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Microcirculatory assessment of red blood cell transfusion in children with severe anemia

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Index

List of figures

List of tables

1. INTRODUCTION

1.1. Anemia

1.1.1. Definition

Anemia is classically defined as a deficiency of red blood cells or hemoglobin leading to a reduction in the oxygen-carrying capacity of blood. [1]

1.1.2. Etiology

Anemia occurs as a result of excessive blood loss (hemorrhage), impaired production (ineffective hematopoesis) or blood cell destruction (hemolysis). [2]

1.1.2.1. Cancer-related anemia

No consistent definition of what constitutes anemia in pediatric oncology exists.[3] Children with cancer frequently develop anemia both from the disease and chemo- and radiotherapy. Cancer-related anemia is multifactorial and often presents both acute and chronic components.[4,5] Impaired production may be caused by an infiltration of the marrow by malignant cells, which produces a slow decrease in the hemoglobin level.

Suppression of erythropoiesis is often related to iron deficiency and to impaired use of iron stores, but can also be a direct effect of chemotherapy or radiation treatment. Blood loss may be due to hemorrhage (facilitated by concomitant thrombocytopenia), repetitive blood sampling, infection and hemolysis. The anemia is usually normochromic and normocytic with a low reticulocyte count. Children receiving immune suppressive therapies over longer periods of time may experience chronic anemia with little possibility to recover fully between cycles of chemotherapy or radiation.[6]

1.1.3. Epidemiology and Morbidity

A survey was conducted in Europe with the objective of determining the incidence of anemia in pediatric oncology. Results showed that over 80% of patients were anemic (WHO: hemoglobin <11 g/dL) regardless of tumor type; 97% of patients with leukemia, which is the most prevalent type of cancer (34% of the total population), were anemic.

Death due to chronic anemia is extremely uncommon because the cardiovascular system can adapt well to the respective condition. Morbidity is also extremely rare and is normally caused by the primary disease rather than the anemia per se. [5]

1.1.4. Physiology

In order to attain adequate tissue oxygenation, the delivery rate of oxygen transported from the lungs to the peripheral tissues must satisfy the metabolic requirements. Oxygen is delivered by hemoglobin, which is carried by red blood cells, erythrocytes, and transported via bloodstream to the tissue. [7]

Oxygen has a low solubility in plasma; therefore it is specifically RBC flow that determines oxygen delivery. Consequently, the oxygen carrying capacity of the RBC plays a crucial role in the convective transport of oxygen to the organs and tissue. Oxygen binds co-operatively with hemoglobin within the RBC, in a way that changes its tetrameric conformation. As Hb alternately binds oxygen and releases it to the local tissues, it switches from a relaxed, high oxygen affinity structure, to a tense, low oxygen affinity structure. A hemoglobin molecule binds up to four oxygen molecules in a reversible way.

The binding of the first molecule is difficult. However, as more oxygen molecules bind, the affinity of hemoglobin for oxygen increases. When the fourth molecule binds to hemoglobin, the affinity decreases again. The reason is on one hand the crowding of the hemoglobin molecule, on the other hand the natural tendency of oxygen to dissociate. [8]

1.1.5. Signs and Symptoms

The signs and symptoms of anemia depend on the amount of reduction in oxygen-carrying capacity of the blood. More specifically on how much blood volume is lost and in what time frame this changes occur and on how well the cardiovascular and hematopoetic systems are able to compensate for this loss.

Chronic anemia primarily manifests itself with pallor and a gradual onset of fatigue. Fatigue is a frequently unrecognized and untreated complication of anemia. Other symptoms are headaches, dizziness, dyspnea, irritability, faintness, poor feeding, loss of appetite, inactivity, loss of concentration, change in behavior and poor school performance.[9] Cardiac enlargement and signs of congestive heart failure can occur with either blood loss or chronic severe anemia. Other clinical manifestations of modest to severe anemia include tachypnea, tachycardia, prominent arterial pulses and bruits. The increase in cardiac output and heart rate associated with decreased peripheral resistance and decreased blood viscosity may cause hemic murmurs. Gallop rhythm may be present in a hemodynamically compromised state. Normally these signs and symptoms respond quickly to treatment with transfusion.[6]

The longterm effects of chronic anemia in young patients are poorly understood but may include neurocognitive impairment, as well as retardation of growth and development.[10]

1.1.6. Therapy

The most frequent treatment employed for children with severe or chronic anemia is red blood cell transfusion (RBC Tx). Less than 5% of patients receive drug treatment (which consisted mostly of folic acid or iron). Very few patients receive recombinant human erythropoietin (rHuEPO, epoetin alfa) to treat anemia.[5] The purpose of RBC-Tx is to increase the amount of RBCs at the microcirculatory level and thus increase oxygen delivery to parenchymal cells. However, transfusion practices remain controversial, considering its significant risks and limited scientific background. The risks include transmission of infectious agents, immunologic consequences, increased organ dysfunction and acute lung injury, as well as increased mortality. [11] Due to a lack of scientific studies, guidelines for transfusion in infants and children have been established, by taking standards from adult medicine and adapting them to the patient's clinical

status.[12] However, transfusions are given less frequent in pediatrics, because normal hemoglobin values are lower in healthy children than in adults and children are better able to compensate for RBC loss. Adolescents often do not tolerate the symptoms of anemia as well as younger children. A hemoglobin level <7 g/dL with clinical symptoms usually needs an intervention with transfusion support, whereas moderate anemia (>7 g/dL) may only require close monitoring. However, assigning an absolute level at which to transfuse is difficult since the requirement depends on various factors. The need for immediate red cell transfusion is determined by the etiology and expected duration of the anemia. One also has to put in consideration the patient's ability to compensate for the decreased volume and resultant lack of oxygen-carrying capacity. Considerations also include anticipated procedures and risk of prolonged bleeding. Normally children compensate very well and may be asymptomatic despite low hemoglobin values of even 4 to 5 g/dL. No evidence suggests that such low hemoglobin concentrations pose any systemic problems, but low concentrations can be distressing to children and families. [6] Some studies have shown that maintaining a higher hemoglobin level during chemotherapy results in a better quality of life and may affect survival. [13–15]

1.2. Definition of pediatric infection

- Infection

The International Pediatric Sepsis Consensus Conference in 2005, defined infection as: **"**A suspected or proven (by positive culture, tissue stain, or polymerase chain reaction test) infection caused by any pathogen OR a clinical syndrome associated with a high probability of infection".[16] Strong evidence of infection includes positive clinical infectious signs, imaging, or laboratory tests (e.g., white blood cells in a normally sterile body fluid, chest radiograph consistent with pneumonia, petechial or purpuric rash).

The symptoms that result from these infections may be caused by a wide range of bacterial and viral pathogens, their clinical manifestations however, are very similar.[6]

- Sepsis

The International Sepsis Definitions Conference 2001 defined sepsis as a systemic inflammatory response syndrome (SIRS) in presence of a suspected or proven infection. This definition has later been accepted for pediatric cases. [16,17]

- Severe Sepsis

Severe sepsis is defined as sepsis plus one of the following criteria: cardiovascular organ dysfunction or acute respiratory distress syndrome or two or more other organ dysfunctions.[18]

- Septic Shock

Septic shock is a sepsis that causes cardiovascular organ dysfunction, which results in hypotension despite adequate fluid management and resuscitation. [16]

1.3. Microcirculation

1.3.1. In a nutshell

Microcirculatory function is essential for adequate organ function. Although the macrocirculation (compromised of the heart and large arteries) distributes blood flow globally throughout the body, it is especially the microcirculation that coordinates blood flow to tissues and is the principal area of tissue oxygen transportation. Thus, an intact and functional microcirculation is not only a critical element of the cardiovascular system, but moreover it is vital for effective tissue oxygen delivery. Furthermore its purpose also consists in transporting nutrients to tissue cells, ensuring adequate immunological functions and, in disease, to deliver therapeutic drugs to target cells. [19] [20]

1.3.2. Structure of the capillary system

The microcirculation consists of the smallest blood vessels, the arterioles, capillaries and venules. [20] The vessels on the arterial side of the microcirculation are called the arterioles. Arterioles are well innervated, surrounded by smooth muscle cells, and are 10-100 µm in diameter. Arterioles carry the blood to the capillaries. The capillaries are functionally the most important part of the microcirculation, as it is here that oxygen exchange and distribution takes place. Capillaries have a diameter of 6-12 µm, their wall consist of a thin endothelial layer and a basal lamina. They are not innervated and are not surrounded by smooth muscle cells. Blood flows out of the capillaries into the venules, which are 10-200 µm. The peripheral circulation of the whole body consists of about 10 billion capillaries. Through branching and building multiple three-dimensional networks the capillaries have a much bigger surface area compared to arteries and veins. They make up the biggest endothelial surface of the body. This is an important prerequisite for adequate oxygen exchange [21] [22]

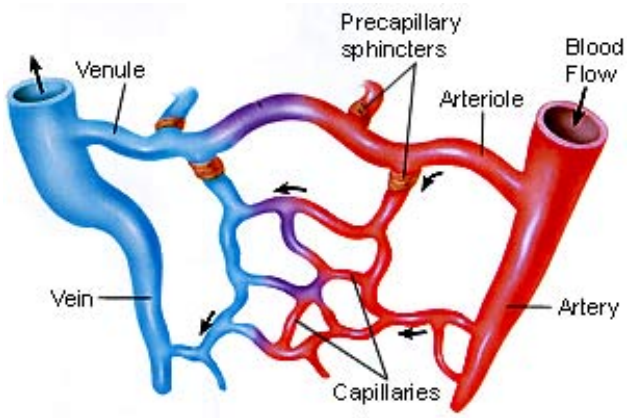
Normally only 25 % of capillaries are perfused in a tissue region. When nutritient- and oxygen demand increases, more capillaries open. [23]

Local blood flow within a tissue is regulated by precapillary arterioles. In contrast to bigger vessels these are not controlled by the autonomous nervous system but rather by local vasodilatatory metabolites, as discussed in the next section. Through change of vascular

resistance of an organ, the arterioles can regulate the total blood flow and oxygen distribution of the organ itself. [24] The structure and function of the microcirculation varies greatly among different organs. The sufficient perfusion of an organ is not primarily determined by the oxygen supply, but depends greatly on the distribution of oxygen within the organ itself.

The main cell types, which constitute the microcirculation, are the endothelial cells (lining the inside of the microvessels), smooth muscle cells (mostly in arterioles), red blood cells, leukocytes and plasma components in blood. [20]

In this study we defined capillaries as vessels with a diameter < 10 µm and arterioles and venoles as vessels with a diameter between 10-25 µm.



1.Figure, Schematic representation of the microcirculation (Source: www.biosbcc.net)

1.3.3. Characteristics of the microcirculation

Further characteristics of the microcirculation are a low partial oxygen pressure and low oxygen concentration of hemoglobin. The microcirculatory hematocrit is much lower than the systemic hematocrit and we find a wide and differing distribution of capillary hematocrit and RBC flow rates along the arteriolar tree. The reduction of the microcirculatory hematocrit is caused by tendency of red blood cells to migrate to the center of the vessel. The heterogeneity of hematocrit is based on the fact that red blood cells distribute unequally along vessel bifurcations.[25] As a consequence oxygen supply is heterogeneous within the capillary network. The diffusion distance of oxygen to the tissue is limited; therefore it is essential that a dense

microcirculatory network controls the supply of nutrients. If tissue cells are not placed in proximity to the oxygen source, the result would be a diffusion limitation of tissue oxygenation [26,27]

The above-mentioned characteristics show that blood flow by itself cannot be used as a good parameter for adequate oxygen delivery to tissue and organs. [24] [20]

1.3.3.1. Regulation of microcirculation

The regulation of tissue perfusion occurs in the microcirculation. Arterioles control the blood flow to the capillaries. They can contract and relax as the vascular smooth muscle cells respond to diverse stimuli. As a consequence microcirculation blood flow is normally steady, despite a wide range of systemic perfusion pressures. This is called autoregulation.[28] At the arteriolar level we find the greatest blood pressure gradient between the arterio-venous system. As mentioned above only 25% of all capillaries are generally perfused, depending on the oxygen and nutrition demands. The metabolic theory states that the degree of opening and closing of the metarterioles and precapillary sphincters is mostly determined by nutrition demand of the surrounding tissue.[29] To achieve this degree of control, the entire microvasculature must be highly sensitive to changing conditions (e.g. increased oxygen demand, reduced oxygen delivery). [30]

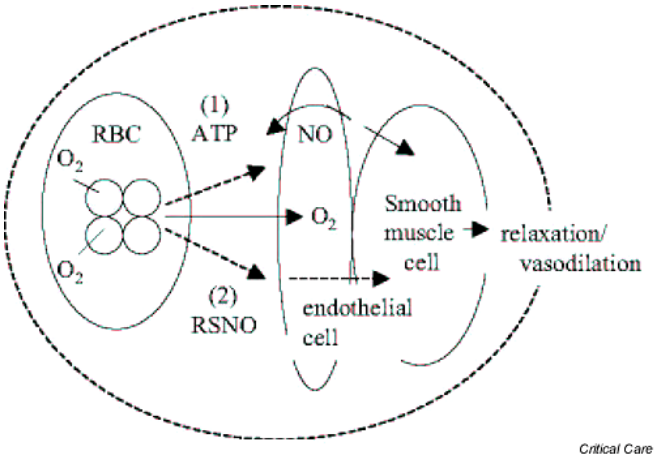
Another important component, which plays a crucial role in the regulation of the microcirculatory network, is the endothelium. The endothelial cells conduct and integrate stimulatory signals, such as changes in vascular blood flow and local shear stress, via cell-to-cell communication across the microvascular bed. [31,32] This means that the vascular endothelium has the capacity to transfer a dilatory stimulus from one region of the capillary bed, to the supplying arterioles of these capillaries and thereby increasing blood supply. Larger arterioles respond to these changes by dilating and restoring local shear stress back to baseline and thereby contribute in further reducing vascular resistance. [33] Vasodilation is achieved by the vasodilatory molecule nitric oxide (NO), which is produced by the enzyme nitric oxide synthase (eNOS) located in the endothelial cells. [34,35] There have been extensive reviews on the central role and vital importance of nitric oxide in maintaining microcirculatory blood flow, especially when the microcirculation is harmed (such as in sepsis), as discussed later on. [36]

1.3.3.2. ATP, NO and the role of RBCs in regulation of oxygen delivery

Another important vasodilator is adenosine triphophosphate (ATP), which is released by RBCs in hypoxic regions. When released, ATP causes vasodilation and thereby increases blood flow and improves local oxygen delivery.[37,38] ATP release is linearly related to hemoglobin oxygen saturation. [39]

Additionally RBCs play a crucial role in regulating oxygen delivery through the transport of nitric oxide (NO). [40] NO is released by hemoglobin molecules when hemoglobin oxygen saturations falls. It has been postulated that deoxygenated hemoglobin itself acts as an enzyme called nitrite reductase that converts nitrite to NO. Through that mechanism it is possible for RBCs to cause a vasodilatation of arterioles in response to local hypoxia. [41]

Through the above-mentioned methods RBCs are able to monitor and regulate oxygen delivery at a microcirculatory level. [42]



2.Figure, Schematic representation of ATP dependent vasodilation (Source: Bateman e al.43)

Essentially RBCs and the endothelium of vessels play a crucial role in regulating and coordinating the arteriolar response to changes in oxygen demand and delivery. As long as the microvascular network is functional and capillary density is sufficient, oxygen will be delivered properly within an organ. [24] The past few decades have shed great light on the flow regulation of the microcirculatory network and highlighted the need for further mathematical and computational approaches to this complex phenomenon. [28]

1.3.4. Dysfunction of the microcirculation

In the critically ill patient there are different global parameters of hemodynamic and oxygen transport that can be assessed and provide important information on the status of the cardiovascular system. These include cardiac output, arterial pressure, vascular resistance, blood gases, oxygen consumption, oxygen extraction and lactate. These parameters are easily measured and therefore used to judge the circulatory function in clinical settings. However, these parameters remain inadequate and unreliable when estimating the hemodynamic situation, especially during critical illness, irrespective of the physician's experience. [44,45] The problem is, that global oxygen transport parameters, as named above, fail to evaluate the status of the microcirculation, which is necessary for adequate organ function. It is on the microcirculatory level that oxygen, nutrients and inflammatory, as well as coagulation factors are delivered and distributed. It is the microcirculation that removes metabolic waste products, heat and carbon dioxide. [43]

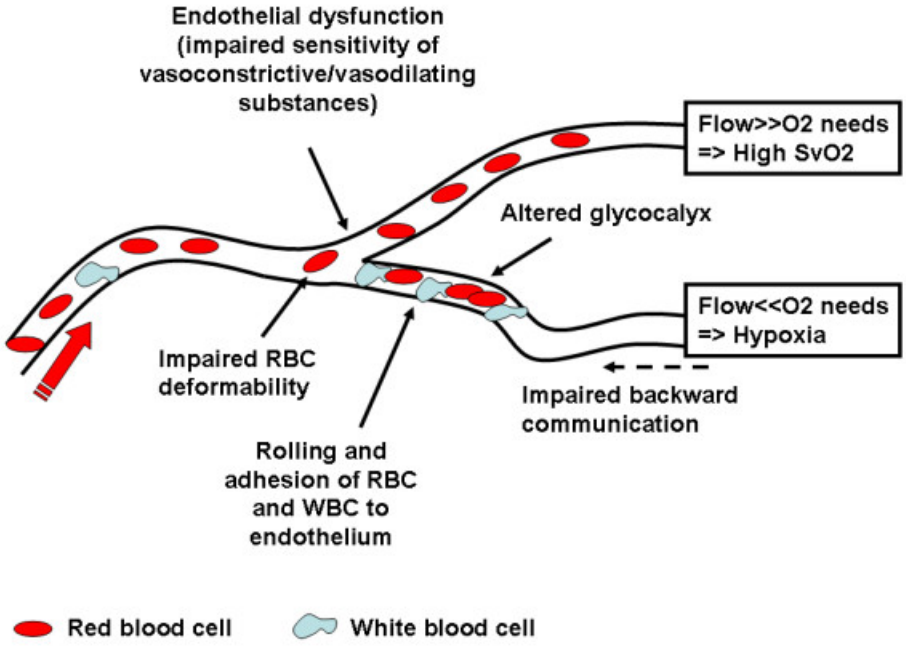
The recent development of new medical imaging techniques, in combination with data from clinical research, has helped to identify microcirculatory dysfunction as a key factor in the pathophysiology of a variety of systemic pro-inflammatory states and shock etiologies including septic shock, cardiogenic shock and ischemia/reperfusion injury. [46,47]

Numerous experimental studies have investigated the microcirculation during sepsis and found out that evaluation of sublingual microcirculatory blood flow is prognostic of outcome and may provide important and specific physiological information that macrocirculatory parameters cannot. They concluded that disturbance and alteration of microcirculatory blood flow appear to be the critical pathogenic event in sepsis and has been linked to acute multiorgan failure and mortality. [20,48–50]

Microcirculatory alteration in sepsis is multifactorial in nature and includes: autoregulatory dysfunction, heterogeneous expression of NO, increased RBC aggregation and impaired RBC deformability, increased leukocyte expression, as well as formation of microthrombi and capillary leakage.

