Transcapillary fluid balance in children

Methodology and new clinical studies
To the interstitial space – and back again

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Dissertation for the degree of philosophiae doctor (PhD)
at the University of Bergen

2016

Dissertation date: 29 April 2016

Year: 2016

Title: **Transcapillary fluid balance in children**

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Print: AiT Bjerch AS / University of Bergen

Scientific environment

The present studies were completed as a part of the PhD programme at the Department of Clinical Medicine, University of Bergen and were carried out during the years 2007-2015.

The foundation of these studies were conceived at the Department of Paediatrics, Haukeland University Hospital and research and experiments were primarily conducted in Haukeland University Hospital, Department of Paediatrics, and Department of Ear, Nose and Throat. Some patients were recruited from Akershus University Hospital, Department of Ear, Nose and Throat.

All laboratory work was performed at the Department of Biomedicine, Cardiovascular Research Group, University of Bergen. Parts of the work were done in collaboration with the Department of Clinical Medicine, Faculty of Medicine, University of Oslo. The financial support for this work was provided by the Western Regional Health Authority.

Acknowledgements

I am deeply thankful for being given the opportunity to be part of a scientific environment that appreciates integrating clinical activity and research. This work would not have been possible without financial support from the Western Norwegian Regional Health Authority (Helse Vest) and the generosity and positive attitude of Britt Skadberg, Head of the Department of Paediatrics, Haukeland University Hospital.

The conceptualisation of my work was borne and developed with great enthusiasm and a curiosity-driven spirit of my supervisor Professor Ansgar Berg, and I am truly indebted to him for introducing me to clinical research and involving me in his projects. He has inspired me with his knowledge for details and a comprehensive fundamental overview, and he trusted me with the responsibility of doing my own research throughout my PhD work. Despite periods of hardship, he pushed me forward and boosted me with enthusiasm.

I would also like to express my gratitude to my co-supervisor Professor Helge Wiig for patiently educating me in laboratory work at the Department of Biomedicine. He contributed much value to all of my published manuscripts, was always available, sharing his vast knowledge of physiology.

I am grateful for fruitful discussions and statistical help from my co-worker Torbjørn Nedrebø, and equally thankful to Marianne Indrebø, Gunnar Norgård, Olav Tenstad and Jan Kristian Damås for advice and valuable feedback. Throughout my thesis work and writing, many of my colleagues -both in and out of office- have contributed to its completion by helpful comments, critical questions and distractions, which all have been invaluable.

Finally, if it were not for Ingrid, my lover, best friend and rock-solid companion, this accomplishment would not have been possible. Her continuous and patient support is beyond words. She has kept our little family on track, supervising me and our beautiful daughters, Lotte and Elly. I really owe her everything!

Background

The cellular components of the human body, from a single cell to a cluster of cells in a complex organ, need to be embedded in fluid to maintain proper and precise functionality. The fluid within the human body accomplishes many tasks whether in its involvement with intracellular communication, removal of waste products or supply of nutrients to the tissue. Any disturbances in interstitial fluid homeostasis may lead to organ dysfunction, and can even be fatal. Fluid balance and composition of body fluids in different compartments are strictly regulated to ensure stability in response to fluctuations in the outside environment. Powerful control mechanisms within the kidneys and endocrine and autonomic nervous systems help to maintain a relatively constant volume and stable composition of body fluids. Due to the complexities of the constant fluid flux among the different compartments of the body, microcirculatory and capillary-interstitial fluid exchange have been topics of particular interest for many decades. Continuously improving knowledge of fluid balance by studying the forces that act on the walls of capillaries and by examining structures connected to fluid and protein transport can enhance our comprehension of fluid physiology further.

Existing information on fluid balance has been derived from research on cell cultures, animal models, human clinical trials, and from clinical experience, together with theoretical knowledge on fluid balance and therapy. In humans, what we currently know about fluid balance is partly based on studies of adults rather than children, leaving an unfilled gap in knowledge about fluid dynamics from the period covering birth to late childhood[1-3]. The reason for this lack of knowledge is not due to a failure in realising the importance of fluid balance in the paediatric population, but due to difficult, suitable methods to study fluid dynamics in this population and stringent research legislation safeguarding children. The strictly regulated laws concerning paediatric clinical research are meant to protect a vulnerable population incapable of protecting their own interests and therefore to avoid potentially harmful and unnecessary incidents[4]. The Norwegian Health Research Act upholds these laws but

grants permission for clinical research under certain conditions[5]. Several unanswered questions remain about fluid balance in children but can now be more safely addressed within the framework of current laws and ethics.

This thesis highlights important clinical issues concerning fluid balance in children. Classic physiological methods were employed, and the research was conducted with close cooperation between basic research and clinical practice. Furthermore, the thesis includes results from studies that may provide new insight into the mechanisms of fluid regulation across the lifespan. These studies propose the adoption of new values for colloid osmotic pressure in healthy children that may enable clinicians to readily recognise pathological processes involved in vascular regulation in conditions in which tissue fluid retention occurs.

Abstract

Objective: The capillaries represent a semipermeable barrier between the blood and the interstitium, where there is a continuous exchange of fluid and solutes. Numerous factors affect the tight regulation of this movement across the microvasculature, with transmural pressure gradient being a key element governed according to the Starling equation. Colloid osmotic pressures, acting on both sides of the capillaries, are one class of forces defining the movement of fluid between the capillaries and interstitial spaces, and these are influenced by age, maturation, health, and disease. There is limited knowledge on the capillary-interstitial exchange in children that favours extravasation of fluid. Our understanding of paediatric fluid balance is to a certain extent based on practical experience, observation, and extrapolation of data obtained in the adult population, rather than on precepts or theory. The studies included in this thesis work were aimed at evaluating a method for sampling subcutaneous interstitial fluid, studying the interstitial and plasma colloid osmotic pressure in the paediatric population, and at gaining a better understanding of local inflammation during asphyxia, in paediatric subjects.

Methods and Results: In healthy adults, the colloid osmotic pressure of subcutaneous fluid sampled by implanted nylon wicks was similar to that reported in previous studies using other sampling methods. Comparing plasma and interstitial fluid using high performance liquid chromatography excluded the possibility of contamination with haemoglobin and other macromolecules in wicks, and indicates that this sampling method is only mildly traumatic. Colloid osmotic pressures obtained from saline-soaked wicks implanted for different time periods indicate that an optimal implantation time is between 75-90 minutes. There was no significant difference in colloid osmotic pressures obtained by dry or wet nylon wicks. Interstitial colloid osmotic pressure also did not differ for implantation times of 60 or 90 minutes in children. Sixty minutes of topical application of anaesthetic cream before insertion of wicks reduced the pain experience, and did not influence colloid osmotic pressure. This result argues that wicks could likely be used reasonably in the paediatric

population. Boys and girls between 2 and 10 years old had colloid osmotic pressure in plasma similar to that measured in adults, showing increasing age-specific values for interstitial and plasma colloid osmotic pressure. The altered colloid osmotic pressure gradient that occurs during the ages of 2 to 7 years may facilitate fluid transport into the capillaries and reduce lymphatic absorption for the purpose of preserving homeostasis. The colloid osmotic pressure gradient for asphyxiated neonates was unaltered throughout therapeutic hypothermia treatment, showing decreased interstitial and plasma colloid osmotic pressure compared to healthy-term neonates. Cytokine levels during therapeutic hypothermia, as measured by magnetic bead immunoassay, were elevated for IL-1α in tissue and reduced in serum for IL-1RA, IL-6, IL-8, and IL-10, suggesting a balanced inflammatory stimulus. This is underscored by a decreased white blood cell count, which is known to be beneficial for recovery after brain injury.

