper highlights the differences between macrocirculation and microcirculation. Research in adults already demonstrated that the microcirculatory alterations could occur despite a stable macrocirculation (23, 24).

A study which resembles our study in chapter 4 was conducted by Kroth et al (25). OPS imaging was performed from day 3 to day 30 of life in 25 preterm infants (median

gestational age 28 weeks, birthweight 900 grams). Results from 3 days were pooled for each week (days 6–8 for week 1, days 13–15 for week 2 etc.). Functional small vessel density (FSVD), RBC-velocity and vessel diameter were the only outcome parameters reported and no information was given on the clinical status of the infants. FSVD decreased significantly from week 1 to week 4 (236 cm/cm^2 to 207 cm/cm^2). Contrary to the transfusion study, a correlation of ρ=0.76 with hemoglobin levels was found. Based on this univariate correlation analysis, the authors conclude that the decrease of FSVD was caused by the decrease in hemoglobin level.

The cohort of preterm infants in the aforementioned study was also used by Weidlich to analyze changes in the microcirculation as an early marker for infection (26). Infants were retrospectively categorized in a positive or negative infection group, according to laboratory parameters. Microcirculatory movies prior to the onset of the infection were analyzed. Despite an almost identical median value, the authors claim a significant decrease of FSVD between day 5 and 1 day prior to infection (no p-value given). The results of this study have to be taken cautiously, as the study methodology is poor. For instance, video images were analyzed by two operators, despite poor reproducibility (15). Subjects were used multiple times in the same group and no positive blood cultures were found in the 'positive infection' group. Hemoglobin, in their previous study the explanation of the decrease in FSVD, was not used as a confounding variable in the analysis.

Hiedl reported on the effects of patent ductus arteriosus on the microcirculation in preterm infants (27). Patients suffering from patent ductus arteriosus (PDA) were compared to a group of patients without PDA, before and after treatment. There was no significant change in functional vessel density before and after treatment in the PDA group. Patient in the PDA group had a lower FVD before treatment compared to non-PDA. The authors do not give an explanation why the microcirculation is unchanged (and not higher) after successful treatment of PDA.

That mean arterial blood pressure is not leading for microcirculation perfusion was shown by Schwepke et al. (28). Hypotensive preterm infants requiring inotropic support (dobutamine) had a significantly higher FVD at 6 hours of age compared to a control group, despite a lower MAP. No information on other microcirculatory parameters, like MFI or HI, was reported. The higher FVD was explained by a loss in microvascular tone, resulting in flow redistribution. Nevertheless the authors state that the link between blood pressure and perfusion remains unclear.

The relation between infection and microcirculation has been extensively studied in adults (16, 17, 19, 24, 29). Beside the study of Weidlich et al. in preterm infants (26), one other study has focused on newborns and infection (30). The quality of this study is however publishing unworthy. A parameter 'proportion of vessels with continuous blood flow' was created (most likely to demonstrate a significant difference between groups), no information on 'conventional' outcome parameters was given and a supplementary movie is unfocused and shows a clear pressure artefact. It

is astonishing that this paper, published in 2013, comes from the researchgroup of Genzel-Boroviczény, a group that publishes paper on the microcirculation for over 10 years. Especially this supplementary movie, makes one wonder if the quality of the data used in this research group meets the quality criteria for analysis.

The previous research performed in adults do point out an interesting matter: the microcirculation is altered in a state of sepsis. If there is a difference between sepsis and 'normal state' the next logical step would be to recognize the onset of a sepsis in an early state. Early intervention and treatment with antibiotics would lead to a decrease of mortality rate, especially in vulnerable preterm infants. The observational study in chapter 4 can be seen as the foundation for future research in this as its provides reference values of healthy preterm infants. The fact that this study only found a difference in MFI on day 1 between healthy and non-healthy infants, does not mean there is no difference. It was not the primary aim of the study and numbers of sick infants was relatively low.

Research field in its infancy

The research field of microcirculation is still in its infancy. This is reflected by the lack of clarity in published manuscripts, both in the adult as pediatric/neonatal field. A variety of outcome parameters have been created, leading to indistinctness and incomparable results.

Outcome parameters based on density (total vessel density), flow (proportion of perfused vessels, microvascular flow index) and heterogeneity (heterogeneity index) were created. Also parameters combining density and flow were created (perfused vessel density or functional capillary density) These parameters could be separated for 'small vessels' and 'non-small vessels'. This already leads to 15 different outcome parameters. This raises the question what 'the microcirculation' really is. For some authors this long list of parameters wasn't enough. One of the founders in microcirculatory research, dr. De Backer from Belgium created the 'perfusion heterogeneity of the proportion of perfused vessels' (PPV HI) (31). Furthermore, in the neonatal field, the manuscript of Ergenekon has the outcome parameter 'percentage of vessels with sluggish flow'(32). Lastly, Nussbaum reported on 'percentage of vessels diameter coverage'(33). One would wonder why these outcome parameters have been created.

Although the results of the studies are different to compare due to different outcome parameters, analyzing techniques and site of measurements, several studies report on the vessel density. Some results are remarkable. For instance, there is a 80-fold difference in capillary density in the paper of Top et al (34) (FCD 2.5cm/cm^2) and Genzel-Boroviczeny et al (25) (FCD 206 cm/cm^2). From the same research group, Lee et al (35) reported a FVD of 30 cm/cm^2, which is about an factor 10 smaller than Hiedl (27) (FVD 260 cm/cm^2).