Normally the microcirculation, with an intact regulatory system (such as vascular endothelium and RBCs) and sufficient capillary density, can deliver oxygen to specific places where it is needed within an organ. Microcirculatory dysfunction is characterized by decreased capillary density and heterogeneous abnormalities in blood flow.[43,51] The pathophysiological change and critical factor in early sepsis is the inability of the microcirculatory network to compensate for the loss of functional capillary density. The impaired ability to control local oxygen distribution results in severe tissue hypoxia, even when oxygen supply to the organ is adequate. [24]

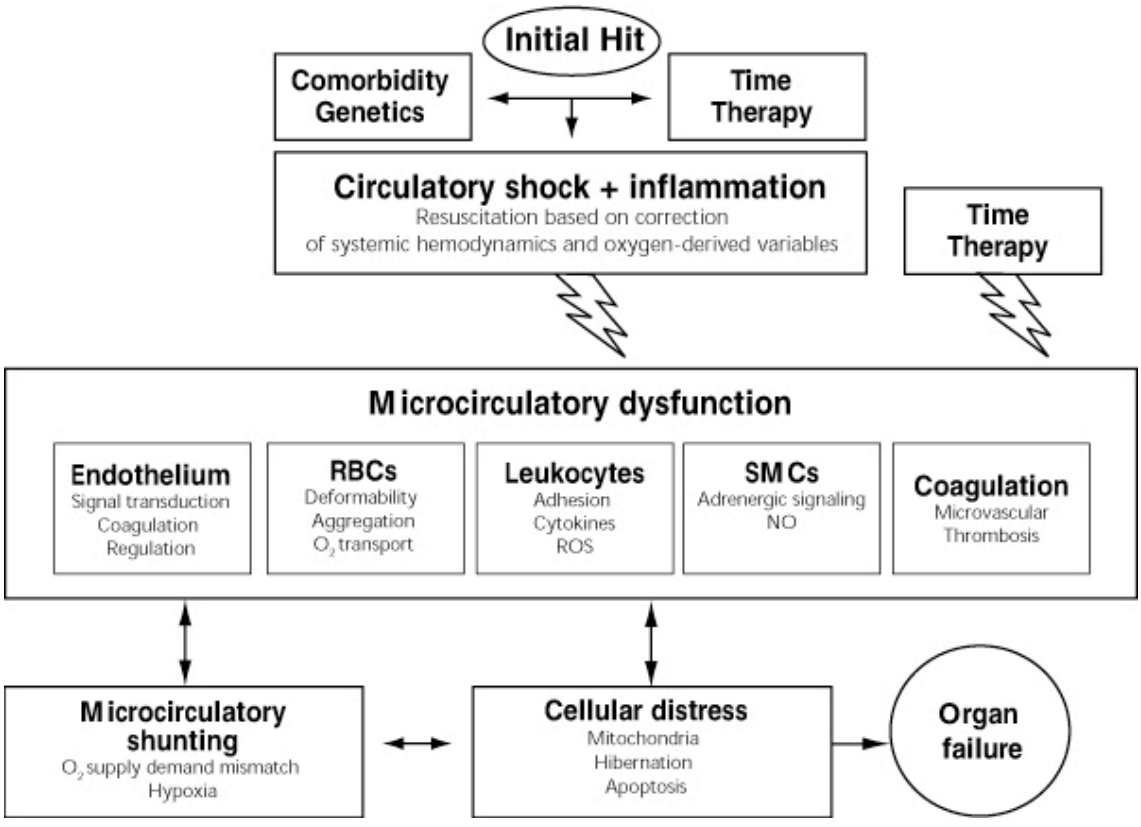
Left uncorrected, microcirculatory dysfunction causes respiratory distress of parenchymal cells. This in consequence leads to organ failure. [20] A study by Sakr. et al demonstrated that changes in perfused microvessel density during the first 24 hours of sepsis were predictive of outcome and mortality. [54] Other studies have shown that microcirculatory flow is more notably deteriorated and more heterogeneous in sepsis nonsurvivors when compared with sepsis survivors. [46]



3.Figure, Principal mechanisms implicated in the development of microcirculatory alterations (Source: de Backer et al. 55)

In regard to children, Top et al. described such microcirculatory alterations in the buccal mucosa of children with sepsis. They were able to show that persistent microcirculatory alterations in children with septic shock are linked with a poor clinical outcome. [56] Similar changes were observed in the skin of premature neonates with infection using OPS imaging. The authors assumed that the observed alterations might be predictive of infection, even before clinical suspicion emerges. [57]

Collectively, all the data indicates that early deterioration of microcirculatory blood flow is correlated with lower survival, thus making the assessment of microcirculation in resuscitation and goal-directed cardiovascular support extremely important.



4.Figure, Development of microcirculatory dysfunction (Source: Ince et al. 20)

1.3.5. Microcirculation of the pediatric patient

The structure of the microcirculation experiences great developmental changes within the first few weeks of life in a healthy neonate. In the first month of life the capillary density decreases significantly. The change in FCD correlates with the decrease of hemoglobin that happens during this time period. [58,59] The microcirculation reaches an adult pattern at the age of approximately 3 months. [60]

1.3.6. The effect of blood transfusion on the microcirculation

In clinical settings, global parameters of perfusion (such as blood pressure, base deficit, hemoglobin and hematocrit) are usually measured to assess the response to transfusion. However, only a few studies have investigated the effects of RBC transfusion on peripheral microcirculation. Until now there have been no microcirculatory investigations in anemic children and adolescents.

Sakr et al. found no consistent effect of blood transfusions on the sublingual microvascular perfusion in a group of patients with severe sepsis, however considerable interindividual variability. [61] In a cohort of trauma patients the microvascular results were quite variable and depended greatly on the baseline perfusion.[62] Yuruk and colleagues came to similar conclusions using near-infrared spectroscopy (NIRS).[63] Contrary to these results, SDF imaging after blood transfusions in non-anemic cardiac surgery patients demonstrated improved microcirculatory parameters and microcirculatory oxygen saturations.[64] Genzel et al. found an improved microvascular perfusion in anemic preterm infants after RBC transfusion.[65]

The contrasting results of these studies will be further evaluated in the “discussion” section of this thesis and compared to the microcirculatory data of our study.

1.3.6.1. Transfusion of stored red blood cells

During the past few decades many researchers have tried to evaluate the effect of stored red blood cells in transfusion therapy. Weinberg et al have repeatedly documented significant correlation between RBC age and adverse clinical outcomes. In his studies the transfusion of older RBCs was associated with an increased risk of mortality. [62,66,67]

It has been shown that storage leads to a decreased RBC pH and ATP levels, which alters the shape and rheological properties of the RBC. [68] The changes mainly involve a loss of deformability, which again impact the flow in the microcirculatory network. [69,70] Additionally it has been shown that stored RBCs can occlude the microcirculation by adhering to the endothelium. [71,72]

As stated above, Nitric oxide (NO) plays a fundamental role in maintaining normal vascular function. Normally, the intact RBC membrane acts as a diffusion barrier and thereby restricts NO scavenging by intra-erythrocyte hemoglobin, allowing sufficient NO escape for vasodilatation. [73] During storage the integrity of the RBC membrane is reduced, which causes the cells to break down (hemolysis) and consequently leads to the formation of cell free hemoglobin. It has been postulated that this hemolysis of stored RBCs may be the most fundamental storage lesion, causing disruption of the NO-mediated vasodilation and potentiate vasoconstriction, in a manner similar to pathologic hemolytic conditions. [74–76]

1.3.7. Diagnostics in microcirculatory assessment

Various diagnostic approaches can be used to assess perfusion within the capillary network:

Testing the capillary filling time is an easy tool to assess the microcirculatory function in any setting. However, the information value is very limited, due to subjective evaluation and external influences. Furthermore, an elevated level of lactate can be a good marker for an anaerobic metabolic state and an indicator for disturbed oxygen supply to the tissue. [77] Thus, blood lactate levels can be seen as an indicator for altered microvascular perfusion. However, serum-lactate levels can be influenced, especially by liver- and renal failures.[78] Therefore, a higher lactate level cannot be taken as a specific sign indicating tissue hypoxia.

The following modern technological developments have made the direct assessment of microcirulatory parameters in patients possible [79,80 81]:

- **Laser- Duplex- Fluxometer (LDF)**

  The LDF is a noninvasive method that emits laser light, which is scattered and reflected in the tissue. Moving objects (e.g. red blood cells) cause a change in frequency through doppler effect that is proportional to the amount of red blood cells and their velocity. However, this method presents some major disadvantages, one being the lack of penetration depth of laser light. Other shortcomings are the great variability of the evaluated parameters and the lack of absolute values.

- **Venous compression-plethysmography**

  The circumference difference of an extremity after venous congestion can be measured and consequently the hydrostatic microvasculatory pressure can be calculated. However the long duration of this procedure and the likeliness of movement disturbances are reasons why this method is not being used to evaluate a patient's microcirculation in clinical settings.

- **Near-Infrared Spectroscopy (NIRS)**

  This method measures the oxygen saturation in tissue through laser light. A major disadvantage is the high variability of the calculated results.

- **"Invasive" intravital-microscopy**

  This is a method that requires the use of fluorescence light as a marker. Therefore it is primarily used in animal experiments, e.g.: the dorsal skinfold of hamsters.

- **"Non-invasive" intravital-microscopy**

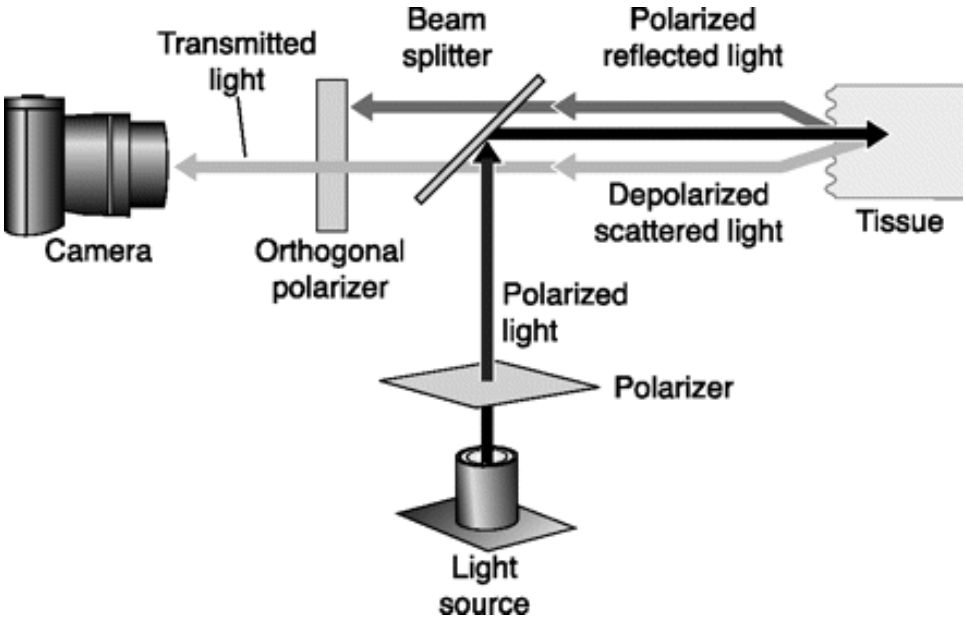
  Noninvasive devices for the measurement of the microcirculation include the newly developed, hand-held microscopes with Orthogonal Polarization Spectral (OPS) Imaging and Sidestream Dark Field (SDF) Imaging. These methods offer a great opportunity to view the microcirculation in vivo without the use of contrast agents.

  These novel techniques will be further discussed in the following section.

1.3.8. Imaging technique

A few decades ago, capillary microscopes were used to perform direct intravital observations of the microcirculation in humans. These had to be applied to the nailfold capillary bed, thus markedly hindering the microcirculatory investigation in clinical settings.[82] Through the introduction of Orthogonal Polarization Spectral (OPS) imaging by Slaaf et al., the investigation of human microcirculation in exposed organ- and tissue surfaces became possible. This method provides a functional image of the microcirculation by using orthogonal polarized light.[83] Compared to conventional, noninvasive capillary microscopes OPS imaging offers a considerable improvement in image quality.[84] Various studies have been performed in different clinical scenarios where cardiovascular function is at risk, e.g. during cardiac surgery.[85] Studies have especially been made in exploring the microcirculation in emergency- and intensive care medicine[20,46,48], as well as during sepsis, shock and resuscitation. [20,48,49,86] Different medical centers and researches have shown that OPS imaging of sublingual perfusion can provide more sensitive and specific information on outcome from sepsis and shock, when compared to conventional hemodynamic parameters. [20,86]

The OPS technique consists of a handheld device that illuminates an area of interest with polarized light. Within the tissue the light is scattered and depolarized, only on the skin surface the light remains polarized. The remitted light goes through a second polarizer (analyzer), oriented orthogonal (90 degrees) to the area of illumination. This analyzer blocks the undepolarized light, which is reflected by the tissue surface. By eliminating the reflected light, the camera recognizes only the scattered, depolarized light in the depth of the tissue. By blocking the reflected and polarized light, the reflections of skin and mucous membranes is eliminated. The backscattered light can be imaged and subsurface structures, such as the microcirculation, can be pictured.[87] [88] If one chooses a wavelength that lies within the hemoglobin absorption spectrum (548nm), red blood cells will appear dark against a lighter background. This happens because hemoglobin absorbs the reflection of green light, and only the depolarized reflections of the surrounding tissue and vessels are captured by the video camera. The pictures obtained are black and white, one-dimensional images and present a "negative" image of the microcirculation. Through OPS imaging it is not possible to visualize the vessel walls directly, their imaging depends on the presence of red blood cells in a vessel lumen.[50]



5.Figure, Schematic representation of OPS imaging (Source: Vollmar et al. 89)

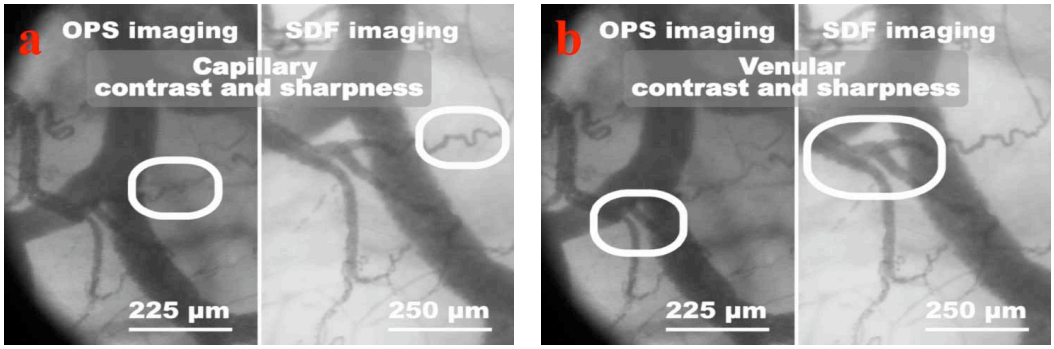
This method has been validated in animal models against intravital fluorescence microscopy.[83,90] A comparison of fluorescence intravital microscopy with OPS imaging in the awake Syrian golden hamster showed equivalence in measured physiological parameters, such as functional capillary density (FCD) and showed no significant difference in contrast between the two methods.[83,91,92] These results were also ratified through other experiments on liver surfaces of rats[93], on pancreatic glands of rats [94] and on the dorsal skinfold chamber of hamsters.[95]

OPS imaging has contributed immensely in the field of intravital microcirculatory imaging, however several shortcomings have been noted.[87,88,96] Both reflected and emitted light pass down the same light guide (mainstream), OPS imaging is therefore highly sensitive to internal scatter of light. As a consequence this can lead to blurring and limited visualization of the capillaries, and the measurement of blood cell velocities is hindered. The technique also requires high-power bulky light sources, thereby reducing its applicability in difficult circumstances, such as critical care or intensive care medicine.

Driven by the success of OPS imaging, Ince et al. developed a new imaging modality for the microcirculation, which they named Sidestream Dark Field (SDF) imaging.[88] This new approach was directed at improving the above-named shortcomings of the OPS imaging. One thing they

changed was the lens system, by optically isolating it from the outer ring and adding stroboscopic LED ring-based sidestream dark field (SDF) illumination to depict the capillary network. Thus impaired images due to tissue surface reflections are minimized and better image quality with more detail, capillary contrast and less motion blur is offered.

In the materials and method section (2.3.1) the Sidestream Dark Field technique and its development and functioning principle will be further discussed in detail.



6.Figure (OPS imaging versus SDF imaging of the sublingual microcirculation) (Source :Goedhardt et al. 88)

1.4. Aims of dissertation

In both children and adults, the main goal of red blood cell transfusion is to provide sufficient cells to prevent or reverse tissue hypoxia due to limited oxygen delivery. The effects of blood transfusions on the microcirculation and tissue oxygenation are still poorly defined.
To our knowledge, no studies have yet assessed the microcirculatory response to RBC transfusion in anemic children outside of the neonatal period.

Until recently, evaluation of the microcirculation in clinical practice has not been possible and was reserved for animal studies. However, the invention of novel noninvasive tools in the past decade, such as SDF imaging, have helped clinicians and researchers to better understand the microcirculatory network and have aided in shedding light on the pathologies of several disease states. Through a better understanding of underlying principles and through the mentioned new technologies the microcirculation can be monitored more intensively and tissue hypoxia can possibly be detected and corrected earlier. The aim of this study is to better understand the effect of RBC transfusion on the microcirculation and to thus possibly contribute valuable information to ameliorate pediatric transfusion policies. The following questions, regarding the microcirculatory response to RBC transfusion, will be analyzed and evaluated:

- Do red blood cell transfusions improve microvascular perfusion of severely anemic children?
- How do concomitant infections influence the response to transfusion?
- How do microcirculatory parameters of anemic children differ from the microcirculatory parameters of a healthy control group with normal hemoglobin levels
- Does the severity of anemia (hemoglobin level) correlate with the functional vascular density?
- What role does RBC storage time play? Does RBC age matter?
- What are the implications of our studies regarding future approaches in transfusion therapy?

2. MATERIALS AND METHODS

2.1. Patient population

2.1.1. Recruitment

The study population consisted of children < 18 years of age who were diagnosed with a hematologic or oncologic disease and treated at the “Kinderklinik und Kinderpoliklinik des Dr. von Haunerschen Kinderspitals” in Munich between August 2009 until July 2010. Nineteen children who required red blood cell transfusion due to anemia were studied. The control group consisted of children who underwent minor plastic or reconstructive surgeries at the same clinic and could be considered healthy individuals. Exclusion criteria for the control group were systemic diseases, congenital diseases or any other severe mental or physical disorders.