Conclusions: The wick method is a feasible method for sampling native interstitial fluid in adults and is now demonstrated to be applicable also in children, if performed with topical anaesthesia. Implantation times of 60-90 minutes are sufficient for sampling fluid in both adults and children to avoid cellular inflammation due to implantation trauma. The optimal harvesting time within this time frame is uncertain. Asphyxiated neonates have lowered interstitial and plasma colloid osmotic pressure and reduced colloid osmotic pressure gradient, which may be a normal physiological and beneficial response to therapeutic hypothermia treatment, combined with an overall immunosuppressive effect. The finding of comparable colloid osmotic pressures in children between 2 and 10 years of age and in adults, and for infants over 2 months of age, suggests a change occurs as part of the circulatory transformations that take place after birth.

List of publications

This thesis is based on the following papers, which are referred to in the thesis by the Roman numerals listed here.

I. Guthe HJ, Nedrebo T, Tenstad O, Wiig H, Berg A (2012)

Effect of topical anaesthetics on interstitial colloid osmotic pressure in human subcutaneous tissue sampled by wick technique

PLoS One 7(2): e31332

II. Guthe HJ, Indrebø M, Nedrebo T, Norgård G, Wiig H, Berg A (2015)

Interstitial fluid colloid osmotic pressure in healthy children

PLoS One 10(4): e0122779

III. Guthe HJ, Nedrebo T, Wiig H, Berg A (2015)

Transcapillary fluid flux and inflammatory response during neonatal therapeutic hypothermia: an open, longitudinal, observational study

BMC Pediatrics (2018) 18:82

Abbreviations

CFC	Capillary filtration coefficient
COP	Colloid osmotic pressure
COP_{if}	Interstitial colloid osmotic pressure
COP_g	Glycocalyx colloid osmotic pressure
COP_p	Plasma colloid osmotic pressure
CPB	Cardiopulmonary bypass
EC	Endothelial cells
EG	Endothelial glycocalyx
ECV	Extracellular fluid volume
ECF	Extracellular fluid
ECM	Extracellular matrix
EMLA	Eutectic mixture of local anaesthetic
FCD	Functional capillary density
GAGs	Glycosaminoglycans
HI	Hypoxic ischemia
HIE	Hypoxic ischemic encephalopathy
HPLC	High performance liquid chromatography
ICV	Intracellular fluid volume
IF	Interstitial fluid
IFV	Interstitial fluid volume
MFI	Microvascular flow index
NICU	Neonatal intensive care unit
Pc	Capillary hydrostatic pressure
ROS	Reactive oxygen species
SD	Standard deviation
TBW	Total body water
TH	Therapeutic hypothermia

Contents

Nå er vi i slutten av en deilig sommer,

og slutten er alltid at noe annet kommer.

Så da er vi igjen, midt i begynnelsen…

deLillos, Vakre dager, Midt i begynnelsen, 2002

1. Introduction

The normal cell is a highly complex unit in which the various organelles and enzyme systems continuously carry out metabolic activities that maintain cell viability and support its normal functions. The normal function of cells largely depends on the integrity of the interstitial tissue that makes up the immediate microenvironment of the cell. Interstitial tissue is composed of cells, water, electrolytes, ground substances and fibrillary elements. Transport of ions, nutrients and waste products (metabolic substrates) in the extracellular fluid is life supporting for cellular functions and reproduction. This internal environment or 'milieu intérieur' was already described in the mid-eighteenth century by the French physiologist Claude Bernard[6]. Dr Berhard described in his lectures from 1857 how the human organism tries to maintain equilibrium of body fluids for optimal function, in other words, homeostasis.

1.1 Body water and its compartments

Total body water (TBW) comprises almost 60% of the body weight of an adult man and is distributed between the intracellular and extracellular space. These spaces are separated by a cell membrane, across which the composition of the two fractions are very different[7]. The extracellular fluid is distributed in the interstitial compartments and plasma, separated by the capillary wall, and is thought to be approximately 33% of TBW. The typical adult human has an extracellular volume of 15 litre, for which plasma volume represents 3 litre[8]. The total water content in the body varies considerably, according to a number of factors such as gender, age, and content of body fat. Since TBW, as a per cent of body weight, will decrease with increased body fat[9], a hypothetical adult female will contain about 50% of her body weight as water. This lower percentage is due to a higher body fat content. Interestingly, longitudinal studies indicate a concurrent increase in body water volume with increasing epidemic obesity and overweight together with an enlarged proportion of extra cellular water to TBW[10].

In the foetus, TBW, together with extracellular fluid, declines during pregnancy. By contrast at term, intracellular fluid comprises approximately one half of TBW, the latter presumably reflecting cellular growth and accretion of protein and minerals in addition to accumulation of body fat[11]. At birth 75-80% of body weight is represented as TBW, a percentage that rapidly declines during the first 12 months. A more stable level is reached before 3 years, and around 9-10 years of age, a TBW similar to that of an adult individual is achieved[12] (Figure 1). Regardless of technique used for foetal or neonatal measurement, reported variations in proportions of TBW are small amongst various studies[13]. The brisk reduction of TBW and ensuing foetal weight loss in newborns is normally 5-10% of body weight[14], increasing to 15% in premature infants[15]. The neonatal weight loss can be partially explained by a loss of extracellular fluid, which is greater than the increased volume of intracellular water. Although this phenomena is not fully understood, this may be due to the physiologic cardiopulmonary adaptation that occurs postpartum[16, 17] and to postnatal diuresis, together with a brief negative net water and sodium balance occurring after birth[18-20]. The reduction of TBW throughout childhood is therefore primarily due to a gradual decrease of extracellular water content from approximately 40% of body weight in a neonate to 20% at puberty[11]. With advancing age, muscle mass is reduced and fatty tissue may be increased by 5-10%. This fatty tissue increase is associated with a concomitant progressive decrease in TBW[21], mainly from the intracellular compartment[22].