Also, sublingual TVD values in healthy control groups differs between studies. Tytgat et al. report a TVD value of 42.6 mm/mm^2 (36) and Nausbaum et al.found

a TVD of 19.2mm/mm^2 in a healthy control group (33). These differences in values and study results are difficult to explain.

Other techniques for microcirculatory measurements

Several techniques are used in the field of neonatal care to measure perfusion and oxygenation. **Near-infrared spectroscopy** (NIRS) is a technique that utilizes near-infrared light to measure oxy- and deoxyhemoglobin in tissues. This technique is first described in 1977 (37). The fractions of oxy- and deoxyhemoglobin are used to calculate tissue O2 saturation (rSO_2). The rSO2 reflects a regional balance between oxygen supply and demand for the underlying tissue , and fractional tissue oxygen extraction can be estimated (FTOE = [SaO2-rSO2] / SaO2). Increased FTOE reflects higher oxygen consumption in relation to oxygen delivery to the brain tissue (38). The NIRS signal analyses arterioles, capillaries, and venules but as 75% of the blood in a skeletal muscle is venous, NIRS rSO_2 measurements mostly represent local venous hemoglobin O2 saturation (39).

This technique is particularly interesting as it provides quantitative information on microvascular function within seconds. Multiple observational studies have been performed in preterm infants (40) and in specific research fields like congenital heart disease, transition, hypoxic-ischemic encephalopathy (41). It demonstrated differences between healthy and sick infants, but consequently there is a large overlap between these groups. Reference ScO2 values for healthy preterm infants range between 45% to 90% . Also, data reported with different devices vary up to 15% (42). This absence of standardization may limit comparisons of results from different trials and application in the clinical setting. A benefit is that it is easy in use and can be used non-invasively in preterm infants. An important trial involving NIRS is the SafeBoosC trial (43). The primary objective of this is to examine if it is possible to stabilize the cerebral oxygenation of extremely preterm infants during the first 72 hours of life through the application of cerebral NIRS oximetry and implementation of an rStO2-specific clinical treatment guideline. It is hypothesized that by using the specified treatment guideline to respond to cerebral monitoring readings outside the target range, the burden of hypo- and hyperoxia is reduced and thereby brain injury. Preterm infants will be randomized into one of two groups (experimental or control). Common is that both groups will have a cerebral oximeter monitoring device placed within three hours after birth. In the experimental group, the cerebral oxygenation reading is visible, and the infant will be treated accordingly using a defined treatment guideline. In the control group, the cerebral oxygenation reading is NOT visible, and the infant will be treated as usual. The concept of this trial will discover if NIRS is of real benefit for preterm infants.

"Oxygen to see" (O2C) is a device that combines laser Doppler flowmetry (LDF) and visible white light spectroscopy (VLS) technology in one probe. LDF uses the Doppler shift to give a value of the mean erythrocyte flux in the tissue under investigation and has been used extensively for noninvasive measurement of microcirculatory changes in

human skin. The O2C-devices transmits continuous wave laser light (830 nm) and white light (500-800 nm, to tissue where it is scattered and collected on the surface at fibers in the probe. Data are analyzed by comparison with prerecorded deoxygenated and oxygenated hemoglobin spectra (44). VLS measures microvascular oxygen hemoglobin saturation in the superficial tissue layers, such as the skin and mucous membranes. Because most of the blood in the measured regions is in the mucosal capillaries, the HbO_2 measured by VLS yields a reliable estimate of the oxygen saturation of hemoglobin in mucosal capillaries, and reflects the balance between oxygen influx and consumption at the microcirculatory level.

The outcome parameters are mixed venous oxygen saturation (in %), flow, velocity and relative amount of hemoglobin (in Arbitrary Units). The relative amount of hemoglobin (rHb) represents an excellent discriminator between arterial occlusion and venous congestion. Arterial occlusion would cause a decrease in this value, whereas a venous congestion would cause an increase (45). Flow and velocity parameters can, in combination with mixed venous oxygen saturation, provide the clinician with useful information on peripheral perfusion. Its use in preterm infants is somewhat controversial. The reproducibility is described in chapter 8 and was found to be poor between different limbs. Also this device can be mainly used as a trend monitor within one individual. To adequately measure flow and velocity no pressure must be applied on the sensor. To keep the sensor in place, strong double-sided adhesive tape is used which is risky for the vulnerable infants' skin. It is up to the clinician to decide if the benefits of the measurement justify its use in preterm infants.

Recommendations

It has been 14 years since the first manuscript of videomicroscopy of the microcirculation in preterm neonates was published. Using the OPS camera, the German group of dr. Genzel – Boroviczény described the cutaneous microcirculation of 28 preterm infants(20). In the meantime several observational studies have been published, most of them pointing out differences between two study groups or described the effects of therapeutic interventions on the microcirculation. Despite technical advances – in 2013 already the third generation of videocamera's became commercially available – there is still a lack of randomized control trials. Furthermore, the bridge to application in clinical care is still lacking. Various reasons can be mentioned as an explanation. They can be categorized in the following groups:

1. Microcirculatory physiology
2. Acquiring and processing of images
3. Technical limitations

One of the most important difficulties is the substantial intra-subject heterogeneity of the vessel density(19). Within one subject, there can be a difference up to 40% in vessel density (unpublished data). This distribution in vessel density complicates the

process of creating reference values. Without reference values it is virtually impossible to correctly interpret the outcome of microcirculatory videos on an individual level. To deal with the problem of distribution, it is the general consensus to average the outcome of 3 to (preferably) 5 videos(46). This makes acquiring of videos time-consuming, which is acceptable for the researcher but hinders the clinician in quickly assessing microcirculatory state. Besides the time-factor, obtaining high quality images is operator dependent and requires an extensive period of training and exposure (11).