The study protocol was approved by the ethics committee of the medical faculty of the Ludwig-Maximilians University in Munich prior to the implementation of the study.

2.1.2. Informed consent

The parents of the patients were informed and instructed extensively about the procedure before participation. For this purpose a comprehensive brochure was given to the parents and children that explained the scientific background of the study, as well as its practical implementation. A written consent signed by one of the parents (or the patient >15 years of age) was obtained prior to participation. Parents were allowed to be present during all the measurement procedures. The patients took part in this study voluntarily. Therefore they were allowed to brake off the measurements at any time and end the participation in case of reconsideration.

2.2. Study design

The prospective, controlled, observational study used Sidestream-Darkfield Imaging (SDF) to directly visualize the sublingual microcirculation in 19 children [Mean (95% CI) age: 10,2 years (8,3-12,1years)] who required red blood cell transfusion due to anemia. One child received two blood transfusions within one week and was measured twice, increasing the number of transfusions to 20. Decision to treat was independent of the study and up to the discretion of the attending oncologist. As such, the study had no protocol to interfere with the indication for transfusion. The clinical condition of the child and the blood hemoglobin level was evaluated prior to transfusion therapy. The general indications for RBC transfusion (RBC Tx) were an Hb level <7 mg/dL in oncology patients and < 10mg/dL for children with hemoglobinopathies, who received RBC-Tx at defined time periods. The measurements of the sublingual microcirculation in the anemic children [Mean (95%CI) Hb: 7,2 g/dL (6,6-7,9)] were conducted before RBC transfusion and right after the transfusion. To evaluate the long-term effects of transfusion on the microcirculation, another measurement was performed 48-72 hours after RBC transfusion. All children received a Tx of 200-300 ml [Mean (95%CI): 273 ml (252-293)] over 2-3 hours. A control group of healthy individuals was introduced, to determine potential differences in the microcirculation between anemic children and healthy children. This group included 18 children [Mean (95%CI) age: 10,3 years (8,9-11,7 years)] with normal blood hemoglobin levels [Mean (95%) Hb: 12,9 g/dL (12,3-13,5 g/dL)]. To answer the question whether concomitant infections influence the response of RBC transfusion to the microcirculation, the anemic group was further subdivided into 2 groups:

9 patients with clinical signs of infection and CRP levels > 3 mg/l **(Inf Group)**
11 patients without clinical signs of infection and CRP levels < 3mg/l **(nInf Group)**

All RBC units had undergone prestorage leukoreduction within 24h of collection by high-efficiency filters. The storage duration (days) for each RBC unit transfused was noted.

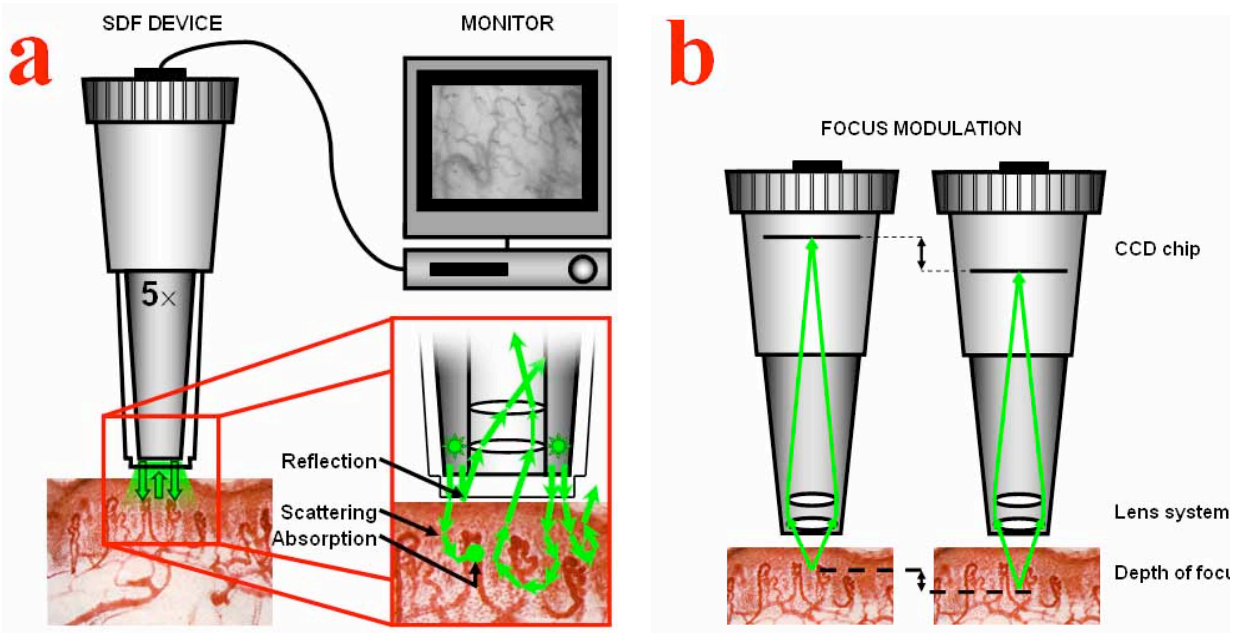
2.3. Assessment of the microcirculation

2.3.1. Functional principle

Sidestream Dark Field (MicroScan, BV Meibergdreef 45, 1105 BA Amsterdam, Netherlands) is an optical, hand-held imaging device that uses a probe with a 5x-magnifying lens to image the tissue-embedded microcirculation. This new approach is similar to its forerunner model: OPS imaging (as described in 1.3.8.), however it provides improved image quality. Due to lower energy requirements the device can be powered by battery. Illumination is provided by concentrically placed light emitting diodes (LEDs). The LEDs, placed at the tip of the probe and protected by a disposable cap, send green light (530 nm) deeper into the tissue then OPS illumination. This allows deeper sublingual arterioles and flowing blood cells to be observed more clearly. As previously stated in the explanation of OPS imaging, the visualization of the microcirculation is based on the fact that hemoglobin from the erythrocytes absorbs green light, whereas the surrounding tissue scatters light. Blood cells are thereby depicted as dark moving structures against a bright background. The light emitted by the diodes produces a wavelength of 530 nm, which equals to the isosbestic point (wavelength at which the total absorbance does not change during physical changes) of deoxy- and oxyhemoglobin. This means that absorption of the light I stable in both oxygenated and deoxygenated states of hemoglobin.

A clear advantage is that the LED lights offer a stroboscopic imaging by using pulsating illumination in harmony with the CCD frame rate, thereby allowing moving structures to be observed more clearly and preventing motion-induced blurring.

The SDF lens system is optically isolated from the illuminating outer ring, presenting another clear advantage through sending illuminated light and reflected light via two independent pathways. Thus, SDF imaging is able to prevent impaired images by tissue surface reflections (which was a common problem of OPS-imaging). A 5 or 10 times magnifying lens is used to project the image onto a video camera, providing clear images of the capillaries and allowing for better computer automatic analysis of the images. Images are recorded using a digital video recorder and visualized on a computer monitor. [88]



7.Figure, (a) The Sidestream Dark Field (SDF) imaging device, with a 5x magnifying objective lens using green-pulsed LED ring illumination Images are recorded using a digital video recorder/computer and visualized on a monitor. When the light reaches the tissue it scatters (indicated with arrows) and is absorbed by RBCs (indicated with dots. (b) The CCD chip can be axially translated with respect to the fixed lens system in the tip of the SDF probe to fine-tune the depth of focus. (Source: Goedhart et al.88)

2.3.2. Experimental setup

The MicroScan Video Microscope System consists of:

- MicroScan Imaging Unit
- MicroScan Battery Unit
- MicroScan Detachable Handle
- MicroScan Calibration Unit
- MicroScan Sterile Disposable Lenses
- Connecting Cable - A/C Adapter

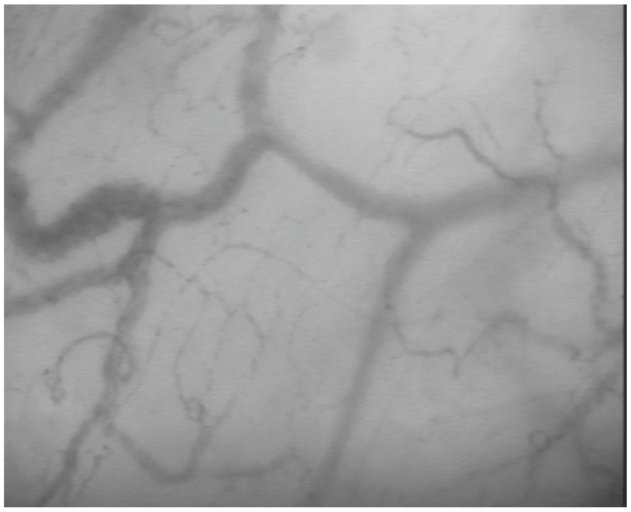
8.Figure, SDF device

The SDF system is in essence a handheld video microscope that uses LED illumination as described in the previous section. It uses a 5x magnification The Battery Unit has a duration minimum of 12 hours when used continually. Measurements can therefore be performed for a whole day, without needing to charge the battery.

The System includes a standard video-output (PAL or NTSC), which allows the user to record the measurement on any recording device with a standard video-input. Through a capture device, the image can easily be converted into a digital signal, which can be converted straight to a computer hard drive.

9.Figure, Microscan

The MicroScan uses a sterile disposable cap, the MicroScan Lens, to protect the device and allow for safe and clean measurements on patients. These can be ordered separately from MicroVision Medical (www.microvisionmedical.com)



10.Figure, Example of sublingual microcirculation as assessed with the SDF-imaging technique

(image taken from own study population)

2.3.3. Measurement procedures

During a 12-month period, measurements were performed at the hemato- and oncologic unit and at the oncologic day clinic, “Dr. von Haunersches Kinderspital”, University Hospital Munich. Children who underwent minor ambulant plastic or reconstructive surgeries served as a healthy control group and were measured at the surgical day care unit of the same hospital. These measurements were performed either before surgical interventions and anesthesia or after surgery, when patients had fully recovered from sedation.

Most of the measurements were conducted in the patient’s bed in dorsal position, since this turned out to be the best way of obtaining qualitatively good SDF video sequences. Only a few were undertaken in upright position, sitting in a chair. The devices were adjusted for optimal focus and contrast. According to the guidelines of a recent consensus report on the performance and evaluation of microcirculation[97], the SDF device was covered by a sterile disposable cap and then gently applied sublingually or on the buccal tissue surface just inside of the lower lip. In each patient 5-10 sites were examined for 15 seconds to obtain a stable image, which was stored. The best site was selected for blinded off-line analysis. Movement of either the tongue or the head limited measurement procedures and image quality, as well as unintended movements by the investigator. Some measurements had to be terminated, due to difficulties with the cooperation of some children. These were generally young children < 5 years of age, who had difficulties to understand the given requirements or agitated and anxious children, who either moved too much or simply broke off participation. All sequences of 5 children who had received RBC Tx could not be analyzed due to bad image quality or movement artifacts and were therefore excluded from analysis.

Best image quality was obtained by holding the microscope parallel to the sublingual mucosal membrane. The probes were carefully placed onto the tissue and then slightly withdrawn until contact was almost lost, in order to prevent pressure application on the image area, which could lead to perfusion alterations. Then the probes were carefully advanced again up to the point where contact was regained and the microcirculation was clearly depicted and in focus of the lens systems. However, the implementation of this theory needs operating-experience from the investigator and good cooperation from the child, since the smallest pressure can alter the

sublingual blood flow. The best way of evaluating whether blood flowed regularly was by looking at the capillary site on the screen, rather than fixing the attention solely on the SDF probe. This way a stagnant blood flow could be detected immediately and consequently moving the probe slightly from the site of interest reduced the pressure artifacts.

2.3.4. Clinical data

Clinical data was collected before RBC Tx, as well as 48-72 hours after RBC Tx. The data included temperature, heart rate and blood pressure. Further clinical data, such as admission diagnose, other co-morbidities, drugs and chemotherapies were also recorded retrospectively. Laboratory data were extracted from the charts with no blood sampling solely for research purposes. Therefore hemoglobin levels were only available before RBC Tx (pre Tx) and 48-72 hours after Tx (pTx2), since there was no routine blood sampling right after Tx (pTx1). The following data were collected: hemoglobin, hematocrit, platelets, white blood cells, red blood cells and C-reactive proteine (CRP) (the detailed parameters of each study patient can be found in the appendix). At pTx2 clinical and microcirculatory data of only 13 out of 19 study subjects could be collected.

2.3.5. Video recordings

The SDF images of all 38 patients were recorded directly with a Notebook (Fujitsu Siemens Lifebook, Microsoft Windows Pofessional XP). Video output was visualized on a monitor and connected to the computer via a signal converter.
The SDF sequences were stored to the notebook in Audio Video Interleaved **(AVI)** format. From the sublingual microcirculation images, the best capillary site from each participant was selected for further off-line analysis.

2.4. Analyzing data

2.4.1. Software configuration

AVA Software was used for image analysis. AVA is short for **A**utomated **V**ascular **A**nalysis and can perform both quantitative and semi-quantitative analysis of various microcirculatory parameters.

2.4.2. Analysis

Randomized numbers were given to the microcirculatory videos of each patient and analysis was performed in a blinded manner. Subsequently, all videos were independently evaluated by a single observer (to avoid inter-individual variability). The observer was blinded to both study patient and image sequence (i.e. pre-transfusion vs. post-transfusion). Additionally, practicing on multiple sequences prior to analysis-start minimized inter-individual variability.

Selected AVI-video-sequences were imported to the AVA program. Images were then stabilized before doing microcirculatory measurements. Normally SDF images show a region of interest of approximately 1000x750 μm. Due to such a small image scale and the use of hand-held instruments, inter-image displacement occurs. AVA performs image registration by shifting image-frames to a best-matching position (stabilization) and by cutting away the individual image edges that do not coincide with others. Stabilized images are stored as new video files (AVI) to disk. After stabilization, image-quality can be enhanced by correcting variations in the background and by adjusting image-contrast. Automated vessel-segmentation was performed with a certain single scale of analysis. In this procedure the program automatically detects vessels in the given area and marks these with defined colors. The examiner then deleted vessels manually, which were wrongfully detected by the program. Vessel segments that were not recognized were added using local image analysis on a selectable scale. After automatic vessel segmentation, quantitative velocity assessment was carried out. It has to be considered that blood flow changes at a vessel bifurcation, thereby also causing changes in RBC velocity. Therefore space-time diagrams have to be determined between vessel bifurcations. Such vessels were selected and the examiner then manually drew characteristic lines in the time-

space diagram. The program automatically creates space- time diagrams by tilting the centerline intensity of a vessel as vertical lines. The line orientation is indicative for RBC velocity. The user can overrule the result of automatic analysis by tracing lines in the space–time diagram interactively. Finally, the acquired orientation is converted to an actual velocity value. [98] In addition semi-quantitative velocity classification per vessel was performed. All vessels were assigned to different categories and marked accordingly. Vessels are considered "perfused" if they are assigned one of the following velocity classifications: sluggish flow, continuous flow and hyperdynamic flow. Other classifications, such as "no flow" or "intermittent flow" are not considered perfused. The reason for including this type of velocity classification while a quantitative method is available, is to provide a method of classification in case other classification fails, e.g. due to a very low image contrast.

The different vessel subgroups where allocated by their diameter and in this study defined as the following:

- 0 – 10 µm : SMALL
- 10 – 20 µm : MEDIUM
- 20 – 100 µm : LARGE
- > 100 µm : VERY LARGE

At last the program presents the evaluated in a microcirculatory report.

The report generator shows all analysis parameters, sorted in tables. The following microcirculatory parameters were evaluated in this study:

Consensus parameters

Included were Functional Vessel Density **(FVD)** (mm/mm^2), the Perfused Vessel Density **(PVD)** (mm/mm2), the Proportion of Perfused Vessels **(PPV)** (%)

Density distribution parameters

Flow classification per vessel

Number of vessel segments within a given diameter range and with a certain velocity classification (semi-quantitative velocity result)

2.4.3. Storing and reviewing analysis results

The results in memory are automatically stored to disk as a logbook file, when the operator switches files, starts segmenting a new image or erases the current results. Logbook files are stored as text files with extension “TXT”, which makes them readable using an elementary text editor, such as Notepad. Retrieving a logbook file can regenerate reports. This feature allows the operator to review the total analysis results, both by visual inspection of the previous results as well as by calling the report generators to get (semi-) quantitative results. It even allows continuing analysis. If changes are made, e.g., by erasing misinterpreted vessels or by adding vessel segments interactively, a new logbook file will be created when the analysis session is closed.

2.4.4. Statistical analysis

Graph Pad Prism 5.0, Version, was used for statistical analysis of data. Descriptive analysis compromised of means ± standard deviation (SD) and confidence intervals (CI) was performed. Results were considered significant at $p < 0.05$. The increase of significance was marked the following way: p=<0,05; p<0,005; p<0,0005; n.s: not significant.

The following statistical tests were used:

Column Statistics: descriptive statistics for evaluating mean, median and confidence intervals for the various measured data

Normality test: we used a D´Agostino & Pearson omnibus normality test for evaluation of normal distribution of a study population

Mann-Whitney test: The Mann-Whitney test, also called the Wilcoxon rank sum test, is a nonparametric test that compares two unpaired groups

Wilcoxon matched pair signed-rank test: a nonparametric test that compares two paired groups

Friedman test: a nonparametric test that compares three or more paired groups

Statistical data was graphically presented using Box- and Whisker plot. The box indication the first and third quartile and a bar indicating the median. The whiskers present the maximum and minimum values. Outliers are presented as dots.

Descriptive statistics were performed for the full and subgroup samples to assess similarities in patient characteristics, including age, gender, infection versus no infection and age of blood. Changes in hemodynamic and other observed measurements taken before (pre), 1 hour after (pTx1) and 48-72h after (pTx2) the transfusion were assessed by an unpaired t test. Mean, standard deviation and p value were reported for each comparison. Analysis for the full sample and subgroup were conducted separately.

3. RESULTS

3.1. Study population

Characteristics	Tx-Group	Control-Group
Age (years)	10.2 (8.3-12.1)	10.3 (8.9-11.7)*
Sex (male:female)	12:7	11:7*

1. Table, Patient characteristics, * n.s.

Forty-two children were recruited for the study. As previously described, the sublingual microcirculation was recorded in all of these children. The video sequences of five children were excluded due to bad image quality, leaving thirty-eight study subjects for further analysis. In sections 2.2 and 2.3.3 the criteria for participation and the measurement procedure has already been explained.