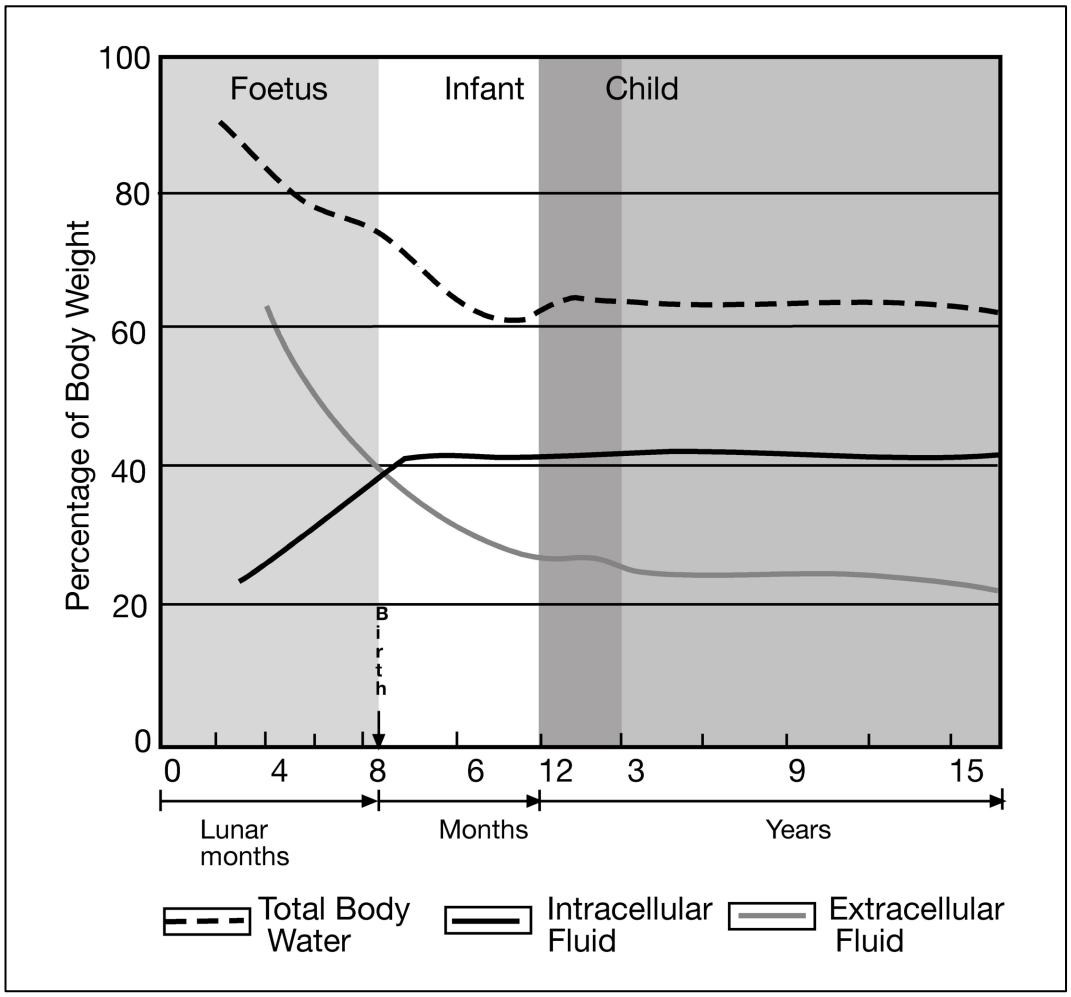

Figure 1. Variation in body water volume with increasing age. (Redrawn from Barsness[23])

From a functional and clinical point of view, total body fluid volume can be roughly thought of as being divided into two separate compartments, the extracellular fluid volume (ECV) and the intracellular fluid volume (ICV). ECV accounts for one-third of TBW (20% of body weight), and ICV accounts for two-thirds of TBW (40% of body weight)[7](Figure 2). With increased muscle mass occurring during puberty in males, their ICV will be typically greater than that of females. A small fraction of volume, called transcellular fluid, includes the volume contributed by the cerebrospinal fluid and synovial fluid. Transcellular fluid has features and content that differs from ECV and ICV and normally represents less than 3% of all body fluids[24]. Solute composition in ECV and ICV differs. While potassium and magnesium are the major cations and proteins and organic phosphates are the major

anions in ICV, the major cation and anions of the ECV are sodium and chloride, respectively, together with the anion, bicarbonate. Cellular ATPase is involved in maintaining this asymmetric transmembrane distribution of cations, in which net flux of water is driven by ECV osmolality. The plasma and interstitial fluid (IF) accounts for 25% and 75% of ECV, respectively. The dynamic exchange of fluid between the plasma and interstitial spaces takes place across the capillary membranes and depends on its permeability properties, restricting protein access to the IF. As a result, IF and plasma will have a similar ionic composition, although plasma will normally contain a higher protein concentration than IF. IF, then, is engendered properties of a fluid buffer in the body, serving to maintain proper fluids and volume in the intravascular space[7]. The plasma volume represents about 5% of body weight in all age groups although may markedly change under pathological conditions[25].