Some researchers avoid the heterogeneity of the microcirculation by performing continuous monitoring of one region of interest (47). Fixation of the video microscope is essential and stabilizers are commercially available (48). For research purposes, this is an elegant option to bypass intra-subject heterogeneity and evaluate the effect of interventions on the microcirculation. Practical issues however make this option hard to apply in clinical practice. For instance, in order to measure the exact same region of interest in adults, sedation of the patient is required. For preterm neonates, a sensor should be developed that could be placed and fixated on the upper arm. This will not only avoid the problem of the heterogeneity of the microcirculation, but also ease the process of obtaining good quality images.

The lack of clarity in outcome parameters is another topic which needs to be tackled. The 'De Backer Score', an alternative analysis method emerged to speed up the offline analysis, can be cast aside in the presence of automated computer analysis. Further improvement is correlated with technical improvements on flow measurements. If flow can be measured accurately, other outcome parameters can be deleted. First of all, the subjective parameters MFI and HI can be replaced by a outcome parameters 'velocity' (in μm/s). It can be argued that the vessels with the smallest diameter are the most important ones, as gas exchange takes places in this area. Therefore larger vessels should not be taken into account. A subdivision can be made between small vessels (diameter ≤ 10 μm) and large vessels (diameter between 10-25 μm).

Chapters 6, 7 and 8 of this thesis found questionable results for reproducibility of new non-invasive diagnostic tools in preterm infants. This demonstrates that results of validation studies performed in healthy adults cannot be extrapolated to preterm infants. Also, most devices produce a wide range of values, within and between subjects. Reference values are therefore hard to create and devices can only be used as trend monitor within a patient. This interferes implementation in clinical care. Clinicians should only use diagnostic tools which are properly validated in (preterm) infants. Also, only devices with clear reference values should be used.

Future perspectives

Beyond all the (technical) difficulties, the visualization of the microcirculation can help other fields in medicine, mainly the oncology & hematology department. In the oncology department, leukemia is characterized by leukocytosis (49). Hyper leukocytosis can lead to the hyper viscosity syndrome (50). Hyper viscosity syndrome lacks a concrete

definition and is based on clinical symptoms. Visualizing the microcirculation could help define hyper viscosity syndrome, based on flow rates of the microcirculation. This could help identify patients who benefit from treatment and prevents overtreatment. Also, it is a great diagnostic tool for follow up of the treatment.

Another potentially interesting disease to study is sickle cell disease, a disease characterized by vascular occlusion and crisis of onset of pain in bones and joints. A previous study in 7 patients suffering from a sickle cell crisis, no differences were found in MFI. It should be noted that the MFI in the healthy control group showed remarkable low values (MFI 2.7) which is close to be considered abnormal (51). The sublingual microcirculation might be to 'central' to visualize this disease, but one can imagine that videomicroscopy could help as a diagnostic marker in this disease.

Other interesting research questions to be answered can be found in treatment of the microcirculation. Multiple studies (both in adults and children) have shown that raising blood pressure does not improve the microcirculation (52-54) . Other studies using vasodilators to improve the microcirculation showed conflicting results. (55). In patients with septic shock a positive effect on the microcirculation was found using nitroglycerin (56), levosimendan (57), dobutamine (29) and ketanserin (58). On the other hand, no improvement was found in studies using nitroglycerin (55), magnesium sulfate (59) and dobutamine (60, 61). Finding strong evidence for improvement of microcirculatory flow will accelerate clinical trials investigating the benefits of visualization of the microcirculation.

In general, hemodynamic monitoring of preterm infants might transform into a more multimodal approach using not only blood pressure, but combining multiple diagnostic techniques. Instead of 'old fashioned' outcome parameters like capillary refill, urine output and heart rate, NIRS, O2C and cardiac output measurements will continuously monitor the patient. In order to accomplish such a model, physicians must collaborate with specialist from technical universities and for now remains a distant prospect. For it to be effective 'big data' needs to be gathered, processed, analyzed, and presented in a understandable manner. The complexity of biology in this age of "big data" requires diverse teams in order tackle such vast amounts of data and to make sense of it all. New technologies that crunch data faster and more efficiently also permit researchers to re-analyze existing datasets. This task is outside the capabilities of the physician. Big data can compare multiple variables with complex relationships to provide the most comprehensive set of predictions possible. This approach makes the comprehensive hemodynamic monitoring and data acquisition system more powerful and informative (62). This concept is better known as systems biology (63). Systems biology is based on the understanding that the whole is greater than the sum of the parts. It is **collaborative**, integrating many scientific disciplines – biology, computer science, engineering, bioinformatics, physics and others – to **predict** how these systems change over time and under varying conditions. A nice example of an effective model is the 'HeRo' heart rate characteristics monitor. This software analyzes electrocardiogram

data for decreased heart rate variability and transient decelerations associated with sepsis and converts these changes into a score. This score is the fold increase in probability that a patient will have a clinical deterioration from sepsis within 24 hours. It has proven to be effective, reducing mortality rate in very low birth weight infants (64). Especially the predictive capabilities of this model in combination with a simple and effective outcome (one single score) is interesting for clinical use.