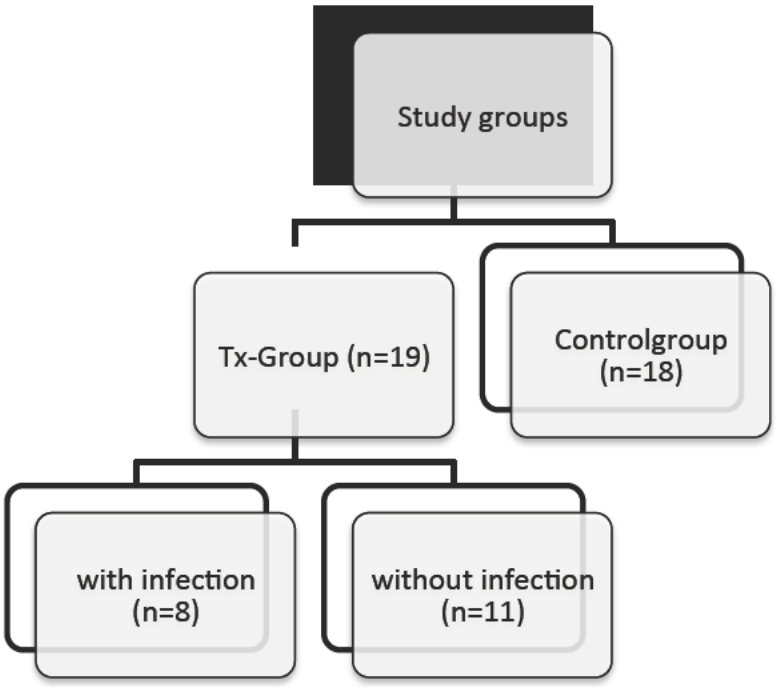
The **Tx-Group** consisted of 19 children with anemia, who received RBC transfusions. The patient characteristics, including age and gender distribution are presented in chart 1 above. There was no significant difference in the mean age or sex among the transfusion group and the control group. Within both groups the youngest patient was 4 years of age and the oldest patient was 18 years of age.

One patient was newly diagnosed with leukemia and admitted to the hospital with a blood hemoglobin level of 4,4 mg/dL. She was given a RBC Tx at admittance and subsequently another RBC Tx the following day (increasing the number of transfusions given to 20). SDF measurements were carried out before and after each of the subsequent transfusions. These measurements were analyzed separately and consequently the results were counted as two individual study observations. Among the Tx-Group 12 patients had cancer and 7 patients had hemoglobinopathies.

For an overview of patient diagnoses see Chart 8 in the Appendix.

Among the anemic children with cancer, 8 children developed an infection. We defined infection as consisting of elevated CRP-levels (> 3mg/dL), in combination with clinical signs, such as: fever, chills, myalgia, arthralgia, headache and sore throat. Therefore another subdivision was made, - separating the Tx-group into patients with infections **(Inf Group)** and patients without infections **(nInf Group).**

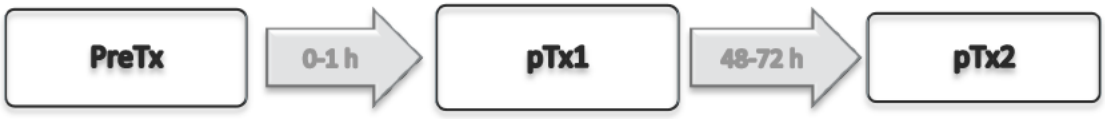
The control group **(CG)** consisted of 18 children matched for age and sex who underwent minor plastic or reconstructive surgery and had no medical problems, such as systemic diseases, congenital diseases or any other severe mental or physical disorders.



11.Figure, Overview of study groups

3.2. Clinical data – Group Analysis

As previously described, the microcirculation of patients receiving RBC transfusion was measured 0-2 hours before transfusion start (preTx). Further microcirculatory data was obtained 0-1 hour after Tx (pTx1). The third SDF measurement was performed 48-72 hours after transfusion therapy (pTx2). Eight patients were either treated at the day clinic or were discharged early and therefore left the hospital before the third measurement could be performed. In this case, only two sets of data could be analyzed (right before and right after transfusion).



12.Figure, Time chart of measurements

Clinical data, including diagnosis, age, gender and weight is listed in the Appendix (7.2)

3.2.1. Laboratory Data

	Pre-Tx (n 19)	**Post-Tx 2** (n12)	**CG** (n 18)
Hemoglobin g/dL	7.2 (6.5-7.9)	8,0 (7.3-8.6)	12,9 (12.3-13.5)
Hematocrit %	0,21 (0.2-0.2)	0,23 (0.2-0.3)	0,39 (0.4-0.4)
Erythrocytes T/l	2.4 (2.1-2.7)	2,7 (2.4-2.9)	4,6 (4.4-4.8)
Leukocytes G/l	9.7 (2.6-16.7)	7.0 (1.9-15.9)	6.1 (5.4-6.9)

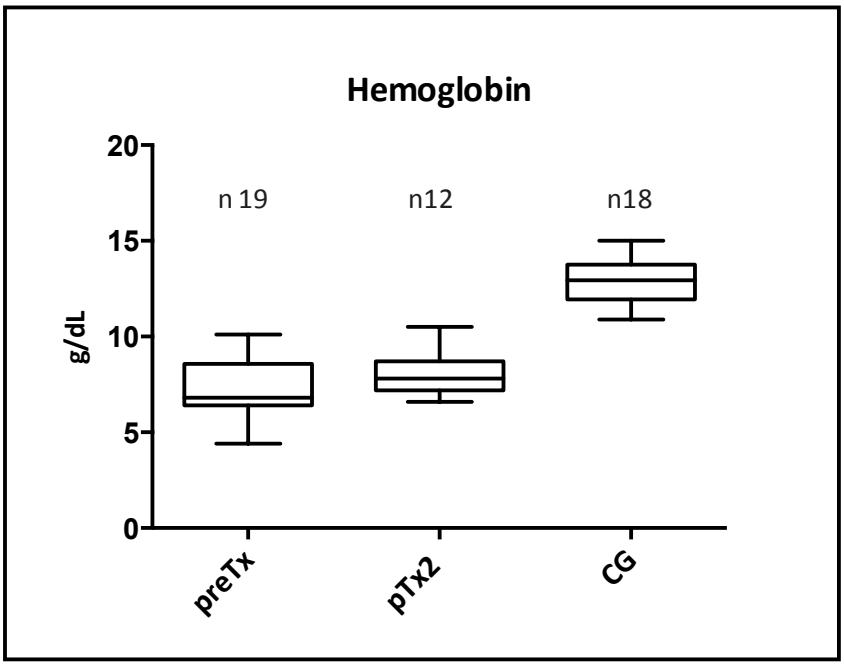
2. Table, Laboratory data presented as mean values and 95% CI,

n indicates the number of patients that had blood samples taken

(We did not include laboratory data at **Post-Tx1** due to limited numbers**.** Blood samples were only taken once a day, -usually in the morning. Only five children in the transfusion group had blood samples taken within the first 12 hours after transfusion. Due to low patient number and consequently low representative significance for the whole group, these values were not further included in statistical analysis. Post-Tx 2: 48-72 hours after transfusion.)

3.2.1.1. Hemoglobin (Hb)

Within the Tx-group hemoglobin values increased significantly 48-72h after transfusion (pTx2). Hemoglobin content increased from 7.2 g/dL [CI: 6.5-7.9] to 8.0 g/dL [CI:7.3-8.6] at pTx2 (p=0.0002). Hb values before Tx and after Tx (post Tx 2) were still significantly lower than Hb-levels of the healthy control group (CG) (p< 0,0001).



13.Figure, Box and Whisker plot (median, first and third quartile, minimum, maximum) Hb values of the anemic study group before and 48-72h after transfusion in comparison with Hb values of control group (CG)

We further subdivided the Tx- group into patients with infections and patients without infection and found that hemoglobin levels before transfusion differed significantly between the 2 groups. 48-72 h after transfusion values did not differ significantly between the two subgroups. The increase of hemoglobin 48-72 hours after transfusion was not significant in the subgroup without infection (nInf-group), however only 3 values were obtained from this group at this point of time. Hemoglobin values of both groups differed significantly from the healthy control group at all times of measurement ($p<0.0001$).

	preTx	**pTx2**
Infection	6.2 (5.5-6.9)	7.8 (7.2-8.4)
No Infection	8.2 (7.3-9.1)	8.9 (5.4-12.4)

3. Table, Mean and 95% CI of hemoglobin values of subgroups

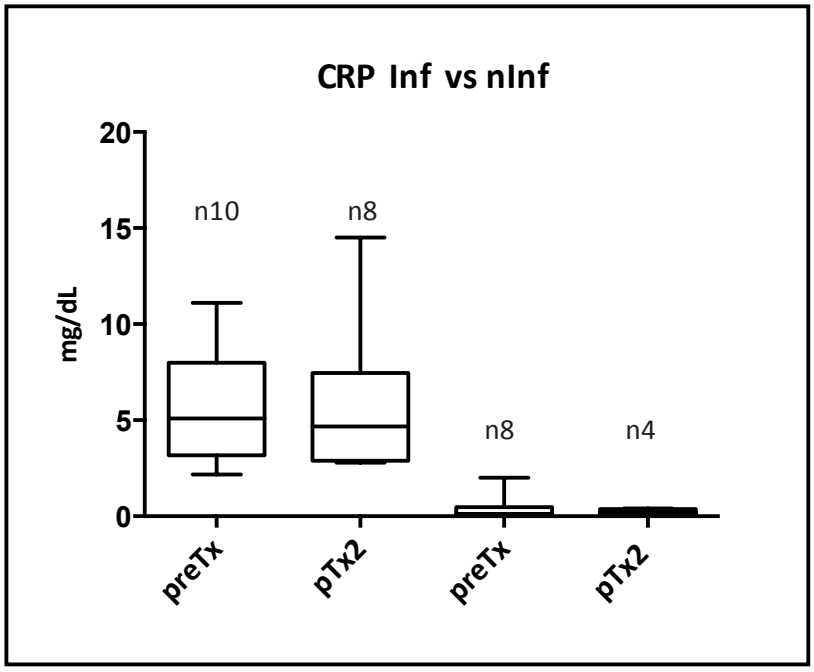
* Hemoglobin values at pTx1 were not included because of low numbers
(This does not apply to microcirculatory imaging results, which were performed on all patients at pTx1)

3.2.1.2. Hematocrit (Hct)

The **Hematocrit (Hct)** is a measure of the fractional level of red cells in the blood. Strictly speaking, anemia is defined as a decrease in total body red cell mass and can therefore be indicated by a low Hct. Consequently, when Hct is below normal, the erythrocyte count and hemoglobin level will also be low. In our anemic study population both Hematocrit and Erythrocyte count were significantly ($p<0,0001$) higher 48-72 hours after transfusion (postTx2). However, these elevated values still differed significantly ($p<0,0001$) from the normal values of the healthy control group (CG). (Comparable to Hb-values on Fig. 11)

3.2.1.3. CRP

As described in 2.2 the Tx-group was further divided into a group with elevated CRP levels >3mg/dL and clinical signs of infection (Inf) and a group with normal CRP levels <3mg/dL and no infection (nInf).



14.Figure, Box and Whisker plot (median, first and third quartile, minimum, maximum) Comparison of CRP values between anemic patients without infections and with infection

With a mean CRP of 0,4 mg/dL (95%CI: -0,03-0,8 mg/dL) the nInf-group differed significantly ($p<0,0001$) from the Inf-group where the mean CRP value was 5,7 mg/dL (95%CI: 3,6-7,8mg/dL) prior to Tx. 48-72 hours after Tx values were still significantly different between the two groups (p 0,0264). The patient number of the nInf-group was much lower at post Tx2. However it can be assumed that the CRP levels of these patients did not increase after 48-72 hours. The reason for this assumption is the following. The n-Inf consisted mainly of patients with hemoglobinopathies, who received RBC transfusions at the day clinic at defined time periods. Apart from the typical signs of anemia, as described in 1.2.3, these patients showed no clinical signs of infections and were in good health. It is unlikely that their condition changed within 2 days.

3.2.2. Vital signs

3.2.2.1. Heart rate (HR)

	preTx	pTx1	pTx2
Heart rate (bpm)	100 (91-105)	86 (82-91)	85 (78-91)

4. Table, Mean and 95% CI of heart rate

The results of the sub-groups were similar and did not differ significantly (p>0,05, n.s.). Therefore the whole Tx-group (without separating into Inf and nInf-Group) was included in the statistical evaluation of vital signs. Within the whole Tx-group the mean heart rate before Tx was 100 bpm (95% CI: 91-105). After Tx the HR decreased significantly (p<0,05). The mean HR at pTx1 was 86 bpm (95% CI: 82-91) and did not change significantly at pTx2, where the mean value was 85 bpm (95% CI: 78-91).

3.2.2.2. Temperature

	preTx		pTx2	
	Tx-group		Tx-group	
	37,1		36,8	
Temperature (C˚)	**nInf**	**Inf**	**nInf**	**Inf**
	36.5	37.7	36,5	37.1
	(36.2-36.8)	(37.2-38.1)	(36.2-36.9)	(36.2-37.9)

5. Table, Mean and 95% CI of temperature in the Tx and subgroups

Temperature was measured before RBC Tx and 48-72 hours after Tx. For statistical evaluation the Tx-group was again divided. Individuals with elevated CRP levels (Inf) had a significantly higher temperature than children without signs of infection ($p<0,0001$). The mean temperatures within the infection group (Inf) and patient group without infections (nInf) are presented in the table above. The temperature in the Inf-group decreased 48-72 hours after Tx. At this point the values did not differ significantly ($p=0,2797$, n.s.) from the ones obtained of patients without infection 48-72 hours after Tx.

3.2.3. Blood Units

Packed RBC units were obtained from the blood bank of the Red cross of Munich. Mean storage time of the RBC units was 13.3 (95% CI 11,9-14,6) days. All children received 200-300 ml (Mean 273 ml, 95% CI 252-293) of packed, irradiated and leucocyte reduced RBCs over max. 3 hours.

3.3. Microcirculatory Results – Group Analysis

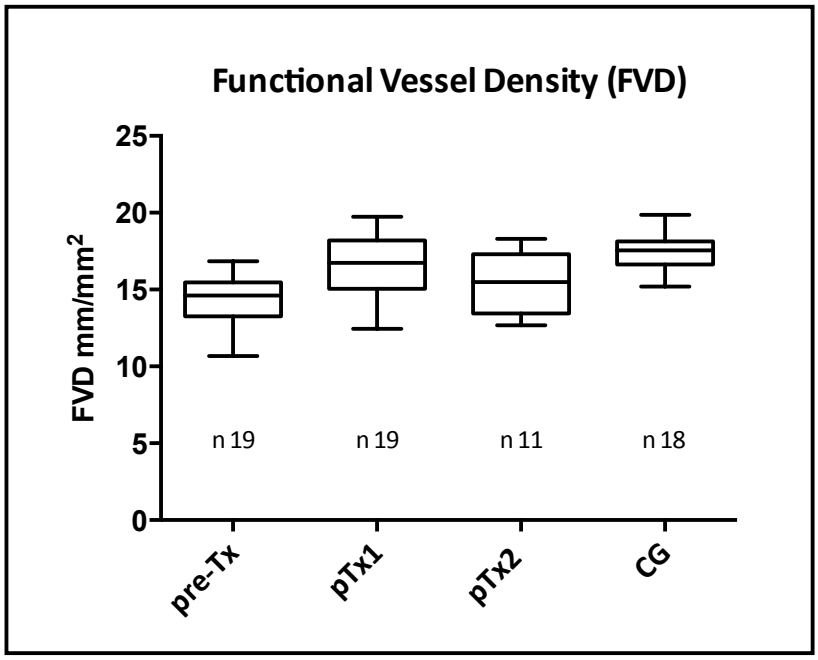
3.3.1. Functional Vessel Density (FVD)

3.3.1.1. Transfusion group (TxG) versus Control group (CG)

	preTx (n 19)	**postTx1** (n 19)	**postTx2** (n 11)	**CG** (n 18)
Mean	14,3	16,4	15	17,5
95% CI	13,5-15,0	15,5-17,4	13,7-16,2	16,5-18,1
SD	1,65	2	1,7	1,3

6. Table, Mean, 95% CI and Standard Deviation (SD) of FVD (mm/mm$^{2)}$

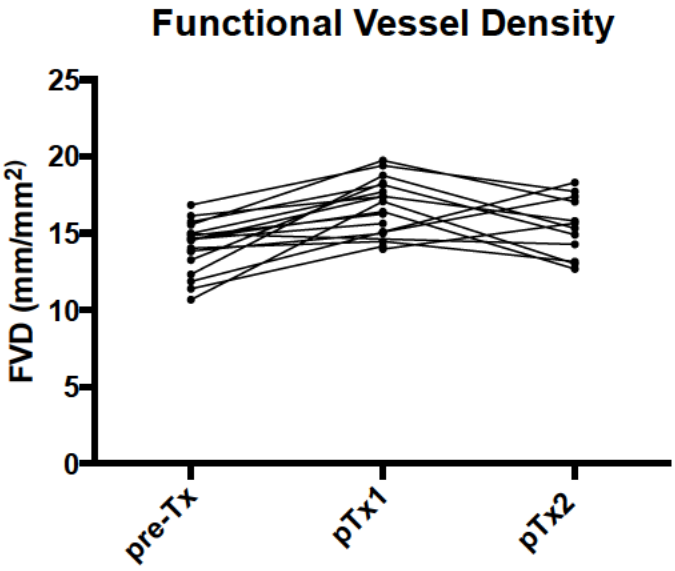
(n indicates the number of patients for each subgroup)



15.Figure, Box and Whisker plot (median, first and third quartile, minimum, maximum) showing the FVD values from the Tx-group before (preTx), 0-1 h after (pTx1) and 48-72 h after RBC transfusion, compared to the FVD of the control group (CG)

A Wilcoxon matched pairs test was performed to compare the FVD values of the anemic group before (preTx) and after transfusion (pTx1). It showed a significant result ($p < 0.0001$, one tailed). The same test did not yield a significant difference between FVD values at pTx1 and pTx2. It has to be noted however, that the mean value at pTx2 was higher compared to preTx, as seen in table 6. It is likely that the Wilcoxon matched pairs test was not able to detect this difference, due to a lower patient number 48-72 hours after transfusion (pTx2). At this point only 11 samples were obtained, compared to 19 samples right after transfusion (pTx1).

Compared to the anemic children, the healthy control group had a much higher Mean-FVD, as seen in Table 1. The Mann-Whitney test was used to analyze the means of the unpaired groups and confirmed this significant difference ($p<0.0001$). The difference was especially significant prior to RBC Tx ($p<0,0001$). Right after transfusion the FVD parameters of the Tx group did not show a statistically significant difference from the control group, although the mean value and confidence intervals was notably lower in the anemic group also after transfusion. At pTx2 the mean FVD of the Tx group dropped and was again significantly lower compared to the control group.