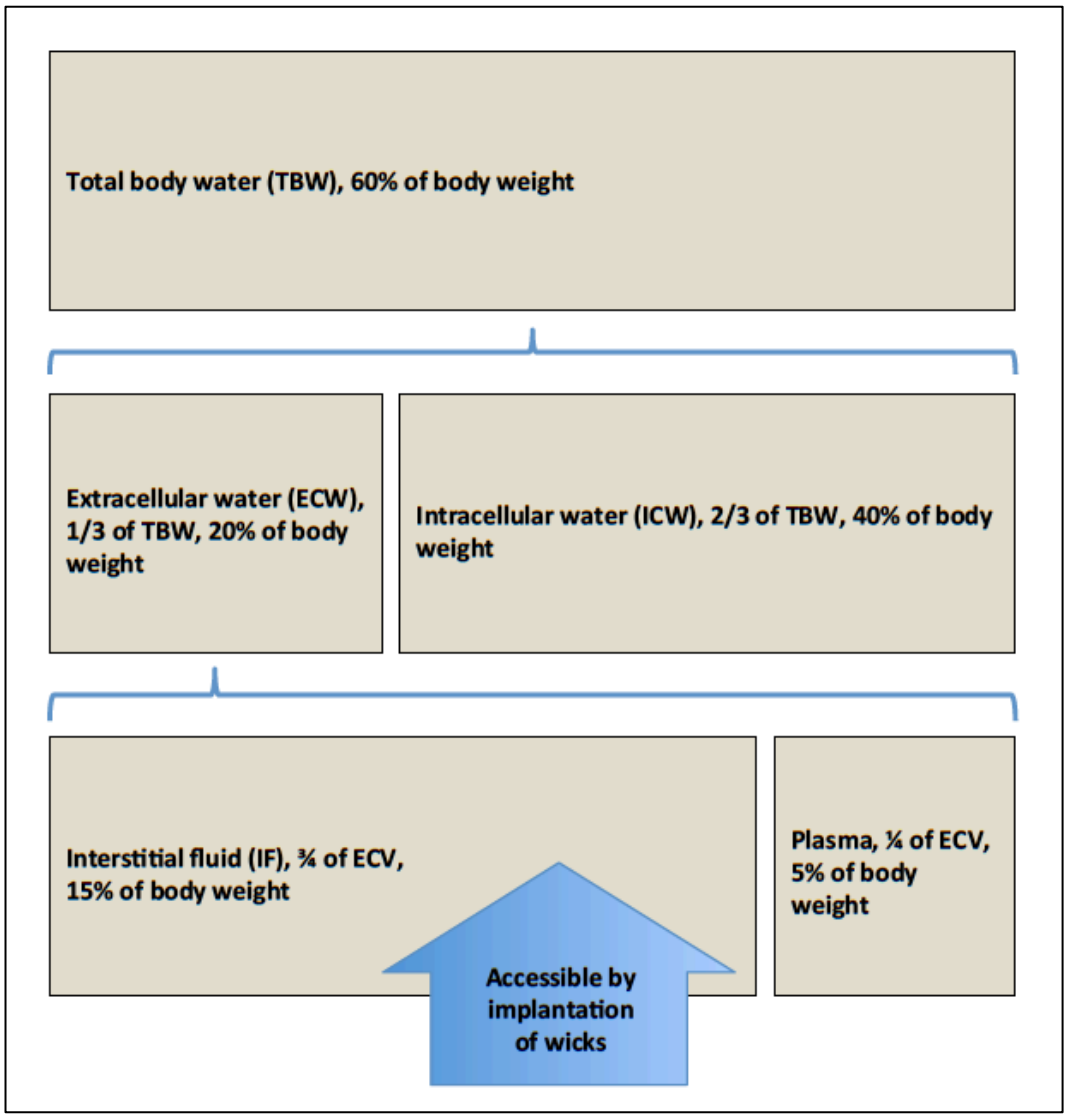

Figure 2. Body fluid compartments

1.2 Macro, - and microcirculation

The circulatory changes during birth represent a change from aquatic to terrestrial life. Clearance of water from the lungs with a concomitant rapid decrease in pulmonary vascular resistance allows increased pulmonary blood flow to fill the left atrium and close the foramen ovale. Obstruction of the intra-atrial connection, in turn, increases left ventricular preload almost simultaneously with cardiac output, a series of events that normally terminates foetal circulation. Due to the increasing systemic pressure over the previous placental pressure (which is lower), systemic vascular resistance rises after birth and is followed by a gradual decrease within the first five years of life. Both stroke volume and cardiac output gradually increase to meet increasing metabolic needs[26].

Although maintenance of blood flow throughout the body is accomplished by the macrocirculation and is vital for homeostasis, functional auto regulation of microcirculation and organ perfusion is a prerequisite for this to happen. Analysing tissue perfusion, and hence the microcirculation, is possible by means of different imaging techniques, like orthogonal polarization spectral imaging and sidestream darkfield imaging, in which microcirculatory variables are measured by a handheld optical device.[27, 28]. Two parameters defining the microcirculation are: (1) functional capillary density (FCD) that serves as a marker of tissue perfusion and (2) microvascular flow index (MFI), a measure of convective flow[29]. FCD in buccal mucosa in neonates younger than 7 days is significantly greater compared to FCD in later infancy. This is related to postnatal adaptation, with initial increased cardiac output partially due to, in general, elevated oxygen consumption, temperature control, and stimulation of gastrointestinal function[30]. This is in line with similar changes in skin found in premature babies, suggesting a correlation with circulatory transition after birth rather than advancing age[31]. A low FCD during disease suggests that tissue perfusion is reduced, and is exemplified by tenacious depressed FCD in children suffering from septicaemia, which has a pessimistic outcome[29]. Similarly, a decrease in axillary microcirculatory blood flow accompanied by lower MFI during therapeutic hypothermia (TH) is reversed after rewarming in neonates suffering from

asphyxia[32]. Whether the reduced tissue perfusion is due to partially microvascular shut down (lowered capillary hydrostatic pressure (Pc)) or to hyperviscosity (as seen in TH) is unknown.

The vessels that permit exchange of fluid and nutrition between cell and blood are part of the microcirculation. These consists primarily of capillaries less than 10 μm in diameter[7]. The capillaries, composed of a unicellular layer of adjacently packed endothelial cells (EC), are encircled by a basement membrane and interspersed by infrequently positioned smooth muscle cells. ECs function as 'gatekeepers', allowing fluid to pass across overlapping intercellular junctions that act as pores between endothelial cells (paracellular route)[33]. These pores can function as signalling complexes that communicate cell position, growth, and apoptosis, and thus regulate vascular homeostasis[34]. Therefore, EC junctions have different characteristics, depending on the target organ and its specific needs. Thus, the endothelium of capillaries can be characterised by plasticity and heterogenic phenotypes[35]. The continuous non-fenestrated endothelium, with 'tight and narrow' junctions, allows only small molecules and water to pass, and is localised to brain, heart, skin, and lung tissue. On the contrary, a discontinuous/sinusoidal endothelium found in liver, bone marrow, and spleen permits nearly all dissolved substances, including proteins, to pass relatively freely. Continuous, fenestrated endothelium has openings throughout the cell layer, which allows vast amounts of fluid and solutes to pass freely, holding back only large plasma proteins. Peritubular renal capillaries, choroid plexus, intestinal and gastric mucosa capillaries, and glands contain this type of endothelium[7].

At a more macro level, the vascular tree is made up of different classes of vessels, continuously patterned and remodelled according to the functions of specific organs. Endothelial heterogeneity across the organs is greatly influenced by epigenetic factors. New insight into vessel maturation and differentiation, pre- and postnatal, reveals that haemodynamic forces[36, 37] (pressure and flow) and presence/absence of hypoxia[38, 39] are important determinants for the continuous development of

components of the vessel wall (i.e., endothelium, smooth muscle and extra-cellular matrix).

1.3 The lymphatic system

The lymphatic system is closely connected to the terminal part of the microcirculation and covers almost all tissues of the body except for the epidermis and cartilage. IF, proteins, immune cells, and other solutes of high molecular weight derived from the arterial end of the blood capillary circulation are absorbed by the lymphatic capillaries and returned to the blood through the lymphatic system. The network of lymphatic vessels therefore guide the immune cells to the lymph nodes, which is important for adaptive and innate immune response in addition for preserving fluid homeostasis and tissue proteostasis[40].

The endothelial cells of the lymphatic capillaries are of different morphological composition than blood vessels, because their function is different from blood vessels[41]. Absorption by lymph is possible because terminal lymphatic vessel walls have spontaneous and stretch-activated vasomotion (lymphatic smooth muscle cells), which serves as ‘pumps’ that favour transport through the lymphatic system. Lymph is emptied into the large veins, accounting for a lymph flow of 120 ml/hour or 2-3 litres/day returned[7, 42]. This is about one-tenth of all fluid filtered by the capillaries.