It is clear that healthcare will evolve towards a more technical approach. As a result the position of the doctor will evolve as well, from a 'classical doctor' towards a 'technical operator'. This requires different skills and education for physicians. Decisions will increasingly be based on computer information and eventually be taken by the computer itself. This scenario is unrealistic for the near future, but eventually will take place. For now, it is our task to critically judge technical applications on their benefit for the patient.

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CHAPTER 10

SUMMARY / SAMENVATTING

SUMMARY

The aims of this thesis are to evaluate the strengths and weaknesses of Incident Darkfield Imaging (IDF) compared to its predecessors; to evaluate the cutaneous microcirculation of preterm infants in the first month of life; to establish reference values for term and preterm infants; and to evaluate reproducibility of newly introduced hemodynamic diagnostic techniques.

Part 1. Microcirculatory imaging using Incident Dark field

In **chapter 1** an overview is given of the responses of the fetus and (preterm) neonates to hypoxia. The human fetus has to cope with extreme hypoxic conditions in utero. This might be a protection mechanism to cope with the malicious effects of oxygen. The delicate balance between hypoxia and hyperoxia is discussed, as well as the role of reactive oxygens species (ROS) in neonatal diseases. The terms hypoxia, dysoxia and DO_2/VO_2 balance are introduced.

The hypoxic environment in utero stabilizes hypoxia inducible factor (HIF), which is a family of transcription factors that play a central role in cellular adaptation to insufficient oxygen. Maladaptation can lead to vascular remodeling, most likely to occur in the lungs. This remodeling is characterized by the combination of hypertrophy and hyperplasia of the cells within each layer of the vessel wall. There is an increased smooth muscle proliferation and muscularization of the normally muscle-free peripheral arteries. These antenatal and postnatal adaptation mechanisms can have life-long consequences; the negative effects of pulmonary hypertension and intra uterine growth restriction on health in later life is notorious. Finally, a brief overview of modern monitoring techniques used in neonatal critical care is given.

The subject discussed in **chapter 2** is probably one of the most important one in this thesis. In this chapter the technique of microcirculatory imaging is discussed in a step-by-step tutorial. Moreover, this tutorial is visualized in addition to support the manuscript. The technique of cutaneous microcirculatory imaging seems easy to apply, however it is quite complex. This tutorial gives future researchers a tool for high quality images, thereby increasing reproducibility and quality of the research field. Five key elements given in the tutorial are: 1) Always perform measurements with two operators. 2) Stabilize the elbow and wrist while holding the videoprobe. 3) Reduce the length of the measurement by combining steps (i.e. finding an appropriate spot and focus depth). 4) Hold the camera perpendicular to the skin. 5) Always capture 8-10 movies, offline analysis often reveals (pressure) artefacts.

Obtaining high quality images is essential for accurate offline analysis, both manual as automated.

Chapter 3 describes a comparison study between the newly available Incident Dark field (IDF) Imaging camera called CytoCam and its predecessor MicroScan, using Sidestream Dark field (SDF) imaging. The new camera has superior technological

hardware. Twenty stable preterm infants were consecutively measured with SDF and IDF imaging. All videos were exported and analyzed offline by one operator. A higher vessel density was found using IDF technology, mainly in small vessels. Surprisingly, a lower PPV was found. Both findings can be explained by the superior image quality of the obtained videos, objectified by the microcirculation image quality score. The IDF camera is easier to handle, which led to more focused images. Therefore smaller vessels can be seen and the flow can be scored more accurately. Although sometimes referred as a validation study and thus validation of the newly introduced IDF camera, this study only demonstrates superior quality of the images of IDF versus SDF technology. The higher vessel density found does indicate that existing reference values, created by SDF, need to be revised.

In a prospective observational study reported in **chapter 4**, we measured the cutaneous microcirculation of 60 preterm neonates born below a gestational age of 32 weeks in the first month of life. This is the largest study of cutaneous microcirculation in preterm infants. Measurement were performed on day 1, 3,5,7, 14 and 28 after birth. Video images were analyzed offline using automated analysis for TVD and manual analysis for MFI. Besides demographic data, several clinical parameters such as heart rate, blood pressure and arterial saturation were recorded during the measurements. The study demonstrated that total vessel density decreases in the first month of life. Using a linear mixed model, only postnatal age had a significant effect on total vessel density. Variables as gestational age, blood pressure or state of health did not had a significant effect on the microcirculation. These results are somewhat surprising, as several studies showed correlation between these variables and the microcirculation. This study however uses a superior statistical approach, combining all variables in one model. Univariate mixed model demonstrated a significant differences of total vessel density on the first day of birth between infants small for gestational age and appropriate for gestational age. This differences could not be seen in subsequent measurements. We hypothesize that this result is explained by more hypoxic antenatal conditions, resulting in an increase in angiogenesis. These results are in line with other studies focusing on the relation of hypoxia/hyperoxia and microcirculation. One of this study is described in **chapter 5**. In this chapter, the cutaneous microcirculation of healthy term infants born at high altitude (Puno 3830m, Peru), an environment with natural hypoxemia, was compared to infants born at sea-level (Rotterdam 0m, The Netherlands). A total of 86 term infants were measured in the first hours after birth. Total vessel density was found to be 14% higher in babies born at high altitude. This effect was mainly seen in small and medium sized vessels. Oxygenation profiles (pre- and postductal SpO2, cerebral and regional NIRS saturation) were significantly lower at high altitude compared to sea-level reference groups. These findings supports the overall hypothesis that antenatal oxygen exposure influences the microcirculation. A subgroup analysis of the high-altitude newborns showed that vessel density was independent of the neonates' ethnicity, however numbers in this analysis were limited.