16.Figure, A before-after graph shows the FVD values of the Tx-group before and after transfusion. This graph plots each subject as an arrow to show the direction from preTx to pTx1 to pTx2

We further used the Friedmans test, which is a nonparametric, repeated-measures test and can control for experimental variability. Since this test only evaluates a set of data consisting of three groups or more, only patients with microcirculatory data from each measurement (preTx,

pTx1, pTx2) could be included in this analysis. Eleven patients had a complete set of data and were further analyzed with the Friedman test. The results showed a significant difference in means (p=0,0115).

3.3.1.2. Infection vs no infection

In order to see if infections play a role in the change of FVD the group was divided into Inf-group and nInf-group for further evaluation.

When analyzing the data it becomes evident that the emphasis of interpretation should be laid on the obtained values before (preTx) and right after RBC transfusion (pTx1). At this point the number of patients is comparable among the groups. At pTx2 the group without infections only includes 3 patients and therefore interpretation would only yield unsatisfactory results and lack substantial statistical significance. Data at pTx2 is included in the graphs but left out of statistical analysis.

Patients with Infections (Inf)

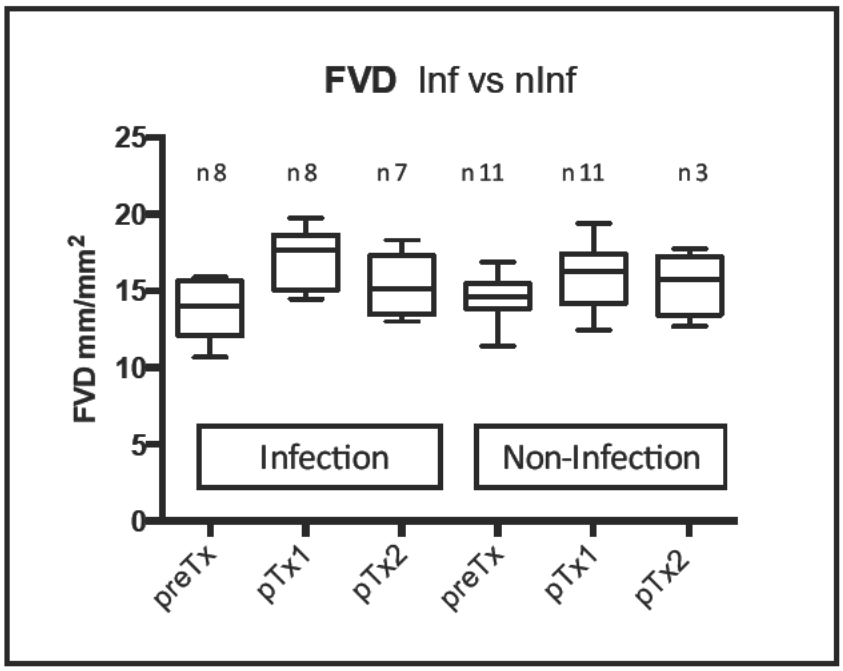
	preTx (n 8)	**pTx1** (n 8)	**pTx2** (n 7)
Mean	13,2	17,5	14,6
95% CI	11,6-14,8	15,8-19,1	13,0-16,2

Patients without Infections (nInf)

	preTx (n 11)	**pTx1** (n 11)	**pTx2** (n 3)
Mean	15,1	16,3	15,5
95% CI	14,4-15,8	15,1-17,6	12,2-18,8

7. Table: Mean FVD values (mm/mm^2) and 95% CI for both groups (Inf and nInf)

n indicates the number of patients in the subgroup



17.Figure, Box and Whisker plot (median, first and third quartile, minimum, maximum) showing FVD values for both subgroups (anemic patients with infection and anemic patients without infection) at each point of measurement

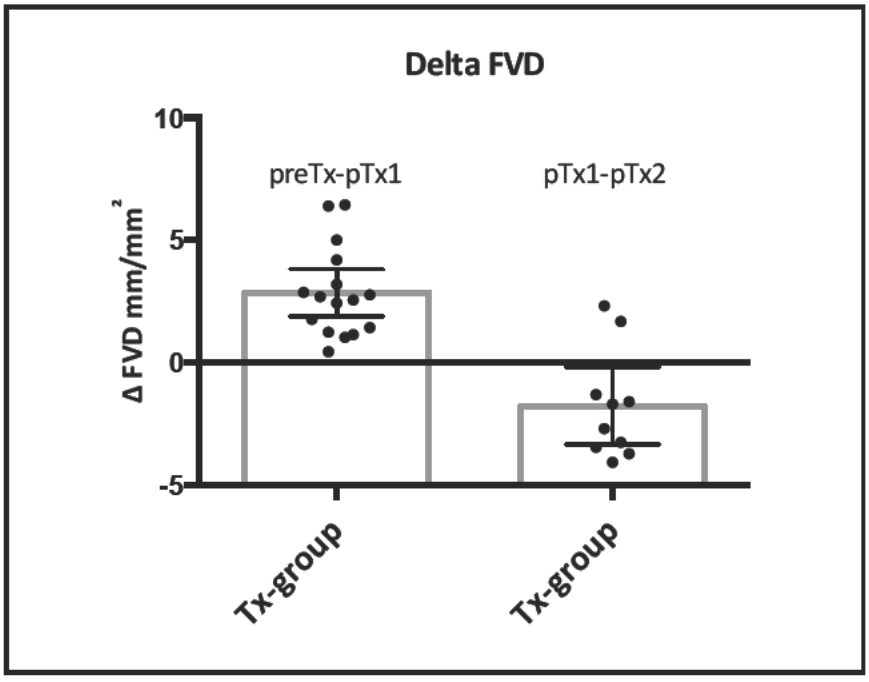
The Wilcoxon signed rank test showed a significant increase of FVD after transfusion in the anemic patients with infections ($p<0.05$), with a median of differences of 3,7. The FVD of anemic patients without infections also rose significantly ($p<0.05$), however the median of differences was only 1,4.

3.3.2. Δ FVD

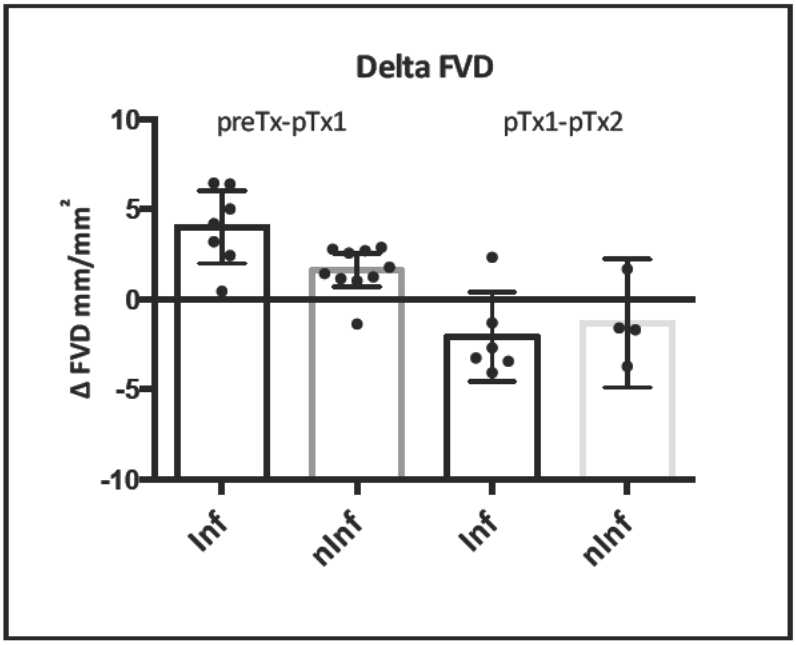
To better evaluate the actual FVD changes, the difference of FVD between each measurement was calculated.

	Tx-Group		Inf-Group		nInf-Group	
	preTx-pTx1	pTx1-pTx2	preTx-pTx1	pTx1-pTx2	preTx-pTx1	pTx1-pTx2
Number	19	11	8	7	11	3
Mean	2,2	-1,3	3,4	-1,3	1,3	-0,5
SD	2,3	2,6	2,6	2,9	1,5	1,9
95% CI	1,2-3,3	-3,1-0,4	1,2-5,6	-4,0-1,4	0,3-2,4	-5,3.-4,2

8. Table: Mean, Standard Deviation and 95% CI of ΔFVD for all groups

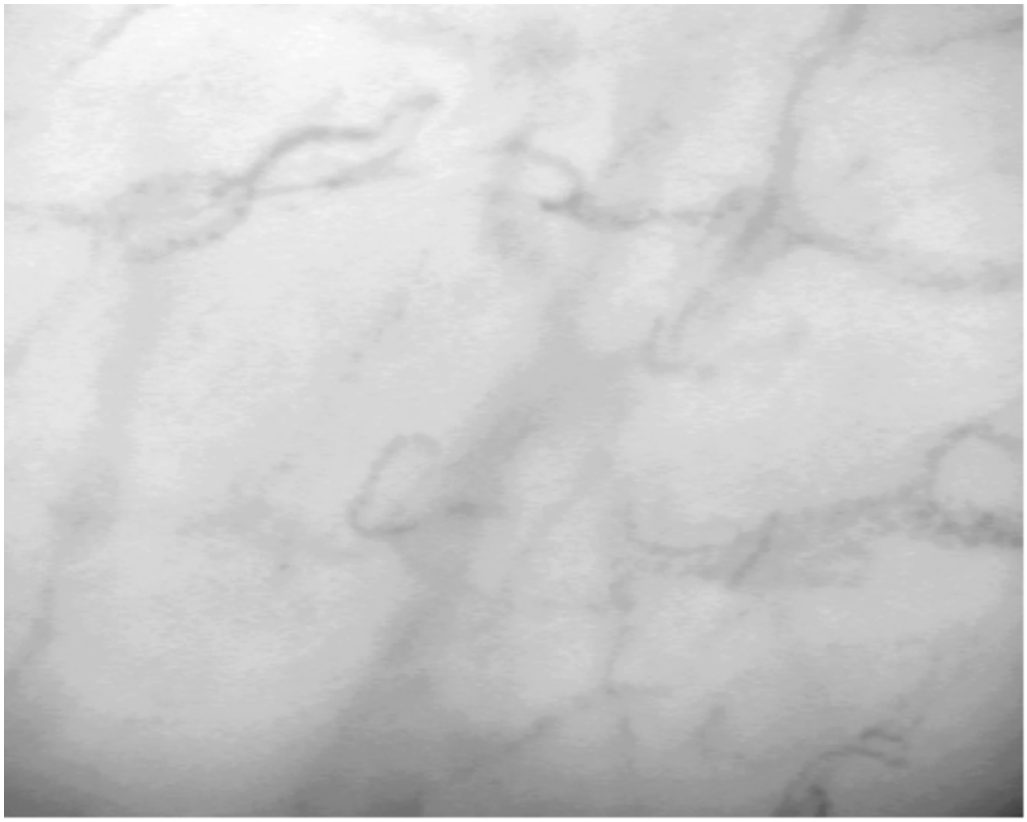


18.Figure, Scatter dot plot (with mean and 95% CI) showing the actual change of functional vess density (ΔFVD) for ALL anemic patients before to right after transfusion (preTx-pTx1) and right after transfusion to 48-72 h after transfusion (pTx1-pTx2)

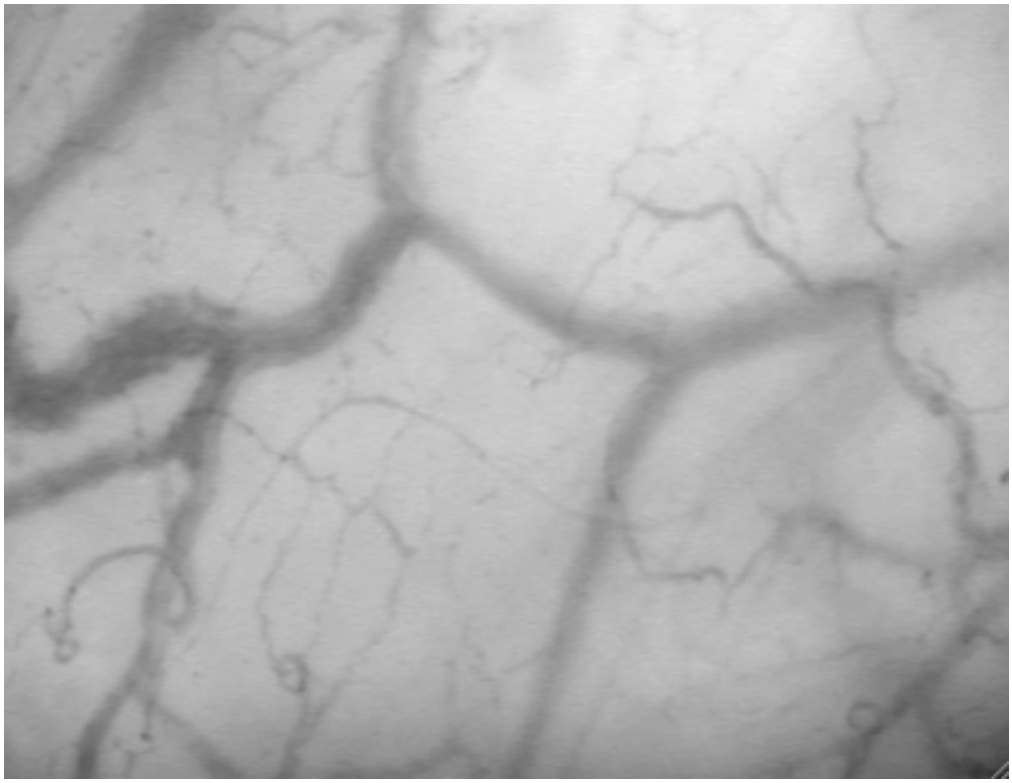


19.Figure, Scatter dot plot (with mean and 95% CI) comparing the ΔFVD between the anemic group with infections (Inf) and the anemic group without infection (nInf)

As seen in the graph above the FVD increased considerably more in the infection group compared to the anemic patients without infections. The Mann-Whitney test confirmed that the change of FVD (ΔFVD) right after transfusion (preTx-pTx1) was significantly greater in the infection group ($p<0.05$). The Mann-Whitney test was not able to detect a significant difference between the two groups in the change of FVD (ΔFVD) after to 48-72 hours after transfusion (pTx1-pTx2). It has to be considered that the number of available data at this point is too low for a meaningful statistical analysis.



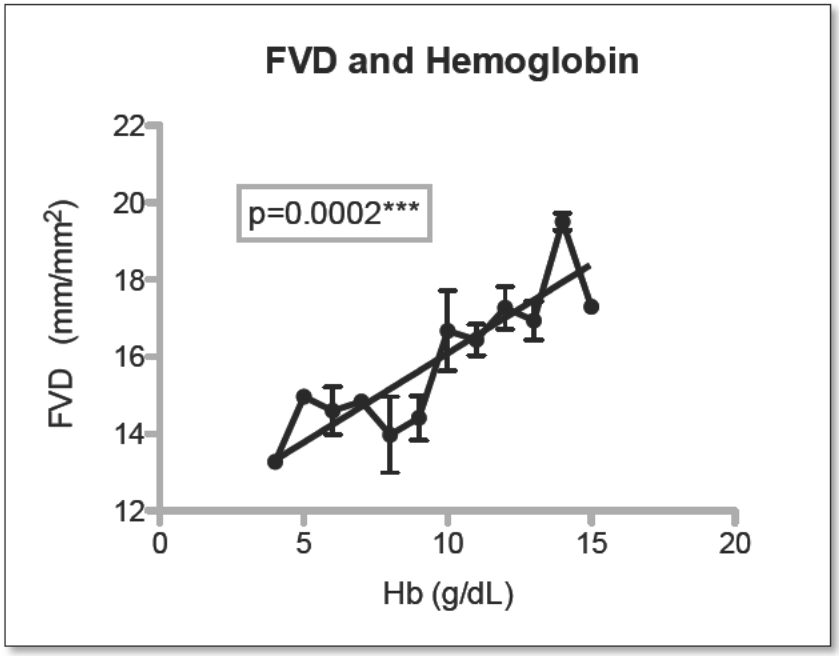
20.Figure: Image of the sublingual microcirculation prior to RBC-Tx



21.Figure: Image of the sublingual mircocirculation right after RBC-Tx

3.3.3. Correlation Hb-FVD

The mean values, SD and 95% CI of hemoglobin (Hb) for both the Transfusion-Group (TxG) and the Control-Group (CG) have already been listed in **Table 2**. Hb levels from severely anemic children were obtained before transfusion, as well as levels from healthy individuals with normal Hb-values. The Hb levels before tranfusion were directly correlated with the FVD values before transfusion. With a minimum Hb of 4,4g/dL and a maximum of 15,0 g/dL the Hb range in this correlation is rather big. The graph below shows a highly significant correlation of FVD and hemoglobin (p=0.0002).

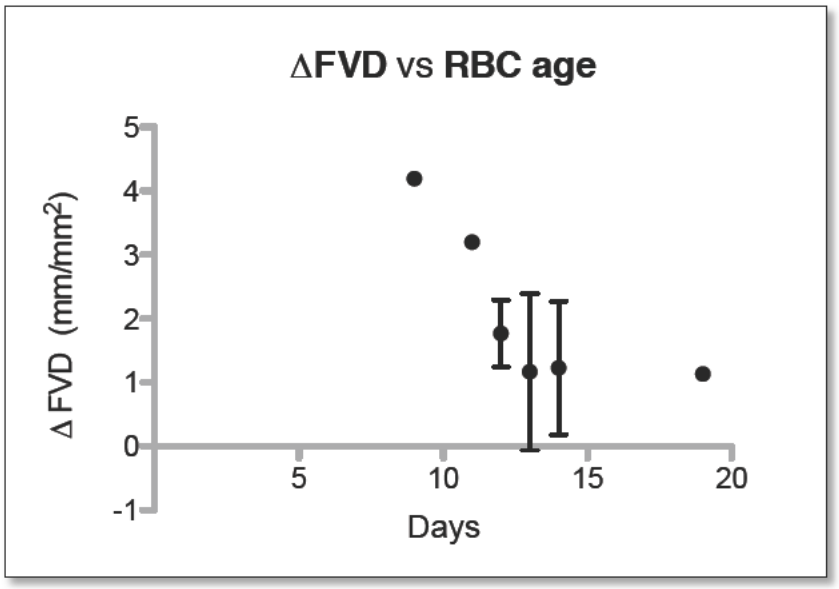


22. Figure, Correlation of FVD and Hemoglobin

3.3.4. Correlation ΔFVD - Age of RBCs

The mean age of RBC-transfusions was 13,3 days [95%CI (11,9-14,6 days)].

As Δ FVD we used the FVD values before and at pTx1 to see if the immediate change of vessel density was effected by the age of the given RBCs. We plotted the value for the age of the blood unit on the x-axis, with the corresponding ΔFVD after transfusion with that given bood unit on the y-axis. The correlation was not significant.