There are major differences in basal lymphatic function between the foetus and adult[43]. For example in newborn lambs, increased lymph flow occurs as a response to fluid expansion[44], potentially serving as an oedema defence mechanism[45]. It is generally accepted that lymph entering the lymphatics has a composition similar to IF, and that sampling of lymph from the lymphatics, or at a point after the lymph nodes, differs in its protein composition, the latter being increased[46]. Probably more important for lymph flow is the hydrostatic pressure within the interstitium, which generates a positive lymph flux. Sub atmospheric pressures down to -6 mmHg has been demonstrated in the leg of a dog[7]. Interstitial pressures are slightly subatmospheric or close to atmospheric and when increased there is a concomitant rise in lymph flow[8]. Interestingly, a rise in interstitial pressure to the level of

atmospheric pressure generates almost a 20-fold increase in relative lymph flow, and then when the threshold for maximum lymph flow rate is exceeded, oedema will occur (called exceeding the oedema safety factor).

Beside interstitial pressure and the lymphatic 'pump', external intermittent compression of the lymphatics favours lymph propulsion[42]. This compression occurs during contraction of surrounding skeletal muscle, body movement and gravity, pulsation of adjacent arteries, and external compression of the body. Additionally, filaments that anchor the walls of lymphatic endothelial cells to the surrounding tissue will stretch with increased IF, facilitating a flux of fluid into the lymphatics[8]. Since the volume of IF is inextricably tied to a rise in interstitial pressure, the lymph flux in the initial lymphatics will be affected by compliance (see 1.4 Transcapillary fluid exchange) of the interstitium defining the interstitial pressure. The lymphatic system is therefore a primary determinant of IF pressure, volume of IF, and protein concentration of IF.

1.4 Transcapillary fluid exchange

Fluid exchange across the capillaries is possible by either diffusion or filtration. Diffusion according to Fick's law states that fluid moves from a region of high concentration to a region of low concentration, having a magnitude that is proportional to the concentration[47]. Since the capillaries consist of a single endothelial layer, bidirectional transport of fluid across such a semipermeable membrane is considered to be a passive phenomenon, and transport of solutes depends on the chemical and physical properties of the properties[35, 48]. This is called ultrafiltration. Filtration, on the other hand, results in a net movement of water that occurs because of an imbalance between the forces promoting outward flow and forces promoting inward flow.

Ernest Starling postulated in 1896 that fluid movement related to filtration across a capillary wall depends on the balance between the hydrostatic pressure gradient and the colloid osmotic gradient across the capillary wall[49]. This theorem has been reviewed many times, referred now to as the "Starling hypothesis"[50]. It was later

expressed formally in the Starling equation[40] (Eq. 1), in which transcapillary transport of fluid (J_v) is determined by the difference between hydrostatic pressure (P) and colloid osmotic pressure (COP). The balance of these forces allows calculation of the net driving pressure for filtration.

Eq. 1. $J_v = CFC ((P_c-P_{if}) -\sigma (COP_P-COP_{if})) = CFC \times \Delta P$ = flow of lymph

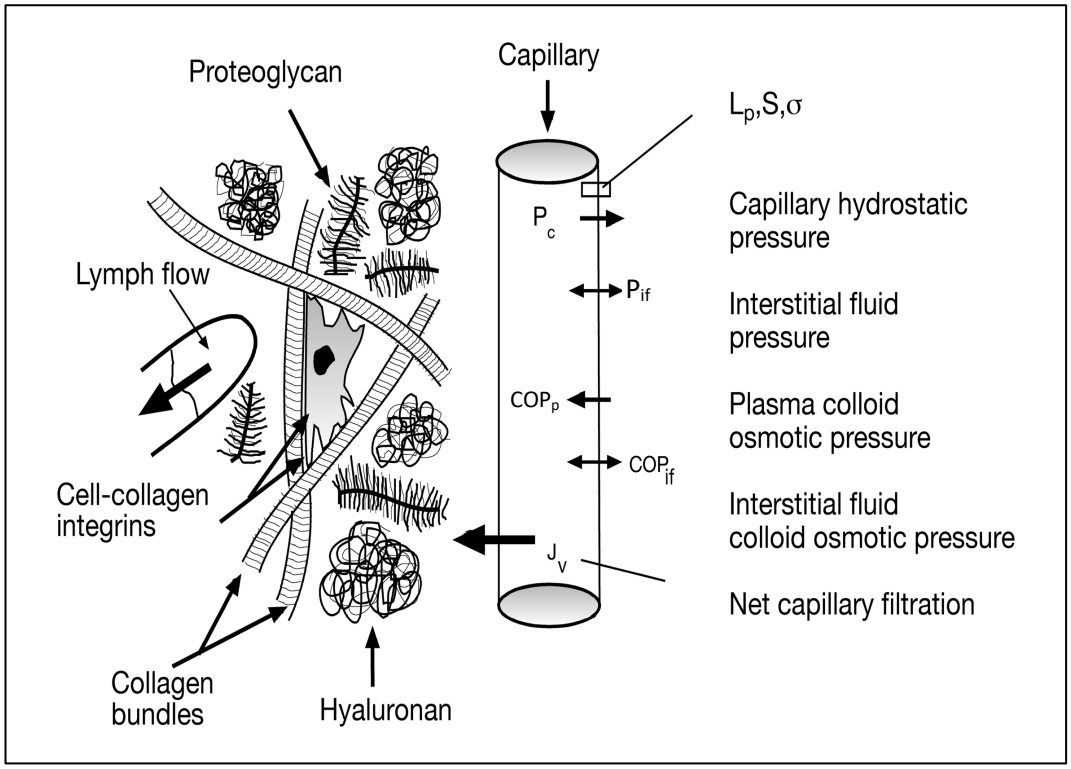

Figure 3 Pictorial representation of the numerical variables of the Starling equation involved in extravasation of fluid. Modified from Wiig et al[51]

The capillary filtration coefficient (CFC) is the product of capillary hydraulic permeability (L_p) per unit surface area and the available area (S) [52,53]. It varies amongst the different vascular beds[54] (i.e., intestine versus skeletal muscle). Capillary hydraulic permeability per unit surface area is often referred to as the hydraulic conductance, which is a measure that describes the efficiency at which water crosses the capillary wall. It follows from Eq. 1 that CFC is a proportional factor related to a given net filtration pressure. A lowered CFC might reduce the net filtration rate that

can be handled by the lymph, but CFC by itself cannot prevent an increase in interstitial volume. CFC is presumed to be constant under different fluid pressures in the same area, given steady state conditions[8]. However, it reportedly shows a moderate increase with systemic low-grade inflammation[55], and gradually decreases in situations of elevated transmural pressure, for example, in the arm or leg, where local vascular response tends to limit excessive filtration [56].