Part II. Reproducibility of modern diagnostic techniques used in critical care

In Part 2 of this thesis we present the results of reproducibility studies of several new technical devices. The reproducibility of manual offline analysis of buccal and cutaneous microcirculation is presented in **chapter 6**. Buccal and cutaneous microcirculation was measured in healthy term neonates using SDF Imaging. Vessel density was independently assessed by two investigators. Reproducibility of vessel density assessment in the buccal area was good, with ICC's for total and perfused vessel density above 0.9 and a near zero bias and acceptable limits of agreement. However, reproducibility of the cutaneous microcirculation was poor with ICCs for total and perfused vessel density below 0.4 and large bias. This study demonstrates the variety of the microcirculation and the differences between two areas. It is harder to meet the quality criteria measuring cutaneous microcirculation, which results in more artefacts and images more difficult to analyse. It also shows the need for standardization in offline microcirculatory analysis, particularly for cutaneous analysis.

In **chapter 7** the reproducibility of the pleth variability index (PVI) is discussed. The PVI is a non-invasive parameter based on the changes of the perfusion index (PI) during a complete respiratory cycle. It is calculated from a standard pulse oximetry measurement. The PVI might be useful in predicting fluid responsiveness and monitor hemodynamic filling state of (preterm) infants. Its repeatability in the specific patient category of preterm infants is however not clear. In 25 hemodynamically stable preterm infants, three five-minute measurements were performed, simultaneously measuring two different limbs. A wide range of PVI values were found, both within as between subjects. Most comparisons showed fair to moderate reproducibility, the consecutive measurement on the right hand showed good reproducibility.

A similar study is presented in **chapter 8**. This chapter focuses on the reproducibility of laser doppler spectrometry measured by the oxygen to see (O2C). In 26 term neonates, two consecutive measurements were performed using two identical sensor. During the first measurement the sensors were placed on the right wrist and left wrist and during the second measurement the sensors were placed on the right wrist and right foot. The results were similar to the reproducibility study of the PVI. Large differences were found within and between subjects, reproducibility between different limbs was poor and reproducibility within the right wrist was moderate to good.

In the general discussion (**chapter 9**) the overall findings of this thesis, general considerations, recommendations and future perspectives are addressed. Each component of microcirculatory imaging is discussed with special attention for the(broad variety of) outcome parameters, quality of the images and analysis of images. The numerous outcome parameters induce 'type 1 errors' and raise questions like "What is the most important parameter". It is a good thing that quality of the video images has secured a more prominent role within the research field. High quality images are essential for reliable results, whether analyzed manual or automated.

Operators should be aware of the difficulties of obtaining images. Two 'quality guides' are currently available: The famous 'Round table conference paper' from 2007 which gives 5 key points and the Microcirculation Image Quality Score designed by Massey and colleagues. For cutaneous microcirculatory imaging, we created a step-by-step video tutorial which contains additional tips and tricks.

The existing literature of microcirculation in preterm infants is limited and comes from one research group. It can be considered as very opaque, for example in every paper different outcome parameters are given. There are serious concerns about image quality and statistical methods used. In this respect the need for more standardization is critical. This is the main recommendation, and there is considerable potential in this area.

SAMENVATTING

Het doel van dit proefschrift is om de sterke en zwakke kanten van Incident Dark field Imaging (IDF) technologie te bestuderen en te vergelijken met zijn technische voorgangers; om de cutane microcirculatie van preterm geboren neonaten in de eerste maand van hun leven te bestuderen; het vaststellen van referentiewaarden voor term en preterm geboren neonaten; het evalueren van de reproduceerbaarheid van nieuw geïntroduceerde hemodynamische diagnostiek technieken.

Deel 1. Microcirculatoire imaging met gebruik van Incident Dark field

In **hoofdstuk 1** wordt een overzicht gegeven van de foetale en neonatale respons op hypoxia. De humane foetus moet in de uterus omgaan met extreem hypoxische omstandigheden. Dit zou een protectie mechanisme kunnen zijn om de kwaadaardige gevolgen van zuurstof tegen te gaan. De delicate balans tussen hypoxia en hyperoxia wordt besproken, tezamen met de rol van zuurstofradicalen in neonatale ziekten. De termen hypoxia, hyperoxia en DO_2/VO_2 balans worden geintroduceerd.

De hypoxische omstandigheden in de uterus stabiliseert hypoxia inducible factor (HIF), wat een familie van transcriptie factoren is, welke een belangrijke rol spelen in cellulaire adaptatie bij zuurstoftekort. Maladaptatie kan leiden tot vasculaire vervorming, welk het meest waarschijnlijk optreed in de longen. Deze vervorming wordt gekarakteriseerd door de combinatie van toename en vergroting in elke laag van de vaatwand. Er is een verhoogde proliferatie van gladde spieren en vascularisatie van de normaal spier-vrij perifere arteriën. Deze antenatale en postnatale adaptatie mechanismes hebben levenslange consequenties; de negatieve effecten van pulmonale hypertensie en intra-uterine groeivertraging op de gezondheid op latere leeftijd zijn welbekend. Tenslotte wordt er een beknopt overzicht gegeven van moderne monitor technieken gebruikt in neonatale intensive care.