23. Figure, Correlation of ΔFVD and RBC age

4. Discussion

As stated in the introduction, transfusion therapy is not free of adverse effects. Additionally clear guidelines regarding transfusion thresholds remain unclear in pediatrics. The goal of this study was to assess the microcirculation of anemic children who underwent RBC-transfusion therapy and thereby provide valuable information on the efficiency of the therapy. I further want to discuss the possible influence of infections on the microcirculatory changes, as well as the potential effect of RBC storage time.

These questions and their results will be analyzed critically below:

4.1. Study design

A prospective, controlled observational study was chosen as a study design. Decision to treat was independent of the study and up to the discretion of the attending oncologist. Measurements were taken at predetermined times for each participant, to make the study uniform.

Measurement intervals have to be analyzed critically. SDF measurements were performed no more than 2 hours prior to RBC transfusion and 0-1 hour after transfusion. However, the time interval until the third point of measurement was 48-72 hours. Such a large time interval was chosen for various reasons. SDF measurements were not to interfere with other clinical procedures. Also the endeavor was to put as little stress as possible on patients and their parents. Some patients were in very poor conditions and preferred measurements at a later point of time. The day of discharge could not be predicted precisely; - some patients went home 2 days after transfusion and were measured before leave. Additionally, it has to be considered that inevitable changes in therapy and procedures could themselves affect the microcirculation. Therefore an interpretation of the variables should only be done carefully (especially 48-72 h after transfusion).

Another possible point for criticism could be the big age difference within the group. The age-range was 4-18 years. Within both groups (Tx-Group and Control Group) the youngest participant was 4 years old, whereas the oldest patients were 18 years old. However, as stated earlier, the microcirculation develops an adult pattern at the age of 3 months. Therefore a direct comparison of patients and microcirculatory variables seemed appropriate. The mean age of the Tx-Group (10,2 years) did not differ significantly from the age of the control group (10,28 years). Therefore a comparison of the microcirculatory parameters between the two groups seemed reasonable.

As stated earlier, limited studies on SDF imaging in children exist. Paize and al. performed SDF measurements on twenty children with severe meningococcal disease treated in the intensive care unit, and compared these directly to a healthy control group. Their control group consisted of anesthetized children between 6 months and 6 years of age that underwent routine surgery and healthy volunteers over the age of 6 years. These were used as a direct control group, similar to our study, with an age span similar to ours.

It has been shown that propofol can have a minor influence on microcirculatory measurements in adults. [99] SDF imaging in our healthy control group was performed after complete recovery of anesthesia and when propofol was likely to be eliminated of the circulatory system.

The process of recruiting participants proved to be rather difficult. Many children who received blood transfusions at the hemato-, oncologic ward or at the day clinic were under the age of 5. Although many measurements were attempted with these young patients, hardly any could be completed or later included in analysis. One of the reasons was a lack of comprehension for the requirements and consequently a poor participation in the measurement procedure. A qualitatively good SDF-sequence is based on a good co-operation and requires the test subject to hold very still while the SDF probe is placed under the tongue or on the lip for imaging. Even slightest movements will cause image blurring and pressure artifacts. Most of the younger children were agitated and anxious and made the recording of good microcirculatory videos impossible.

5 children had to be excluded of the study due to either bad image quality, missed follow up measurements or lacking participation, leaving 37 children for analysis of which one child was analyzed at different points of time.

4.1.1. Statistical analysis

The statistical analysis was to some extent compromised. Other microcirculatory studies, which have been conducted in similarly small patient groups, have used parametric test, such as a t-test, that require a normal distribution of study subjects. [65] The D´Agostino Pearson normality test was performed to test for normal distribution of data in our study. Although we did obtain high p-values when testing for normality (which could indicate a normally distributed data) we decided to use nonparametrical tests. A normality test cannot prove the data were sampled from a Gaussian distribution; all you can say is that the data are not inconsistent with a Gaussian distribution. Especially in smaller patient groups the normality test lacks power. [100] It should be taken to account that it is likely that parametric testing could have been applied for certain datasets in our study and yielded more significant results.

Patient- and microcirculatory data showed noticeable differences and discrepancies and included a few outliers that were mostly visualized in the corresponding graphs. Especially the division of the anemic group into inf-group (with infections) and nInf-group (without infections) was problematic. As accounted for in the results section, the difference at pTx2 between the numbers of patients among the groups was large. The infection group included 7 study subjects at this point, whereas the non-infection group only consisted of 3 study subjects. Statistical testing for this subgroup (at pTx2 between inf- and nInf group) is not reasonable. Other statistical analysis between the whole transfusion group and control group, and between the transfusion group itself (preTx-pTx1) showed similar patient numbers, although numbers were relatively small. It becomes evident that statistical interpretation of microcirculatory findings after 48-72 h (pTx2) is limited and therefore the emphasis of this study should especially be placed on the immediate results of transfusion. Due to quite small samples within the different subgroups of analysis, our methods of nonparametric statistical evaluation might not have had the statistical power to detect differences that are really there.

An extension of the study with an inclusion of more study patients to increase statistical quality and validity was unfortunately impossible due to time limitations. Due to the relatively small number of patients and controls, it is only with great care that our results can be generalized.

It has to be emphasized that this study is useful in showing tendencies and raising questions, rather than assessing severity of illness, evaluating therapies and predicting outcomes.
Our findings need to be confirmed in larger cohort prospective studies.

4.2. Materials and Methods

4.2.1. Measurement

Sidestream Dark Field (SDF) imaging, a stroboscopic LED-ring based image modality, was used for the observation of the microcirculation. As described previously, the device uses green light to illuminates a certain tissue area (up to 3 mm). The scattered light is then absorbed by hemoglobin of red blood cells within vessels and depicted as a black-and white moving image of the microcirculation. [88]

In this study, the buccal or sublingual mucosa was chosen as the site of investigation. The sublingual mucosa has become the most important site for microcirculatory studies and has been used as a representative site for global microcirculation. It has not been proven clearly whether sublingual flow alterations reflect similar microcirculatory disturbances elsewhere in the body. However, it has been postulated that the sublingual or buccal mircrocirculatory network shares similar embryonic origin with the splanchnic mucosa.[101] This finding was confirmed in a recent study by Verdant et al., which showed that sublingual images are a good reflection of gut mucosal perfusion. [102] Transcutaneous measurements are of limited quality, due to the thickness of the skin and based on the skins function as a thermoregulatory organ, a function that isn't shared by other microcirculatory sites. [103] Nearly all studies referred to in the introduction section on microcirculation drew their conclusion on the basis of sublingually measured microcirculatory parameters and showed it's relevance especially in the study of pathologic conditions. [47,54] In addition it is the only site, where investigation of the microcirculation is easy accessible and practical in clinical scenarios.

De Backer et al. described five consensual key points for image acquisition: at least 3 sites per organ should be obtained at evaluation; pressure artifacts should be avoided; adjustments of

contrast and right focusing have to be made; excess fluids between the microscope and tissue should be eliminated; good image quality while capturing should be maintained. [88] All of these criteria were considered in the sublingual SDF measurement. Therefore a good quality of measurement and image acquisition can be assumed.

Video images were immediately captured on a computer using a dedicated video card, and the images were stored at full size as DV-AVI files to allow computerized frame-by-frame image analysis. Recording time was limited to 15 s because it was difficult to maintain a clear and steady image for a longer period.

A possible reason for critique could be the change between indirect digitalization of video-capturing and direct digitalization of measurements on the computer. By converting videos into digital images a loss of information can occur. This error was minimized by only using uncompressed video data for analysis. Thus the mentioned error can be disregarded and should not be held accountable for the changes of microcirculation in our measurements. [97]

4.2.2. Analysis

Different software packages have been developed, that perform FCD calculation and reliable blood flow measurements in individual vessels: The Capiscope program, the grid method by De Backer or by Boerma [50], the Microvision Analysis Software (MAS) and the Automated Vascular Analysis (AVA).

The CapiScope software has been developed for analysis of OPS images. Vessels have to be traced manually and thus FVD, diameters and velocities can be calculated. A clear disadvantage of this method is the long time consumption, as well as the out-dated, slow and complex program.

The method by de Backer and coworkers requires the user to draw three horizontal and vertical lines on the screen, because the concept is based on the principle that vessel density is proportional to the number of vessels crossing these lines. Vessel density can thus be calculated as the number of vessels crossing the lines, divided by the total length of the lines. Functional vessel density (FVD) can be measured by counting the number of grid lines that intersect with the vessels. Another reliable way to measure FVD is by considering the total vessel length, relative to image surface. [104] [97] A clear disadvantage of the score is its

impreciseness and the fact that vessels can potentially cross a line multiple times. Additionally it does not measure the velocity of red blood cells.

The MAS analysis system includes a stabilization image processing, a calculation of FVD and blood flow investigation in individual vessels.

Unfortunately, these programs still require much user intervention, specifically in identifying specific vessels of interest. In addition, the program does not allow for blood flow to be calculated automatically and simultaneously in numerous vessels. Therefore blood flow distribution histograms are difficult to obtain. It is especially difficult and challenging to obtain blood flow in capillaries, which make up the main area of interest.

MAS was later optimized and given the name “Automated Vascular Analysis” (AVA). The program supersedes it forerunners by its ability to reduce the time to determine vascular parameters. A clear advantage is its semi-automatic quantitative vessel detection (segmentation) program. After analysis a detailed report that includes the vessel length-diameter distribution, an area diameter (density) distribution and microcirculatory blood-velocity parameters is provided. The operator can correct falsely detected vessels by deleting the marked artifacts or mark vessels that were not detected by the program. The objectivity of evaluation, the accuracy of the results and the simplified analysis were reasons why AVA was selected for this study.

The following microcirculatory parameters were analyzed in this study: Functional vessel density (FVD), vessel length, vessel surface area and blood-velocity. The duration for image analysis was approximately 40-60 min per video-sequence.

The analysis of further microcirculatory parameters did not seem reasonable. The Microcirculatory Flow Indexes (MFI), which describes the quality of flow, was left out of evaluation. Furthermore the retrospective assessment of flow quality turned out to be rather difficult. The reason was that is seemed impossible to allot a certain etiology to the changes of flow at that point of time. Apart from an impaired microcirculation, pressure artifacts can also be the reason for an altered flow. The perfused vessel density (PVD) was also left out of analysis, since nearly all obtained values were consistent with the functional vessel density (FVD), - indicating a good perfusion throughout.

Offline-analysis is a time-consuming process and still requires lots of user interaction and know-how. Therefore Bezemer et al have started a recent new development for the assessment of

microvascular density and perfusion in sidestream-darkfield (SDF) images, aiming at a fully automatic and rapid analysis. They were able to improve the algorithms of microvascular density assessment previously developed by the same group (AVA) and introduced this new method, which they called tSICA. Although some limitations remain, this method can possibly be used in future to directly analyze microvascular parameters and perfusion at bedside. [105]

4.3. Microcirculatory changes

4.3.1. Does RBC-Tx improve the microcirculation of anemic children?

The effects of blood transfusions on the microcirculation and tissue oxygenation have not yet clearly been defined. To date, only a few clinical studies have evaluated the effect RBC Tx has on the peripheral microcirculation. To our knowledge, no studies have yet assessed the microcirculatory changes after RBC transfusion in anemic children outside of the neonatal period. Yuruk et al. tested the hypothesis that RBC transfusion can improve FVD, perfusion and oxygenation in a relatively healthy host microcirculation. They were able to show that blood transfusions improve the sublingual microcirculatory density and microcirculatory oxygen saturation in cardiac surgery patients and thus have significant impact on the systemic circulation and oxygen-carrying capacity [64]. Similarly we have previously reported that FVD increases in anemic preterm infants after transfusion [65].

Sakr et al. used OPS imaging to assess the effect of transfusion sublingually in septic patients. Contrary to the previously mentioned results, they found no universal changes in FVD and perfusion after transfusion. Parallel to these findings, a recent study also reports no improvement in the microcirculation after transfusion in septic patients. Sadaka et al conducted a similar study in a small sample septic sample population. In their study, microvascular reactivity and sublingual microcirculaton were globally unaltered by RBC transfusion.[106] Similarly, Creteur et al. found no consistent effect of RBC transfusion on microcirculatory oxygenation, as assessed with near-infrared spectroscopy (brief description of NIRS device in appendix-section) in septic and nonseptic intensive care patients. [63] Altogether

these three studies showed no global effect of RBC transfusion on the microcirculatory variables. One reason for the varying results might be that the population in these clinical studies consisted of patients with sepsis, or critically ill patients hospitalized in the intensive care unit. It is therefore hard to distinguish between the results from RBC transfusion at the microcirculatory level and the effects of sepsis itself. More detailed data of these studies and possible explanations will be given in the next chapter.

When comparing the results to previous microcirculatory studies (performed in adults), it has to be taken into account that children cannot be regarded as small adults and published data can only carefully be extrapolated to children. The approach to critically ill children should be made in a way that factors, which distinguish children's physiology from adults, are taken into account (such as different body proportions, a higher metabolic rate and lacking compensatory reserves for respiratory or circulatory distress). [107]

4.3.1.1. Functional vessel density (FVD)

One of the aims of this study was to determine whether RBC transfusions in anemic children result in an improved microvascular perfusion, as indicated by a higher functional vessel density (FVD). (As stated above, FVD is defined as the number of vessels with passage of RBC within a microscopically observed tissue area) Functional vessel density is well validated and at this moment seems to be one of the best quantitative indicators of microvascular perfusion. Kerger at al. showed that a sufficient functional capillary density is the determining factor for survival or non-survival in patients with hemorrhagic shock.[108] Nolte et al. previously proved that the functional vessel density could be used as an indicator for sufficient tissue oxygenation. Furthermore they indicated independence of the parameter for various observers.[104]

Blood transfusion resulted in significant increase in FVD values immediately after transfusion in the anemic study group. However, FVD decreased 48-72 hours after transfusion and did not differ significantly from FVD values before transfusion. At all times the FVD values of the anemic group were significantly lower than the FVD values of the healthy control group. Our

results thereby show a transfusion related tendency towards improved microcirculatory density and oxygenation. The increased capillary density means that intercapillary diffusion distance is minimized and thereby tissue oxygenation is facilitated and ameliorated. It is important to note however, that there was great interindividual variability among the patients. Some had lower FVD values at baseline than others; some showed improvement after transfusion whereas others did not. A possible reason for this phenomenon, such as the presence of infection in some anemic patients, will be discussed later.

In this study, as stated before, the anemic patient group was quite heterogeneous regarding their underlying disease. The number of cases studied was restricted by the availability of suitable patients that received blood transfusions in the study time period. In order to obtain an adequate number of study patients, it was not reasonable to make selections based on the same underlying cause for anemia, as that would have led to a small study population. Especially within the oncologic group we encountered different disease entities (as outlined in 9.2.“Clinical data”). Some patients underwent chemotherapy during the same timeframe, whereas others were currently not receiving any other therapy apart from blood transfusions. To what extent concomitant therapy plays a role and could account for the interindividual variability in microcirculatory observations was not possible to evaluate in this study. Another factor that needs to be considered is that 2 patients were measured in the very beginning, when the diagnosis of acute lymphatic leukemia was set. At this point of time they presented with high leukocyte counts, which could have affected the microcirculatory findings. In these samples leukocytes could be visualized with SDF, rolling along the venular wall, as described in a SDF imaging report. [88] This higher density of filling can affect the microcirculation, and show a static image in some vessels, as we observed in these patients.

One of the reasons for low FVD values in anemia is simply a reduced presence of RBCs at the microcirculatory level. Because a “functional capillary” has at least one RBC flowing through it in a period of 30 sec, at very low hematocrits the number of functional capillaries will be lower, since too few RBCs are travelling through the small vessels. It has to be noted however, that the parameter FVD does not measure the absolute perfusion of a capillary, since a capillary may still be perfused with plasma, even without the presence of RBCs.

Apart from the pressure gradient, mainly the blood viscosity is responsible for the blood flow in the microcirculatory region. [109] The viscosity mostly depends on the amount of solid particles (hematocrit) and to a certain extent also on the amount of protein in the plasma. The significant subjective improvement that is normally seen in anemia after a blood transfusion may be partially caused by an increase in blood viscosity. Higher blood viscosity is responsible for the production of shear stress-mediated endothelial factors. The augmented shear stress on the endothelial further causes vasodilation of the arterioles and a distension of the capillaries though the pressurized viscous plasma. [110]

Saldivar et al. showed in their animal model that FVD was directly related to Hct and the oxygen concentration of the inspired air.[111] At the same oxygen level, the group with the highest Hct had significantly greater FVD values. They furthermore highlighted the fact that higher oxygen levels in the inspired air lead to an increased capillary density, mainly through a recruitment of non-perfused capillaries. These findings would indicate that increased FVD values after transfusion correlate with the improvement of Hct.

4.3.2. Does infection influence the microcirculation?

In human studies, the microcirculation has most broadly been investigated in septic patients. The clinical condition of sepsis represents pathologic processes, which essentially reflect dysfunction of the microcirculation. In sepsis, the microcirculatory endothelial cells loose their ability to communicate through electrophysiological stimulation and loose smooth muscle control, thereby giving up their function as a critical control system. [112] The nitric oxide (NO) system, a critical component in the auto-regulatory control of microcirculatory function, as described earlier in the introduction, is severely altered in sepsis. The disturbance of NO production and release consequently results in pathological shunting of blood flow. Also red blood cells become less deformable, aggregate more easily and experience an increased adhesion to the endothelium. As stated previously, RBCs release NO in the presence of hypoxia and cause vasodilation. This regulatory property of red blood cells may also be affected and altered in sepsis. [113,114] [24]

These severe deficiencies, in combination with the disturbed coagulation during sepsis, further influence and alter microcirculatory perfusion and function.