P_c-P_{if} is the difference in hydrostatic pressure between the capillaries (c in P_c drives filtration) and IF (if in P_{if} partially counteracts filtration), while COP_P-COP_{if} is the correlating difference in colloid osmotic pressure, where p is plasma. The value of P_c is highly variable and depends on tissue type and physiological conditions. It is the most variable amongst the pressures in Eq. 1.[57]. P_c is influenced by distance along the microvasculature, microvascular resistance, arterial (P_a), and venous pressure (P_v), together with gravity. Since P_c must be between P_a and P_y, the effect of pre- and post-capillary resistance (R_a and R_v, defining the ratio of R_a /R_v), together with an alteration in arterial and venous pressure, defines its magnitude[7]. Increments in either P_v or P_a will raise P_c, but an increase in P_v in humans is almost five times as effective as the same change in P_a in altering P_c[58]. This is due to the moderately low venous resistance, in which a change in P_v readily is transmitted back to the capillary. Conversely, a relatively high arterial resistance will not conduct a similar change in P_a downstream, in which the capillary lowers the P_c. Therefore, acute inflammation accompanied by local heat production elicits arteriolar vasodilatation, which lowers the pre- to post-capillary ratio and, consequently, raises P_c, favouring filtration to the interstitium[58]. Conversely, arteriolar vasoconstriction after severe bleeding, for example, elevates this ratio and lowers P_c. This results in a greater value for COP_p, which favours absorption of fluid from the interstitium. P_{if} is marginally subatmospheric in the skin of an adult, given a relative state of interstitial dehydration. This occurs by a small net capillary filtration pressure and removal by lymph[8].

The plasma colloid osmotic pressure (COP_p) in human varies for some age ranges. While children and adults have almost identical values of 25 mmHg for COP_p[59, 60], it

is reduced in younger ages[61], being approximately 19 mmHg[62] and 15 mmHg[63] for term and preterm babies, respectively. It is the plasma proteins that drive plasma colloid osmotic pressure, and albumin is responsible for 80% of COP_p. Large molecular mass and net negative charge (Gibbs-Donnan effect) define its oncotic property[64]. The IF colloid osmotic pressure (COP_{if}) varies in different tissues and under different conditions (between 30-60% of plasma COP), and has been the subject of much debate because different methods for harvesting interstitial fluid have been used to determine its true value[40, 57, 65].

IF is traditionally sampled some distance from the capillary, and according to Levick and Michel[40], the measured COP_{if} may not exert the relevant pressure for capillary fluid exchange. The measured COP_{if} may rather reflect a global COP_{if}. A lower COP close to the barrier of filtration, namely the COP of the glycocalyx (COP_g) creates a higher COP gradient ($COP_p - COP_{if} < COP_p - COP_g$), which in turn determines the fluid balance (Figure 4). A lower COP close to the barrier of filtration may result from the ever-present fluid filtration and dilution of proteins. *In vivo* studies in rats and frogs indicate that this oncotic transcapillary pressure gradient depends on filtration rate, with a global COP_{if} close to COP_g near the endothelial border under normal filtration pressures[66, 67]. With capillaries filtering plasma at a high rate, the anticipated increase in filtration due to elevated COP_{if} may be absent, but can be explained by a COP_g approximating 10% of COP_{if}. Wiig and Swartz have recently extensively reviewed this relation, concluding that 'COP_{if} as determined in global IF is still a major determinant of normal fluid filtration'[46], all in line with COP_{if} mirroring COP_g with slow current of filtrate.

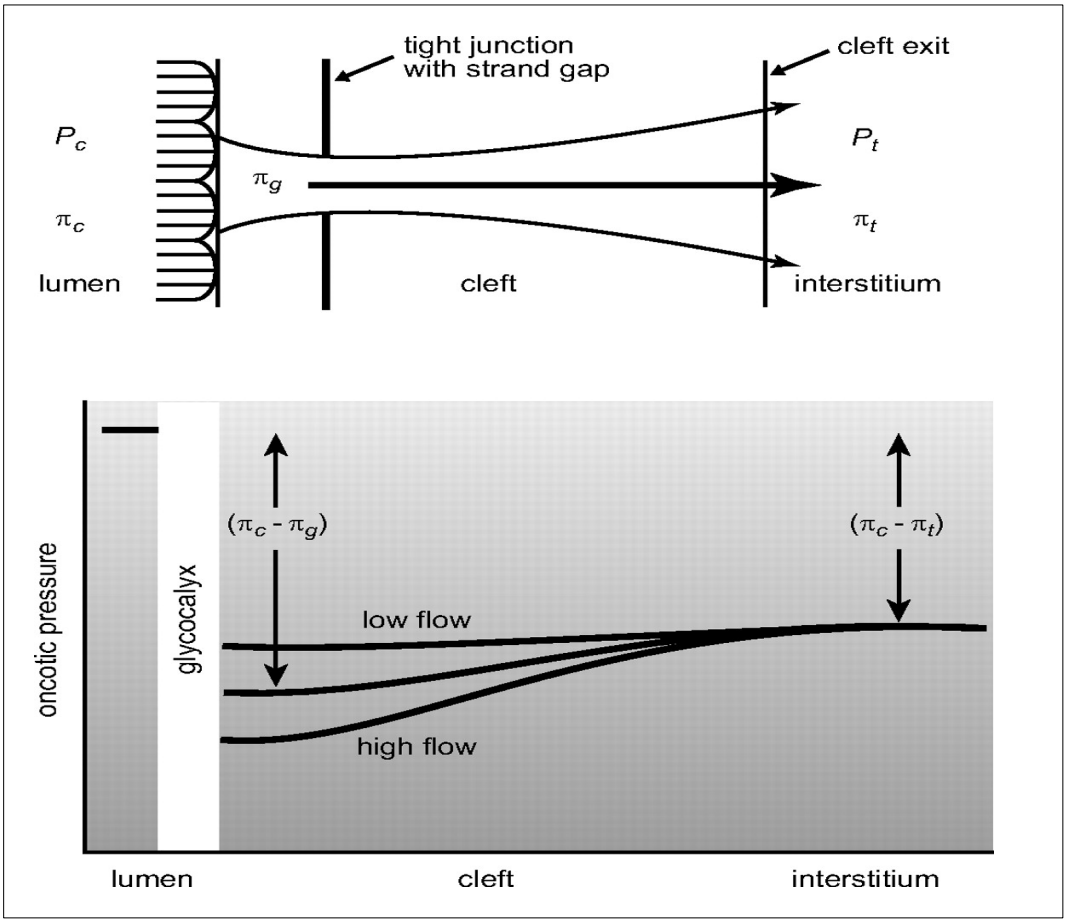

Figure 4. Schematic model illustrating how the effective oncotic pressure in a capillary arises; π_c, π_g, and π_t are the colloid osmotic pressure of the capillaries, the glycocalyx, and the interstitium, respectively. P_c and P_t define hydrostatic pressure in the capillaries and IF. From Wiig and Swartz, with permission[46]

Sigma (σ) is the capillary reflection coefficient for macromolecules and proteins (ranging from 0 and 1), in which an impermeable barrier will have σ = 1, and σ = 0 if the barrier freely allows passage of proteins. Under steady-state conditions, the net filtration of fluid across the semipermeable capillary membrane is balanced by corresponding lymph drainage, and J_v will equal the flow of lymph, which in turn secures steady hydration of tissue. Fluid filtered out from the capillaries and reabsorbed within its proximity is estimated to be 18-27 litres/day[7]. During inflammation, the disruption of the endothelial barrier will change protein permeability and hence reduce the reflection coefficient for proteins causing oedema.