Het onderwerp dat in **hoofdstuk 2** wordt besproken is waarschijnlijk een van de meest belangrijkste in dit proefschrift. In dit hoofdstuk wordt de techniek van microcirculatoire imaging besproken in een stap-voor-stap handleiding. Bovendien is van deze handleiding een instructievideo gemaakt in aanvulling op het manuscript. De techniek van microcirculatoire imaging lijkt eenvoudig om toe te passen, maar is echter complex.

Deze handleiding geeft toekomstige onderzoekers een handvat voor het maken van hoge kwaliteit beelden waardoor de reproduceerbaarheid en kwaliteit van het onderzoeksveld wordt verhoogd. Vijf kernpunten welke worden geven zijn: 1) Voer de meting altijd uit met twee operators. 2) Stabiliseer de elleboog en pols tijdens het vasthouden van de cameraprobe. 3) Verkort de procedure door stappen te combineren (bijvoorbeeld het vinden van een geschikte meetplaats en focusdiepte). 4) Hou de camera loodrecht op de huid. 5) Maak altijd 8-10 filmpjes, aangezien offline analyse vaak een drukartefact laat zien. Het verkrijgen van hoog kwaliteit beelden is essentieel voor accurate offline analyses, zowel handmatig als geautomatiseerd.

Hoofdstuk 3 beschrijft een vergelijkingsstudie tussen de nieuw beschikbare Incident Dark field (IDF) imaging genoemd CytoCam en zijn voorganger MicroScan, welke Sidestream Dark field (SDF) imaging gebruikt. De nieuwe camera heeft superieure technologische hardware. Twintig stabiele preterm geboren neonaten werden achtereenvolgens gemeten met SDF en IDF imaging. Alle video's werden geexporteerd en offline geanalyseerd door één operator. Met IDF technologie werd een hogere vaat densiteit gevonden, met name in de kleine vaten. Verrassend was dat er een lagere PPV werd gevonden. Beide bevindingen kunnen worden verklaard door de superieure kwaliteit van de verkregen videobeelden, geobjectiveerd door de microcirculatie beeldkwaliteit score. De IDF camera is makkelijker in de omgang wat leidde tot meer gefocuste beelden. Hierdoor kunnen kleine vaten worden waargenomen en kan de flow accurater worden gescoord. Ondanks dat deze studie soms wordt gezien als een validatiestudie en dus validatie van de nieuwe IDF camera, demonstreert deze studie alleen de betere beeldkwaliteit van de IDF technologie vergeleken met de SDF technology. De hogere vaatdensiteit die werd gevonden geeft wel aan dat de bestaande referentiewaarden, gemeten met SDF, moeten worden gereviseerd.

In de prospectieve observationele studie gerapporteerd in **hoofdstuk 4**, hebben we de cutane microcirculatie gemeten van 60 preterm geboren neonaten (zwangerschapsduur onder de 32 weken) in de eerste maand na geboorte. Dit is de grootste studie van cutane microcirculatie in preterm geboren neonaten. Metingen werden verricht op dag 1,3,5, 7, 14 en 28 dagen na geboorte. Videobeelden werden offline geanalyseerd met gebruik van geautomatiseerde analyse voor TVD en handmatige analyse voor MFI. Naast demografische data werden gedurende de meting verschillende klinische parameters verzameld, zoals hartslag, bloeddruk en arteriële saturatie. De studie liet zien dat total vessel density daalde in de eerste maand na geboorte. Met gebruik van een lineair mixed model werd gevonden dat alleen postnatale leeftijd een significant effect had op de microcirculatie. Deze resultaten waren enigszins verrassend, omdat verschillende studies correlaties hadden beschreven tussen deze variabelen en de microcirculatie. Deze studie gebruikt echter een superieure statistische benadering door alle variabelen in één model te combineren. Univariate analyse liet een significant verschil zien in totale vaatdensiteit op eerste dag na geboorte tussen neonaten met een te laag en normaal geboortegewicht voor de zwangerschapsduur. Dit verschil werd niet meer gezien in daaropvolgende metingen. We veronderstellen dat dit resultaat wordt verklaard door een meer hypoxische antenatale omgeving, wat resulteerde in een verhoogde vaatgroei. Deze resultaten zijn in lijn met andere studies die de relatie hebben onderzocht tussen hypoxie/hyperoxie en de microcirculatie.

Een van deze studies is beschreven in **hoofdstuk 5**. In dit hoofdstuk wordt de microcirculatie van de huid van gezond geboren neonaten op hoogte (Puno 3820m, Peru) vergeleken met pasgeborenen op zeeniveau (Rotterdam, Nederland). In puno heerst een milieu van natuurlijke hypoxemie. In totaal werden 86 op tijd geboren neonaten gemeten in de eerste uren na de geboorte. Total vessel density was

14% hoger in pasgeboren op hoogte. Dit kwam voornamelijk door een toename in kleine en medium vessels. Zuurstofprofielen (pre- en postductale saturatie, cerebrale en regionale NIRS saturatie) waren significant lager op hoogte vergeleken met zeeniveau controle groepen. Deze bevindingen ondersteunen de hypothese dat zuurstof blootstelling tijdens de zwangerschap de microcirculatie beïnvloed. Een subgroep analyse van de pasgeborenen op hoogte liet zien dat vessel densiteit onafhankelijk was van de ethnische achtergrond, echter de getallen in deze groepen waren klein.