The multiple studies performed in septic patients have revealed highly heterogeneous microcirculatory changes with clear evidence of arteriolar-venular shunting. [20,115,116]

Few studies have been undertaken on children. In one of the few SDF-imaging studies performed in children, Top and al. studied the microcirculation of 21 septic children in pediatric intensive care. They discovered, that FVD increased significantly from day 1 to day 2 in survivors of septic shock compared to nonsurvivors. Even though all patients normalized their systemic hemodynamic parameters, in the nonsurvivors microcirculatory alterations persisted. This was seen as low recruitment of the microcirculation, as indicated by a low capillary density. Thus they concluded that persistent microcirculatory alterations might be prognostic for survival in such a patient population. [56]

An important finding in our study is that anemic patients with infections show significantly better improvement in capillary perfusion after blood transfusion, compared to anemic patients without concomitant infections. Various studies conducted in critically ill and septic patients have shown no global improvement of microcirculation after transfusion.[63,106,117] However, they revealed that the direct effects of blood transfusions are quite variable and depend greatly on the baseline microvascular perfusion. Sakr et al. studied the sublingual microcirculation in 35 severely septic patients using OPS imaging. The measurements were performed right before transfusion and one hour after transfusion of one or two leukoreduced RBC units, with a mean age of 24 days. The principal finding of their study was that patients with an altered perfusion at baseline showed a greater improvement in microvascular perfusion after transfusion than patients with preserved baseline perfusion.[61] Other studies have shown similar results. Sadaka and colleagues performed a prospective, observational study in 21 severe septic patients, where they obtained NIRS derived (near-infrared spectrometry, see appendix) and SDF derived parameters before and 1 hour after transfusion of 1 unit of packed nonleukoreduced RBCs. They found that red blood cell transfusion did not globally affect NIRS-variables or SDF-variables. However, they found that RBC transfusion can improve muscle oxygen consumption in patients with deterioration of these parameters at baseline, whereas the oxygen consumption in patients with preserved baseline tended to decrease.[106] Weinberg et al. found that patients with an altered proportion of perfused

capillaries in the beginning showed an improvement in perfusion after tranfusion, whereas patients with relatively normal perfusion at baseline showed either no change, or even a decline of perfused capillaries. [62]

These statements support our findings. The patients with infections who experienced the greatest improvement of their microcirculation after transfusion were characterized by a significant difference in the baseline capillary perfusion. One critical factor may be the replacement of dysfunctional, rigidified RBCs by cells with a more normal deformability, as suggested by Friedlander et al., who showed that RBC transfusion is associated with a significant improvement in the abnormally low RBC deformability as seen in critically ill patients with systemic inflammatory response syndrome [118]. Elevated concentrations of inflammatory cytokines, as found in patients with inflammation or sepsis, can directly inhibit red cell formation and influence oxygen transport.[119] Reggiori and coworkers confirmed these finding by stating that early alterations of red blood cell rheology are common in patients with critical illness.[120] Other studies support these conclusions. As described more in detail in the introduction, RBC themselves can act as oxygen sensors. In a state of hypoxia RBC are able to release vasodilators, nitric oxide and ATP and thereby modulate tissue oxygen flow variables. As stated above, serious infection can cause a lack of RBC deformability and thereby limit the release of vasodilators and cause an alteration of microvascular density.[38,41]

In summary, the low vessel density of anemic patients with concomitant infection prior to transfusion (preTx) might be related to the impaired microcirculation (due to the above mentioned mechanisms), compared to the other anemic subpopulation with a relatively healthy microcirculation. Through the replacement of dysfunctional RBC in the microcirculatory network in patients with infection, flow through the capillaries can acutely improve and thus lead to an instant increase of vascular density.

Nine patients in our anemic study group presented with concomitant infections, characterized by elevated CRP levels (< 3 g/L), elevated mean temperature and any of the following: myalgia, pain, sore throat, night sweats, chills, headache and other symptoms of apparent infection. Clinical experience and expertise was used to evaluate the patients' condition in combination with the objective clinical signs for infection. If the child fulfilled the criteria for infection, it was

thus allocated to the study population (Inf). No child fulfilled the criteria for severe sepsis. When defining sepsis in pediatrics, physiological variables of the different developmental stages should be incorporated: newborn, neonate, infant, child and adolescent. [16] The age of our study patients varied markedly; therefore it was difficult to establish an objective, similar threshold from when on to speak of infection and especially to asses the severity of it and the possible impact on the microcirculation. However, the apparent clinical deterioration of the patient's status where infection was present made a subdivision of the anemic patients clear. Although most microcirculatory studies were performed in septic patients, impaired microcirculatory parameters have also been shown in adult patients who developed nosocomial (hospital-acquired) infections after abdominal surgery. [121]

Another crucial factor has to be noted when interpreting the microcirculatory results of our study subpopulations: On the one hand anemic patients with concomitant infections had significantly lower hemoglobin values at outset compared to the anemic group without infections. As discussed further on, the low capillary density values (of the Inf-group) at baseline may simply be explained through the positive correlation with the low hemoglobin and hematocrit values at outset. Another important factor regarding the compensatory mechanisms in chronic anemia has to be taken into account. The anemic patient population without infections (nInf-group) consisted mostly of patients with chronic anemia due to hemoglobinopathies, whereas the patients with infections mostly consisted of oncologic patients with more acute onset of anemia. The patients with chronic hematologic illness received blood transfusions regularly (once a month) to compensate for their low hemoglobin values. The transfusion threshold was relatively high for these patients (approximately 10 mg/dL). In this respect, it has been shown that chronic anemia promotes compensatory mechanisms. One mechanism worth noticing is the increased levels of 2,3-diphosphoglycerate acid as seen in chronic anemia, which leas to increased release of oxygen. [122]
Also through this study it is not possible to say to what extent additional therapies, such as chemotherapy, could possibly affect the microcirculation.

4.3.3. Does the Hb correlate with the FVD?

One of the most important findings in our study is a statistically high correlation between hemoglobin levels and functional vessel density (FVD). Hemoglobin (Hb) levels from severely anemic children were obtained before transfusion, as well as levels from healthy individuals with normal Hb-values and then compared to the corresponding FVD values. Kroth et al performed OPS measurements in 25 preterm neonates from week 1 to week 4. They found a decrease of FVD, which correlated directly with hemoglobin levels and incubator temperature. [59] Previous studies have not been able to show this correlation. [65] Top et al. investigated the microcirculatory development in healthy neonates up to children at the age of 3 year olds and could not find a correlation between FVD and Hb levels.[123] However, a clear advantage in our study is the great range of hemoglobin values obtained, with 4,4 g/dL being the lowest value and 15,0 g/dL the highest. Consequently a correlation is more likely to be detected. Other studies support our findings. Saldivar et al. found that in an animal model, FVD was directly related to Hct and the oxygen concentration in the inspired air.[111] As mentioned previously, SDF imaging uses hemoglobin absorption to visualize the red blood cells. Thus, the method could fail at low hematocrits. Harris et al. tested this hypothesis by obtaining microcirculatory parameters of nine awake Syrian golden hamsters during isovolemic hemodilution and low hematocrits. They concluded that OPS imaging (the forerunner model of SDF-imaging) is well validated to measure both diameter and FVD at a wide range of hematocrits. [124] Another study confirms these findings by stating that although the technique is based on light absorption by hemoglobin, it remains valid in anemia, as well as during acute changes in hemoglobin concentration. [125]

4.3.4. Do the mircrocircualtory changes correlate with the age of RBCs?

The microcirculatory results in our study show great interindividual variations with no consistent observable results. One reason for improvement in some patients and microcirculatory deteroriation in other patients could be the effect of RBC storage. Various laboratory studies and an increasing number of observational studies have shown that biochemical changes in RBCs occur during storage (as described in the introduction section, 1.3.6.1.) These changes affect the RBC survival and their ability to deliver oxygen. These changes happen mainly through an increased affinity of hemoglobin for oxygen, which subsequently impairs offloading of oxygen and thus tissue perfusion.[126,127] Other studies postulate that RBC deformability changes during storage; leading to an increased vascular adhesion.[128] These deformed transfused RBCs can then cause microcirculatory occlusion in some organs, which may lead to tissue ischemia.[129] RBC-dependant vasoregulation, which is important for regional O2 delivery, can be compromised by giving processed and stored RBCs.[128] A major problem caused by storage lesions is the increased hemolysis, which inhibits NO dependent vasodilation. [74–76] Various other studies have tried to describe the interactions between RBCs and free hemoglobin with nitric oxide pathways in normal and pathological settings. Ultimately, vasodilation of blood vessels and microcirculatory flow is altered through compromised NO bioavailability from medical storage or disease states. In a recent study Stapley et al. tested the hypothesis that old, stored RBCs inhibit the nitric oxide (NO)-signaling more so than younger cells. They found that NO-consumption rates increased ~40-fold and NO-dependent vasodilation was inhibited 2-4 fold with 42-day old vs. 0-day old RBCs. [130] Gonzales et al observed the intravital microcirculatory dynamics of rat muscle flaps after blood transfusion. They discovered that functional capillary density was greater in the group that received fresh blood, compared to the ones receiving 1- and 2-week banked blood. [131]

It has been suggested that these storage lesions may adversely affect clinical outcomes and exacerbate transfusion associated morbidity and mortality. [132,133] Gauvin et al discovered that stable critically ill children who were given RBC units older than 2 to 3 weeks were more likely to develop new or progressive multiple organ dysfunction. [134] In another recent study on a

cohort of cardiovascular patients, Koch and colleagues suggest that transfusion of RBCs that had been stored for more than 14 days may be associated with a significantly higher risk of postoperative complications as well as a reduced short term and longterm survival. In addition they stated that increased infectious complications in cancer patients might be the result of longer storage times of RBC transfusions. [135]
Other studies, however, have not demonstrated an association between RBC length of storage and outcome. [128,136]

In this study the mean age of RBC transfusion was 13,25 days. Nine patients received RBC with an age of 12 days or lower. Eleven patients received blood transfusions with an age of 13 days or more. Even though the correlation between RBC age and the microvascular perfusion (as indicated by the functional vessel density) was not significant, the graph (Fig. 28) indicates that the administration of fresher RBC (<12 days of age) resulted in a greater improvement of microcirculatory variables, as indicated by a higher ΔFVD. We therefore conclude that a tendency towards a RBC-age related improvement of microcirculation could be assumed in this study.

However, too many limitations remain in this study for a valid conclusion to be drawn regarding blood age and potential risks. The patient group was small and very heterogeneous, both in diagnosis, age and therapy. Some patients received RBC transfusions due to hemoglobinopathies, such as sickle cell disease or thalassemia. These underlying diseases may also lead to abnormal red blood cell (RBC) adhesion to the vascular endothelium and alter vasomotor tone regulation and microcirculatory flow. In these conditions, blood flow is especially dependant on a functioning NO pathway. [137] It can be assumed therefore, that additional transfusion of impaired RBC (potentially causing hemolysis and NO scavenging, as stated earlier) may only exacerbate microcirculatory dysfunction. Another main area of concern is that the study was not primarily conducted to find and evaluate blood age related risks. Only retrospectively were blood age and microcirculatory parameters correlated, and age distribution and underlying sickness was not taken into account.

More randomized, prospective studies are needed to determine the existence of adverse effects from transfusing older, stored RBC. However, various challenges in conducting definitive

clinical trials in this area remain. One problem might be the lack of exposure to “old blood”, since current policies stipulate a distribution of “ younger blood”. A randomization of patients to fresh RBC (3-11 days) is viable, however trial strategies should be designed in way that “old” blood is used as a comparator. [138]

In summary it can be stated that transfusion of stored red cells could be harmful under some circumstances. Further adequately powered, randomized controlled trials are needed to answer the question whether storage age is clinically important.

Various trials are currently being conducted to determine the validity of choosing fresher blood in some patients, as well as the mechanisms for the potential adverse effects. [139,140] If it is possible to clearly demonstrate improved outcomes with delivering fresher blood, current inventory management strategies may need to be revisited.

4.4. Outlook

The data we have provided in this study in combination with similar observations from other studies, suggest the clinical potential for SDF-imaging at the bedside. It's ability to detect subtle perfusion defects and thereby asses the effects of therapies (such as RBC transfusion) make it a valuable instrument.

At this time, however, analysis of the microcirculatory video sequences is relatively arduous and time-consuming and not ideally suited for real-time clinical evaluation and decision making at the bedside.[141] Additionally, software-assisted analysis still requires input from a trained operator. Although SDF microscopy might currently not be suited for clinical settings, it is, however, anticipated that future developments in software-assisted image analysis will enable clinicians to perform relatively rapid evaluation of the patient's microcirculation at the bedside and thus potentially be able to ameliorate subsequent therapies.

5. Conclusion

In conclusion we have shown that blood transfusions increase capillary density in anemic children and thus decrease intercapillary diffusion distance. Consequently the capillary surface area necessary for oxygen diffusion is enlarged and tissue oxygen availability increased. The significant correlation of hemoglobin values with functional capillary density proves that high viscosity seems to have a direct positive effect on peripheral perfusion. Moreover, our observations suggest that anemic children with infections seem to profit most from red blood cell transfusion and might benefit from earlier transfusion thresholds. We found a tendency towards better tissue perfusion with red blood cells stored less than two weeks.

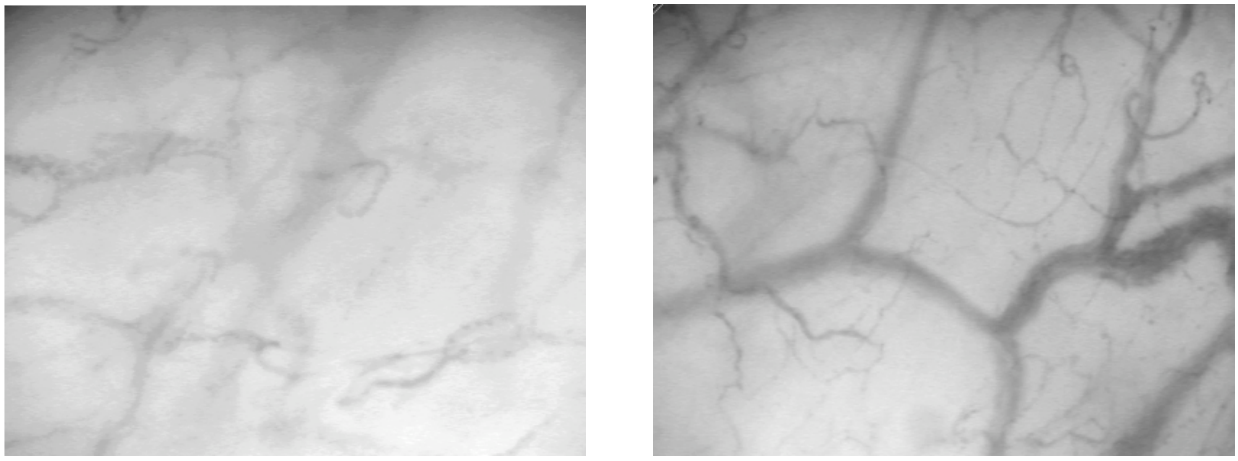
The clinical relevance of our observations regarding the microcirculation and its response to transfusion remains unknown. However, our results highlight the potential for sublingual microscopy to improve clinical decision-making and acknowledge the fact that, given further upgrades in technology, SDF-imaging technique could become a useful tool for assessment of perfusion and it's response to therapy.

Further studies should investigate whether monitoring the sublingual or buccal microcirculation may be useful to adapt transfusion policy and establish standardized guidelines in the treatment of children with severe anemia.

6. Summary

Anemia is classically defined as a deficiency of red blood cells (RBC) or hemoglobin leading to a reduction in the oxygen-carrying capacity of blood. Consequently the purpose of RBC transfusion is to increase the amount of RBCs in the capillaries and to thus increase oxygen delivery to parenchymal cells. However, transfusion thresholds remain controversial due to the significant risks and limited scientific data. Since blindly transfusing to an arbitrarily set hemoglobin threshold may be harmful, better means of identifying the need for transfusion are needed. One way that might be helpful in ameliorating transfusion therapy is looking at its effect on the microcirculation. The microcirculation plays a crucial role in the interaction between blood and tissue, both in physiological and pathophysiological states. It is the microcirculatory network that regulates tissue blood flow and is responsible for vital tissue oxygenation. Analysis of microcirculatory alterations can therefore provide unique perspectives of disease processes at a microscopic level and help to assess therapeutic strategies aimed to improve blood flow through the capillary system.

Our goal was to assess the effect of blood transfusion on the microcirculation and to thus provide valuable information on the quality of tissue perfusion. Only a few studies have investigated the effects of blood transfusion on the microcirculation. We used Sidestream-Darkfield Imaging to directly visualize the sublingual microcirculation in 20 anemic (Mean Hb: 7.2 g/dL, 95% CI 6.6-7.9) children who received red blood cell transfusion. Offline quantitative data of microvascular perfusion was obtained and compared to data of a healthy control group. We found a significant increase in functional vessel density (FVD) immediately after the transfusion with a decrease after 48-72 hours, however with interindividual variation. Capillary perfusion at baseline was lower in anemic patients with concomitant infections but with a larger increase after transfusion compared to anemic children without infections (ΔFVD 3,4 mm/mm^2, vs Δ FVD 1,3 mm/mm^2). Hemoglobin levels and capillary density correlated directly. Whereas conventional monitoring methods may not be able to assess the effect of therapies aimed to improve tissue perfusion, SDF imaging can be helpful to evaluate current guidelines and adapt transfusion policy.



24. Figure; Image of the sublingual microcirculation of an anemic child before and after red blood cell transfusion, as assessed by the side-stream dark field (SDF) imaging technique. AVA Software was used for image analysis. Automatic vessel segmentation and quantitative velocity assessment was performed and presented in a microcirculatory report. Vessels not detected by the program were manually drawn and analyzed. Emphasis was placed on measurement of functional vessel density (FVD). FVD is defined as the length of RBC perfused vessels per observation area and given as mm/mm2.

7. Summary in German/ Deutsche Übersetzung

Die Definition einer Anämie ist ein Mangel an roten Blutzellen, der zu einer Reduktion der Sauerstoff- Transportkapazität des Blutes führt. Der Zweck einer Bluttransfusion besteht darin, die Menge an roten Blutzellen in den Kapillaren zu erhöhen, um eine verbesserte Sauerstoffversorgung an Gewebszellen zu gewährleisten. Aufgrund der erheblichen Risiken und begrenzten wissenschaftlichen Daten sind Transfusionskriterien umstritten, besonders in der Pädiatrie. Da das Unterschreiten eines gewissen Hämoglobin Schwellenwertes als Kriterium für das Verabreichen einer Bluttransfusion nicht ausreicht und in manchen Fällen sogar schädlich sein kann, benötigt man ein besseres Mittel zur Identifizierung von Indikationen für eine Transfusion von Erythrozyten Konzentraten.
Indem man den Effekt von Bluttransfusionen auf die Mikrozirkulation untersucht, könnte man maßgeblich zur Verbesserung von Transfusionskriterien beitragen. Die Mikrozirkulation reguliert den Blutfluss im Gewebe und ist damit verantwortlich für die lebenswichtige Sauerstoffversorgung von Gewebszellen. Die Analyse mikrozirkulatorischer Veränderungen ermöglicht uns wichtige Einblicke in pathophysiologische Vorgänge auf mikroskopischer Ebene und kann somit neue therapeutische Strategien zur Verbesserung des kapillären Blutflusses eröffnen.