ΔP is the net pressure gradient across the capillaries and is estimated to be between 0.5-1.0 mmHg[8] which leads to a net fluid filtration, but this is removed by the lymph

flow. IF volume and plasma volume will therefore be influenced by changes in colloid osmotic pressure, hydrostatic pressure and flow of lymph. These three dynamic parameters must greatly affect each other in order to obtain normal regulation or autoregulation[8] of the IF volume. Although the intravascular forces (P_p and COP_p) are far excessive than the extravascular forces (P_{if} and COP_{if}) (Eq. 1.), the P_{if} and COP_{if} are comparable in magnitude to the difference between P_c - COP_p[57], and therefore may significantly affect the net transcapillary fluid flux.

Hydration of organic matter will normally result in tissue distension. The ability of a certain tissue to resist these changes defines its compliance, and can be expressed as the alteration in interstitial volume due to a corresponding change in IF pressure ($\Delta IF/\Delta P_{if}$). Different tissues have different compliance and consequently, the low compliant tissue will oppose expansion, showing a distinct increase in P_{if}, even at small increments in volume. By contrast, highly compliant tissue may display a considerable volume increase before P_{if} rises. Compliance is also prone to change over time due to stress relaxation. This is exemplified by high compliance in chronic overhydration due to secondary lymphoedema[68]. Composition, change of interstitial content, tissue architecture, and tissue organisation define the compliance and are influenced by both acute and chronic modulation, such as injury, inflammation, and tissue remodelling[46].

Impaired fluid regulation and subsequent swelling of tissue can result from increased capillary hydrostatic pressure or lowered plasma colloid osmotic pressure, resulting in increased transcapillary fluid flux, or result in reduced drainage of lymph[58]. In human subcutis, visible oedema appears with increments of 50-100% in IF volume, with P_{if} being at a slightly atmospheric levels[52]. An overall increased net movement of water with a raised interstitial pressure and/or lowered interstitial COP was first described by Guyton et al. as, ‘oedema preventing mechanisms’[69]. Disturbing this delicate balance of exchange between IF and blood may have profound effects on the human body, and both age-related differences in COP and P, as well as homeostasis or sickness, may influence its outcome.

1.5 The endothelial luminal surface: glycocalyx component

The endothelial glycocalyx (EG) is a multicomponent luminal network of membrane-bound proteoglycans and glycoproteins that cover ECs[70]. Various plasma components incorporate within the EG. A continuous and dynamic equilibrium is present between flowing blood and soluble and bound contents of the EG that continuously affects its extensiveness and composition. Increased vascular diameter appears to increase the thickness of the EG[71]. The EG has a slightly negative electrostatically charged surface and is involved in many functions, including being a messenger for almost all endothelial tasks, adhesion, permeability, mediation of shear stress, and inflammatory processes[72].

The EG serves as a barrier for unintentional adhesion to the vascular wall by leukocytes or platelets. The intact EG hides adhesive molecules and sheds leukocytes before they adhere, thereby protecting the endothelium[73]. This coincides with a potent proinflammatory stimulus, followed by degradation of the EG and releasing the degraded products into the circulation. Also, chronic inflammation and acute injury signalling have been linked directly to glycocalyx functions, being one of the earliest sites for detection of inflammatory mediators. Detection alters glycocalyx structure and function[71]. In pathological conditions like cardiovascular and ischemia/reperfusion disease, the EG is impaired, resulting in changes in permeability[74]. Studying the mesenteric capillaries of rat, Yen et al. found that interrupted or distorted blood flow, which alters the EG, changes regulation of endothelial nitric oxide (NO) synthase and thus vascular NO production; this is accompanied by loss in vascular tone[75]. The EG therefore seems to serve as mechanical sensor and a transducer of blood flow. Endothelial permeability in rat myocardial capillaries, for example, is greatly increased when EG thickness is reduced, leading to oedema. This cannot be explained by the reduction in EG size or steric hindrance alone[76]. Also, a less negatively charged EG due to glycocalyx degradation occurs with increased protein uptake by ECs, promoting oedema[71].

Little is known about EG composition in younger ages, but it seems that senescence of endothelial cells in cell culture increases traction forces through age-related changes in the glycocalyx[77]. The effect on the COP gradient contributed by the glycocalyx was discussed in the previous chapter.

1.6 The interstitium

The space between cells and capillaries is called the interstitium and is surrounded by IF[8]. The interstitium and the IF reside in the extracellular matrix (ECM) [78]. Composition of the interstitium differs in different tissues, as does the amount of wet tissue weight IF, ranging from 10 to 50%[46]. The interstitium is not solely a passive conduit for fluid transport. The complex structure of the constituents of the interstitium allows each constituent to continuously interact with each other, providing a homogenous fluid environment for the surrounding cells, storage of energy, structural support, and cell-to-cell communicating pathways.

In general, the interstitium is made up of glycosaminoglycans (GAGs) and collagens mixed with elastin in a gel phase of proteins and electrolyte-rich fluid. These are passed through the capillary wall and thus reflect the actual capillary membrane characteristics (i.e., the ultrafiltrate of plasma). Together, the collagens consist of three polypeptide (α) chains[79] and are classified according to function and type of homology (there are at least 28 different types of vertebrate collagens)[80]. Beside supporting tensional strength and scaffolding different tissues due to the reduced ability to expand[8], collagen participate in cell communicating and alter paracrine functions through trans membrane collagens[80]. Cells within the matrix itself mainly produce the content of the ECM, and collagen and elastin are secreted by fibroblasts, which are the major connective tissue cell type.

The two main types of GAGs (non-sulphated and sulphated GAGs) are coiled, complex carbohydrate molecules surrounded by a shell of water molecules that have massive hydrodynamic volume in aqueous solutions[81]; these can differ in size because of tissue hydration. Compared to the almost electro-neutral collagen, GAGs are negatively charged and are partly the reason for the negative charge of the

interstitium-attracting counter ions that are responsible for hydration of the ECM by osmosis[52]. Also, both GAGs and collagen are space-occupying masses, which trap plasma proteins, preventing them from mixing with all IF and allowing for a small free-fluid phase. This is called steric exclusion and may amplify the COP_{if} in less hydrated GAGs[46]. This effect may influence collagen matrix deposition *in vitro*. A balanced COP of GAG and free fluid in the IF are thought to reflect COP_{if}[46].