Deel II. Reproduceerbaarheid van moderne diagnostische technieken die worden gebruikt in critical care

In deel 2 van dit proefschrift presenteren we de resultaten van reproduceerbaarheidsstudies van verschillende nieuwe technische apparaten. De reproduceerbaarheid van handmatige analyses van buccale en cutane microcirculatie wordt gepresenteerd in **hoofdstuk 6**. Met gebruik van SDF imaging werd de cutane en buccale microcirculatie gemeten van op tijd geboren baby's. Vaatdensiteit werd onafhankelijk beoordeeld door twee onderzoekers. De reproduceerbaarheid van buccale vaatdensiteit was goed, met intra class coëfficiënten voor total en perfused vaatdensiteit boven de 0.9, een verwaarloosbare bias en acceptabele limits of agreement. De reproduceerbaarheid van de cutane microcirculatie was echter slecht, met een ICC voor total en perfused vaatdensiteit onder de 0.4 en met grote bias. Deze studie laat de verscheidenheid van de microcirculatie zien en de verschillen tussen twee gebieden. Het is moeilijker om aan de kwaliteitseisen te voldoen bij de cutane microcirculatie, wat resulteert in meer artefacten en beelden die moeilijker te analyseren zijn. Deze studie laat ook de noodzaak van standaardisatie van microcirculatoire analyses zien, in het bijzonder voor cutane analyses.

In **hoofdstuk 7** wordt de reproduceerbaarheid van de pleth variability index (PVI) besproken. De PVI is een non-invasieve parameter gebaseerd op de perfusie index (PI) gedurende een complete ademhalingscyclus. Het wordt berekend uit een standaard saturatiemeting. De PVI zou nuttig kunnen zijn in het voorspellen van fluid responsiveness en het monitoren van vullingsstatus van (preterme) pasgeborenen. De herhaalbaarheid van de metingen in de specifiek patiëntencategorie preterme neonaten is echter nog niet duidelijk. In 25 hemodynamisch stabiele preterme neonaten werden drie, vijf-minuut durende metingen uitgevoerd welke tegelijkertijd twee extremiteiten maten. Er werd een brede spreiding aan PVI waarden gevonden, zowel binnen als tussen patiënten. De meeste vergelijkingen lieten een matige-tot-acceptabele reproduceerbaarheid zien, de herhaalde meting op de rechter hand liet een goede reproduceerbaarheid zien.

Een soortgelijke studie wordt in **hoofdstuk 8** besproken. Dit hoofdstuk concentreert zich op de reproduceerbaarheid van laser doppler spectrometrie metingen van het Oxygen to See (O2C) apparaat. In 26 term geboren neonaten werden 2 achtereenvolgende metingen gedaan met twee identieke sensoren. Tijdens de eerste

meting waren de sensoren geplaatst op de rechter pols en linker pols en tijdens de tweede meting op de rechter pols en rechter voet. De resultaten waren soortgelijk als die van de reproduceerbaarheid van de PVI. Grote verschillen werden gevonden binnen en tussen patiënten, de reproduceerbaarheid tussen verschillende extremiteiten was slecht en de reproduceerbaarheid van de rechter pols was matig tot goed.

In de discussie (**hoofdstuk 9**) worden de bevindingen van dit proefschrift, algemene beschouwingen, aanbevelingen en toekomstperspectieven uiteengezet. Elk onderdeel van microcirculatie onderzoek komt aan bod, met speciale aandacht voor (het brede scala aan) uitkomstparameters, kwaliteit van de beelden en analyse van de beelden. De vele uitkomstparameters induceren 'type 1 fouten' roept vragen op als "wat is de meest belangrijke uitkomstparameter?". Het is een goede zaak dat kwaliteit van beelden een meer belangrijke rol is gaan spelen binnen het onderzoeksgebied. Hoge kwaliteit van beelden is essentieel voor betrouwbare resultaten, of ze nu handmatig of geautomatiseerd geanalyseerd worden. Onderzoekers moeten zich bewust zijn van problemen en de moeilijkheid van het verkrijgen van beelden. Twee 'kwaliteits-handleidingen' zijn momenteel beschikbaar: Het bekende 'Ronde tafel conferentie manuscript' uit 2007 welke 5 sleutelpunten geeft en de 'Microcirculation Image Quality Score' van Massey en collega's. Voor cutane microcirculatiemetingen hebben we een stap-voor-stap videohandleiding gemaakt met extra tips en trucs

De bestaande literatuur van microcirculatie bij preterm geboren neonaten is beperkt en is afkomstig van één onderzoeksgroep. Het is onduidelijk en moeilijk te doorgronden, in elk manuscript wordt bijvoorbeeld een andere uitkomstparameter gegeven. Er zijn serieuze zorgen over de kwaliteit van de beelden en de gebruikte statistische methodes. Vanuit dit oogpunt is het zeer belangrijk dat er meer standaardisatie komt. Dat is de belangrijkste aanbeveling en op dit gebied valt nog veel winst te behalen.