Unser Ziel war es, durch die Untersuchung der Wirkung von Bluttransfusionen auf die Mikrozirkulation, wertvolle Informationen über die Qualität der Gewebs-Durchblutung zu gewinnen. Bisher haben nur wenige Studien die Wirkung von Bluttransfusionen auf die Mikrozirkulation untersucht. Wir verwendeten Sidestream-Darkfield Imaging zur direkten Visualisierung der sublinguale Mikrozirkulation von 20 anämischen Kindern (Durchschnittswert Hemoglobin: 7,2 g / dL, 95% CI 6,6-7,9), die Bluttransfusionen erhielten. Die offline-quantitativen Daten der mikrovaskulären Durchblutung wurden mit Daten einer gesunden Kontrollgruppe verglichen. Wir fanden einen signifikanten Anstieg der funktionellen Gefäßdichte (FVD) unmittelbar nach der Transfusion und eine signifikante Abnahme der FVD 48-72 Stunden nach Transfusion, mit interindividuellen Unterschieden. Die FVD anämischer Patienten mit begleitenden Infektionen war vor Transfusion bedeutend niedriger als die von

anämischen Patienten ohne Infektion. Allerdings beobachteten wir in dieser Gruppe nach Transfusion einen größeren Anstieg der FVD als bei anämischen Kindern ohne Infektionen (3,4 ΔFVD mm/mm2, vs Δ FVD 1,3 mm/mm2). Hämoglobinwerte und FVD korrelierten direkt.

Da die Wirkung von Therapien zur Förderung der Gewebsdurchblutung bisher nicht ausreichend monitoriert werden konnten, stellt die SDF Bildgebung ein hilfreiches Tool dar, das zur Evaluierung und Optimierung von aktuellen Transfusions-Richtlinien beitragen kann.

8. Abstract

Background:

Pediatric hematology patients frequently receive red blood all transfusions for severe anemia. Our goal was to assess the effect of blood transfusion on the microcirculation and thus provide information on the quality of tissue perfusion.

Methods and patients:

The sublingual microcirculation was visualized with Sidestream-Darkfield Imaging in 20 anemic (Hb: 7.2 g/dL, 95% CI 6.6-7.9) children receiving red blood cell transfusions and in age matched healthy non-anemic controls. Functional vessel density (FVD) was determined with a semiautomatic program.

Results:

Immediately after transfusion FVD increased (13.4 versus 15 mm/mm$_2$) and RBC velocity (696 (598-792) versus 628 (549-707) μm/s) decreased but FVD was always significantly lower and RBC velocity was always higher than in the age matched control group (FVD 17 mm/mm$_2$; RBC velocity: 486 (441-530) μm/s). FVD at baseline was lower in patients with infections but with a larger increase after transfusion compared to anemic children without infections (ΔFVD 3.4 versus ΔFVD 1.3 mm/mm$_2$). Hemoglobin levels and capillary density correlated well. We did see a larger rise in FVD with transfusion of RBCs aged < 12 days.

Conclusion:

Whereas conventional monitoring methods may not be able to assess the effect of therapies aimed to improve tissue perfusion, SDF imaging can demonstrate improvements after transfusion but also continuous differences to non-anemic controls. In particular, the microcirculation of anemic oncology patients with infection improves after transfusion.

Transfusion thresholds might need to be set higher in in such patients and fresh RBCs < 12 day of storage should be used.

9. Appendix

9.1. List of Abbreviations

RBC-Tx	red-blood-cell transfusion
SDF	sidestream dark field
FVD	functional vessel density
Tx-G	transfusion group
CG	control group
Inf-G	anemic group with infections
nInf-G	anemic group without infections
preTx	before transfusion
pTx1	after transfusion
pTx2	48-72 hours after transfusion

9.2. Clinical Data

Patients	Diagnosis	Gender	Age	Weight
01	Leukemia (AML, CNS +) *	m	5	17,8
02	Leukemia (Prä B-ALL)	m	7	30,7
03	Ewing Sarcoma	f	12	37,4
04	Leukemia (AML)	m	5	16,6
05	Leukemia (ALL)	f	11	39,2
06	Leukemia (ALL)*	f	11	39,2
07	Congenitale anemia	f	4	16
08	β- Thalassemia	m	8	38
09	Leukemia (ALL Relapse)	m	13	53
10	β-Thallasemia	m	8	65
11	Ewing Sarcoma	m	7	79
12	Leukemia (ALL Relapse)	f	17	50
13	Leukemia (ALL Relapse)	m	12	51
14	Osteosarcoma	m	17	64
15	Leukemia (AML Relapse)	m	12	47
16	β-Thallassemia	f	9	25
17	Hemolytic anemia	m	15	50
18	homozygote sicklecell anemia	m	18	45
19	β-Thallasemia	f	8	21
20	Leukemia (Prä B-ALL)	m	15	48

9. Table, Diagnosis, gender, age and weight of each anemic child

***Newly diagnosed (within 48 hours prior to study participation)**

	Hemoglobin		Hematocrit		Plateletes		WBC		CRP	
Patients	preTx	pTx2	preTx	pTx2	preTx	pTx2	preTx	pTx2	preTx	pTx2
01	5,5	7,2	0,17	0,22	95	67	35,1	6	4,1	2,84
02	6,8	8,5	0,2	0,25	10	64	0,4	0,2	0,1	0,1
03	6,1	7,4	0,17	0,21	23	6	0,1	0,2	4,28	3,25
04	6,4	10,5	0,20	0,31	32	37	1,4	2,3	0,74	0,28
05*	4,4	6,8	0,14	0,20	62	53	49,5	41,6	3,37	2,79
06*	5,6	8,6	0,17	0,23	89	51	43,5	30,9	2,57	2,93
07	9,3		0,27		237		5,5		0,1	
08	8,5		0,25		478		9		0,1	
09	6,4	7,8	0,19	0,22	44	130	0,6	1,2	0,15	0,1
10	8,6		0,25		218		5,9		5,9**	
11	6,7	7,4	0,19		19	27	0,1		7,65	
12	7,1	8,9	0,20	0,26	13	36	0,1	0,1	9,02	7,03
13	6,7	8,2	0,192	0,24	37	48	0,6	0,9	6,51	6,14
14	6,4	8,8	0,19	0,26	27	12	0,01	0,5	11,1	14,5
15	6,8	6,6	0,19	0,19	21	23	0,1	0,1	2,18	4,67
16	10,1		0,28		467		11,1		0,1	
17	8,1		0,26		1099		10,8		0,1	
18	9,1		0,27		381		10,6		2	
19	8,9		0,25		233		8,4		0,22	
20	7,2	7,2	0,19	0,21	125	91	0,2	0,1	0,39	0,4

10. Table, Mean laboratory data of Tx-group,(red: subgroup with infections),

*** same patient but different dates of RBC Tx, results were analyzed individually**

**** this patient showed elevated CRP levels, but no clinical signs for infection**

9.3. Vessel length and vessel surface area

	preTx (n19)	**pTx1** (n19)	**pTx2** (n11)	**CG** (n18)
Vessel length	11.3 (10.7-11.8)	12.5 (11.7-13.4)	12.1 (10.8-3.5)	13.2 (12.66-13.81)
Vessel surface area	20.9 (18.9-22.9)	26.7 (24.4-29.0)	22.9 (20.3-25.5)	25.8 (24.5-27.2)

11. Table, Mean and 95% CI of vessel length and vessel surface area

Vessel length is obtained using a drawing tool that allows manual tracing of vessels. As described earlier in the method section, in SDF imaging vessels can only be observed under the presence of RBCs. It is the hemoglobin in blood cells that absorbs the transmitted light from the SDF probe and thereby contrasts the vessels from the background. The vessel walls themselves are actually invisible.

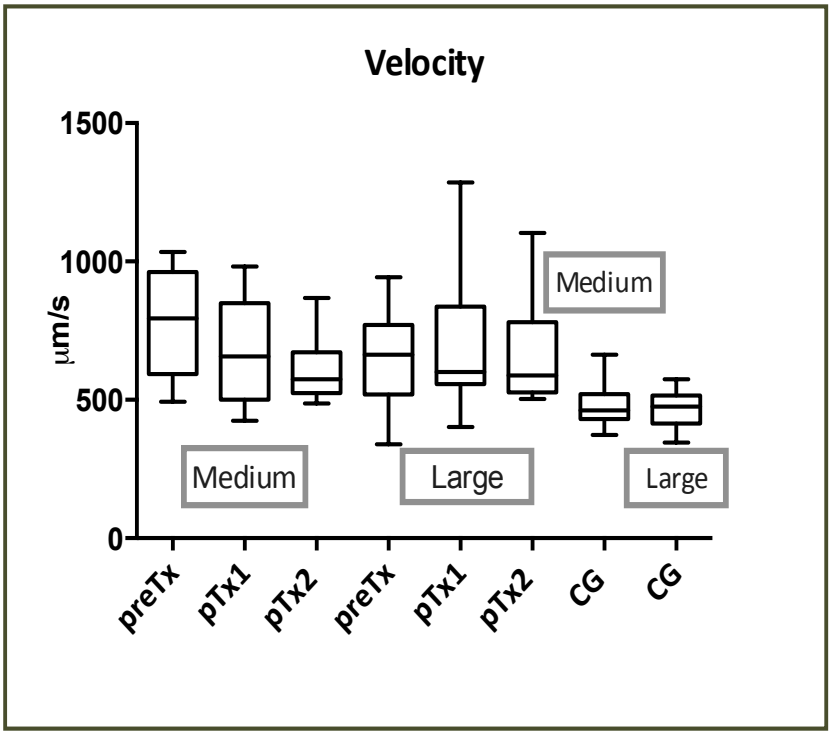
Increased vessel length does not, as the word might suggest, mean that the vessels increase in length. It is the visibility of perfused vessels that increases; therefore the SDF device recognizes more vessels.

9.4. RBC Velocity

		Medium vessels	Large vessels
Control group		477 (433-522)	467 (427-506)
Tx group	preTx n19	707 (611-804)	641 (566-716)
	pTx1 n19	627 (549-706)	699 (586-811)
	pTx2 n10	621 (534-708)	679 (527-832)

12. Table, Velocity values (Mean and 95% CI) for control group and Tx group

The Friedman test was selected to compare the mean velocity values determined at each point of measurement. The Friedman test is a nonparametric test that compares three or more matched groups. It was not able to detect a significant difference in velocity after transfusion for medium vessels or large vessels. The table above shows that the mean values and 95% CI in the control group were much lower for both medium and large vessels.



25.Figure, Box and Whisker plot (Mean and 95% CI) showing velocities for medium and large vessels at different points in Tx group compared to the control group

9.4.1.1. Subgroup sample

For further analysis the whole Tx-Group was again divided into two groups (Inf and nInf), to see if infections influence the microcirculatory velocity results. Statistical analysis with nonparametric tools did not yield a significant difference between the Inf- and nInf-Group.

	non-infection (nInf)	infection (Inf)
preTx	718 (556-880) n 11	694 (563-825) n 8
pTx1	632 (527-737) n 11	620 (459-780) n 8
pTx2	622 (496-748) n 3	603 (469-738) n 7

13. Table, Velocity values (um/s) for medium vessels (Mean and 95%CI)

	non-infection (nInf)	infection (Inf)
preTx	666 (530-801) n 11	652 (588-714) n 8
pTx1	622 (516-728) n 11	873 (495-1252) n 8
pTx2	594 (355-833) n 3	578 (483-673) n 7

14. Table, Velocity (um/s) for large vessels of subgroups (Mean and 95%CI)

9.4.1.1. How was velocity affected?

Vessels were classified according to their diameter into small (< 10 µm), medium (10- 20 µm) and large (> 20 µm). The AVA program is not able to analyze velocity values in small vessels, - therefore only medium and large vessels are taken into consideration.

In this study it was not possible to draw substantial conclusions from the velocity assessments. The clearest finding was that the anemic patients had greater velocity values than the control group, even after RBC transfusion.

In a clinical study performed in pediatric sickle cell patients, the observers used intravital microscopy to evaluate the real time effects of transfusion on the microcirculation. Transfusion resulted in improved tissue perfusion, as indicated by augmented appearance of capillaries and arterioles. They found a decrease in red cell velocity after transfusion. They further postulated that the decrease in velocity might be due to the transfusion related rise in hematocrit and

blood viscosity. However, the decreased velocity might promote vasoocclusion in these patients and thereby impair oxygen delivery.[145] Yuruk et al. showed that perfusion velocity remained unchanged in cardiac surgery patients who received blood transfusions, as analyzed using microvascular flow index (MFI).[64] They further documented the mechanisms by which RBC transfusion improves oxygen transport to the tissue. Apparently the delivery of RBCs to the tissue is not accomplished by increased flow velocities, but by filling "empty" capillaries and thereby diminishing the oxygen diffusion distances among tissue cells. Nitric oxide scavenging and altered RBC deformability might again play a major role in regulating flow velocities, as further described by Horn et al. in an animal model. [146]

Some authors reported that cells can regulate oxygen extraction even under conditions of variable flow and thus the homogeneity of perfusion plays a greater role in assuring tissue oxygenation than flow velocity does. [97]

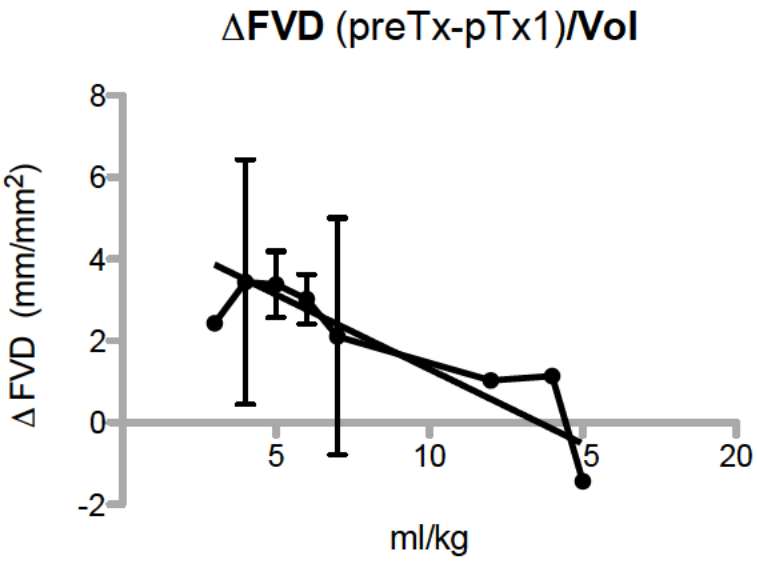
The patient group was too diverse and too small to lead to any applicable assertion in regards to differing velocity tendencies among the patients. It is crucial to note that speed determination of RBCs is uncertain.

One important drawback has to be considered when evaluating velocity scores. Excess pressure applied to the microscope might easily collapse the microcirculation and therefore velocity scores obtained from this area can become unreliable. Capillaries and venules are the most collapsible and react sensitively to pressure. This can result in altered flow, especially in large venules, which manifests itself as sluggish, absent, or alternate. Effort was made to avoid pressure artifacts. However it was hard to distinguish altered or sluggish flow caused by application error from flow alterations as a result of leukocytosis or hemoglobinopathies (e.g. sickle cell anemia). Also movement of the subject or the hand-held imaging device can result in unstable images that disturb vessel recognition and velocity measurements.

Another limitation is that venules and arterioles cannot be differentiated in SDF imaging. It is a known physiological fact that different velocity values are present in arterioles and veins. The observer tried to chose a vessel with fast visible blood flow; also multiple vessels were selected in one image for velocity analysis and a mean value was obtained. It cannot, however, be flawlessly stated which side of the capillary bed (arteriole or venule) was analyzed.

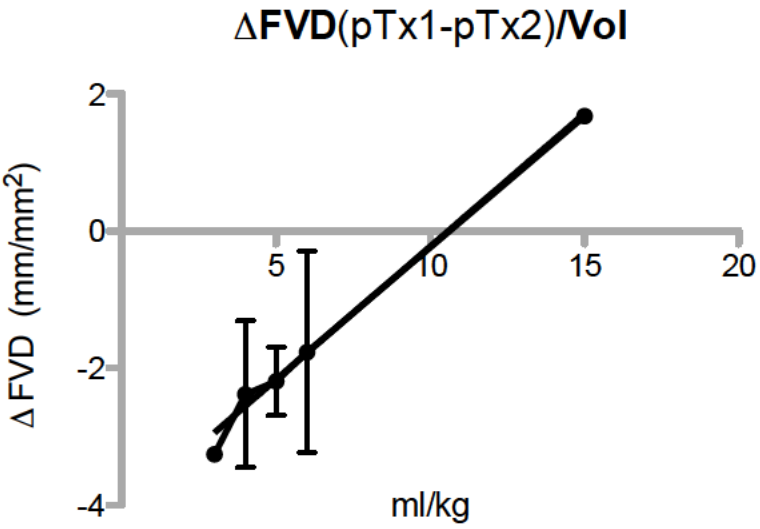
9.5. ΔFVD/Volume

We wanted to see if the change of FVD (ΔFVD) is dependent on the amount of blood transfused (ml) and to thereby hoped to derive helpful indications about ideal transfusion thresholds for the future. However the interpretation of the resulting statistical data is not possible due to too little variance in the obtained variables that would make a statistical calculation viable. Especially the data for children receiving >10 ml/kg bodyweight was limited, due to a lower participation of very young (and light bodyweight) patients. It was ethically difficult to impose multiple transfusions or different amounts of volume unnecessarily. Administration of a certain number of RBCs was left to the discretion of the treating physician; these corresponded to current practices and guidelines. However, the obtained results might raise helpful questions and be assisting for future studies in this area.



26.Figure, Linear regression and correlation of ΔFVD (preTx-pTx1)/Vol.

The correlation is statistically significant (p=0,0044, r^2=0,7659) and would possibly indicate that the change of FVD (ΔFVD) right after Tx depends on the amount of ml/kg transfused. This would mean that the increase is bigger the less blood is given. However, the validity of that argument needs to be proven through a study that specifically aims at this question.



27.Figure, Linear regression and correlation of ΔFVD (pTx1-pTx2)/Vol.

The correlation is statistically significant (p=0,0003, r^2=0,9914)

Whereas the increase of FVD preTx-pTx1 was much higher for patients receiving <10ml/kg blood, patients receiving >10ml/kg RBC clearly experienced a greater change of ΔFVD at pTx1-pTx2.

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12. Curriculum vitae

CARINA MADELEN SCHINAGL

***** 26.11.1986 in Oslo, Norway

Nationality: Norway, Austria

Languages: fluent in German, Norwegian, English, Conversational French

EDUCATION

11/2014 Submission of **doctoral thesis at the Ludwig Maximilians University** (LMU) "Microcirculatory evaluation of red-blood-cell transfusion on severly anemic children, as assessed with the Sidestream-Darkfield Imaging technique¨, under Prof. Genzel-Boroviczeny at the Dr. von Haunersches Kinderspital, Munich

Since 09/14 Pediatric resident at Oslo University Hospital (OUS, Ullevål sykehus)

11/2012 Pediatric resident at Haugesund sykehus
-08/2014

2005-2012 Medical studies at Ludwig Maximilian University (LMU)
08/2007 First part of the state medical examination (Physikum)
06/2012 Second part of the state medical examination (Staatsexamen)

06/2005 General qualification for university entrance

2004-2005 Seminar Schloss Bogenhofen, Austria

2003-2004 Monterey Bay Academy, California, USA

1997-2003 BG/BRG Bruck an der Mur, Austria