APPENDIX

LIST OF ABBREVIATIONS

AGA	Appropriate for Gestational Age
BW	Birth Weight
cFTOE	Cerebral fractional tissue oxygen extraction
CI	Confidence intervals
CPAP	Continuous Positive Airway Pressure
CVP	Central Venous Pressure
GA	Gestational Age
ICC	Intra-class Correlation Coefficient
IDF	Incident Dark Field
iNO	inhaled nitric oxygen
MAP	Mean arterial blood pressure
MFI	Microvascular Flow Index
NICU	Neonatal Intensive Care Unit
NIPPV	Nasal Intermittent Pressure Ventilation
NIRS	Near Infrared Spectroscopy
OPS	Orthogonal Polarization Spectral
PaO_2	Partial Pressure of Arterial oxygen
PDA	Patent Ductus Arteriosus
PE	Pre-eclampsia
PEEP	Positive End Expiratory Pressure
PI	Perfusion Index
PP	Pulse Pressure
PPV	Pulse Pressure Variation
PVD	Perfused Vessel Density
PVI	Pleth Variability Index
$rScO_2$	Cerebral oxygen saturation
SD	Standard Deviation
SDF	Sidestream Dark Field
SGA	Small for Gestational Age
SIMV	Synchronized Intermitted Mandatory Ventilation
SVI	Stroke Volume Index
TEWL	Transepidermal water loss
TVD	Total Vessel Density

PHD PORTFOLIO

Name PhD student:	Hugo A. van Elteren
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PhD period	2012-2016
Promotor	Prof. dr. I.K.M. Reiss
Copromotors	dr. R.C.J. de Jonge

1. PhD training	Year	ECTS
General courses		
BROK ('Basiscursus regelgeving Klinisch Onderzoek')	2012	1.0
Cursus Integriteit in Wetenschappelijk onderzoek	2012	1.5
NIHES CCO2 (Biostatistical Methods for data analysis)	2012	5.7
CPO (Centrum voor patient gebonden onderzoek)	2013	0.6
Methodologie van Patiëntgebonden Onderzoek en Voorbereiding van Subsidieaanvragen	2013	0.3
CRM training (Crew Resource Management)	2014	1.0
Specific courses		
Video Microscopy training, Rotterdam	2012	1.0
Oral presentations		
Joint Researchmeeting Erasmus MC – Sophia, Rottedam	2013	0.3
ESPNIC, Rotterdam (invited speaker)	2013	1.0
Refereeravond Neonatologie, Rotterdam	2013	0.3
Neonatal Fellow days, Nijmegen	2014	1.0
International Fluid Academy Days, Anterwerpen	2014	1.0
NVK Congres, Veldhoven (invited speaker)	2015	1.0
2nd Round table conference in microcirculatory research, Amsterdam	2015	1.0
Poster presentations		
PICC Istanbul, Turkey	2014	0.6
PAS joint meeting, Vancouver	2014	0.6
Committees		
ESPNIC Neonatal and Pediatric Microcirculatory Research Working Group	2013	-
Seminars and workshops		
Study Design: Beyond simple randomization, Rotterdam	2012	0.3
Cerebral and somatic oxygenation, Groningen	2012	0.6
ISICEM Brussel, Belgium	2013	1.0
Sophia Research day, Rotterdam	2013	0.3
Themaweek Hemodynamiek en microcirculatie, Rotterdam	2014	1.0

Weekly researchmeeting neonatology	2009 - 2015	1.0
Erasmus MC PhD day, Rotterdam	2015	0.1

2. Teaching

Supervising medical student master's thesis

V.J. van den Berg	2013	1.4
W.J. den Boogert	2014	1.4
H. Yska	2015	1.4
N. N. Gassmann	2015	1.4

Lecturing

Clinical lectures for nurses and medical staff	2013 - 2015	2.0
Microcirculatory training for multiple researchers	2013 - 2015	2.0
Minor for medical students 'mystery of creations'	2015	0.3

ECTS = European Credit Transfer and Accumulation System
1 ECTS represents 28 hours

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CURRICULUM VITAE

Hugo Adriaan van Elteren was born on June 22, 1985 in Voorburg. He grew up in Moerkapelle and completed secondary school in Gouda at the Coornhert Gymnasium in 2003. Afterwards, he started his medical training at the Leiden University Medical Centre (LUMC). After a successful and entertaining period, he completed his study in 2010. He did residencies in pediatrics and neonatology at the Reinier de Graaf Gasthuis in Delft and Sophia Children's Hospital in Rotterdam. In 2012 he started his thesis in the department of pediatrics, division of neonatology of the Sophia Children's Hospital (supervisors Prof. dr. I.K.M Reiss and dr. R.C.J. de Jonge). During this research, he became an expert in microcirculatory measurements in the neonatal and pediatric population. He trained and educated several staff members, residents and medical students. After his PhD he became medical liaison at Amgen, serving the patients from a biopharmaceutical perspective.

In 2015 Hugo married Thirsa Visser and they live happily together in Den Haag. In 2016 they became proud parents of their daughter Rosa Bodine van Elteren.

The Microcirculation in Preterm Neonates

Preterm neonates are arguably the most vulnerable group of patients in healthcare. Their immaturity and small size make invasive diagnostic tools hard to apply. Hemodynamics is therefore a neglected topic in the field of neonatal intensive care. Non-invasive monitoring techniques are urgently needed to improve quality of care. Imaging of the cutaneous microcirculation is a promising technique that might add value to neonatal critical care. In this thesis, the application of microcirculatory imaging in term and preterm infants is further explored. It comprises reproducibility studies, tutorials on neonatal microcirculatory imaging and results of the largest observational study performed in preterm infants.

During his PhD, Hugo van Elteren performed thousands of microcirculatory measurements at the neonatal intensive care unit and maternity ward. He became an expert in the field of cutaneous microcirculatory imaging and published his results in leading medical journals.