Other glycoproteins, such as fibronectin, laminins, and matricellular proteins, are part of the ECM and function as either structural components or regulatory molecules[82]. The interstitium is also a reservoir for other ECM components. Different growth factors are induced by a wide array of processes in the ECM and may signal synergistically with integrins[83]. Integrins are transmembrane receptors anchoring collagen fibres to cells, bridging cell-to-ECM interactions. Using an integrin-deficient mouse model, Svendsen et al. linked reduced collagen contraction with lowered IF pressure, supposedly occurring through hydration of GAGs and concomitant development of oedema[84]. Beside 'wash out' of structural components of the ECM due to raised fluid flux, a continuous remodelling of ECM is partly managed by different proteinases degrading and altering the substances of the ECM. These are involved in processes like wound repair and neovascularisation[85].

Beside fibroblasts, osteoblasts, and chondrocytes, endothelial, epithelial, and smooth muscle cells are part of the permanent connective tissue cellular network surrounded by the ECM. Cells outside the ECM responding to inflammatory stimuli, migrate from the blood into the tissue. These include leukocytes, lymphocytes, and other plasma cells. Either transient or stationary residence of immune competent cells, their inflammatory response to cellular activation precedes production and secretion of chemokines and cytokines[86]. In different animal models of pathological conditions accompanied by oedema, these inflammatory agents lower IF pressure together with local inflammation[87]. This demonstrates that the constituents of the ECM, and possibly expression of hyaluronan, are capable of actively enhancing fluid filtration[88].

Transport of water and nutrients through the ECM depends on interstitial hydration and its intrinsic permeability, which is described as hydraulic conductivity[78]. Therefore, over-hydrated tissue, constituting increased permeability and area for transportation, will augment hydraulic conductivity, producing more rapid transportation of water and solutes. Conversely, dehydration and its associated reduced interstitial volume and compressed ECM hinders movement because of reduced hydraulic conductivity. Because intrinsic properties of the gel-like composition of the interstitial matrix hamper free flow of fluid, diffusion by kinetic movement allows swift carriage of electrolytes, water, nutrients of small molecular size and waste products between capillaries and cells. In addition, small vesicles and pockets of water are found within the interstitium, being able to expand immensely and to flow freely if tissue oedema develops[7]. The interstitium of skin (i.e., subcutaneous tissue), being the outermost body organ and readily available, has been thoroughly investigated in rats and mice[89, 90], pigs[91, 92], and human subjects[93-95]. They have a relatively high fluid content (approximately 35% of IF)[52]. Due to this, and the fact that clinical subcutaneous oedema is an early and important marker of many diseases, sampling of IF from the subcutis is a preferred approach.

1.7 Regulation of body fluid volume

A practical approach to fluid regulation is to separate the external fluid balance, represented by intake and output, from the internal fluid regulation between the different body compartments.

The constancy of body fluids is linked to release or conservation of water in response to either solute or water deficit or excess. This exchange is taking place both within the body and with the external environment, representing a disparate milieu. Intricate regulation of ingested fluid and fluid synthesised from metabolic processes, together with loss of water from the urine, faeces, sweat, skin, lungs, and metabolic consumption varies both in health and disease. There are also huge inter-individual variations. National guidelines for adequate intake of total water differs widely[96], and the estimated total water intake of approximately 2.3 litres/day in adults[7] diverges

dramatically from that recorded in a recent study done in France. In that study, the daily total fluid intake was 1.0, 1.1, 1.3, and 1.2 litres/day for children, adolescents, adults, and seniors, respectively[97].

Despite huge variability in water intake, powerful mechanisms regulate and stabilise the extracellular volume within narrow limits, despite frequent and wide-ranging fluctuations in salt and water intake and losses[8]. The water regulatory mechanisms are controlled by changes in intravascular volume or plasma osmolality due to cellular size alterations. These are activated by fluctuations in tonicity sensors in the hypothalamus (plasma and in the cerebrospinal fluid), releasing a messenger hormone (antidiuretic hormone [ADH] or vasopressin) that communicates with the kidney to either excrete or reabsorb water; thirst is also stimulated[98]. An augmented release of vasopressin as a result of high plasma sodium will cause distal segments of the nephron to be more permeable to water. This is accomplished by an increase in the number of water channels, which as a result also concentrates the urine[99]. Similarly, other regulatory mechanisms maintain arterial pressure, acid-base balance, and body fluid osmolality through close interaction of pressure and volume receptors in the autonomic nervous system, endocrine systems, and central circulation[99]. A necessary loss of urine due to a required removal of solutes from the body is present under normal conditions, and the kidneys' ability to concentrate urine, and therefore manage water homeostasis, differs according to age. Both neonates and elderly people have reduced ability to concentrate urine and therefore require a higher intake of a minimal fluid amount[100].

Balance of the internal fluid systems related to fluid movement across the capillaries and between the body compartments (i.e., the ECV) is driven by the equilibrium between oncotic pressures and hydrostatic pressures. The cell membrane separating the smallest compartment is permeable to water, unlike impermeant solutes (like Na^+ and Cl^-) that create an osmotic gradient between plasma, interstitial, and intracellular compartments. This can potentially cause huge changes in cell volume if osmotic equilibrium is not achieved. Therefore, the potential osmotic activity between the compartments can exert tremendous osmotic pressure if not corrected and, for the

same reason, improper fluid treatment can lead to excessive osmotic pressures. The osmotic equilibrium will normally not be affected by isotonic fluid in the intravascular space, but adding saline solution to the extracellular space without calculation of the tonicity of a given treatment solution, physical state, and volume of needed fluid, will cause fluid shifts.

1.8 Microvascular fluid exchange and inflammation

Acute inflammation is an early response to tissue injury. It is nonspecific, typically of short duration, occurs before the immune response becomes established, and is aimed primarily at removing the injurious foreign agent. Acute inflammation is characterised by exudation of fluid and emigration of leukocytes from the microcirculation to the area of injury; these latter cells release different inflammatory mediators[101]. These mediators may create change also in the surrounding healthy uninjured tissue, which is clinically characterised by *rubor* (redness), *calor* (increased heat), *dolor* (pain), *tumor* (swelling), and *functio laesa* (loss of function). These are known as the five cardinal signs of inflammation[102]. The vascular bed responds to inflammation, with (1) vasodilatation leading to increased local flow of blood; (2) increased capillary permeability, with leakage of cells, fluid, proteins, minerals, and salts from plasma into the interstitium; and (3) adjacent swelling of tissue due to the migration of cells and accumulation of fluid with concomitant inflammatory responses, all leading to oedema[101].

Normally, absorption of a circulatory volume increment of 0.5 litres in the IF will take place within 15 to 30 minutes[40], whereas excessive microvascular filtration may lead to oedema due to alteration of the variables in the Starling equation (Eq. 1), changed permeability of the endothelium, or reduced drainage by the lymphatics[103]. Depending on the location of the inflamed tissue, oedema may be life threatening, compromising vital circulation or narrowing of the airways. This can lead to organ failure when local oedema-opposing mechanisms fail. It follows from Eq.1 that a decreased ΔCOP (reduced COP_p and increased COP_{if}) and increased ΔP (increased P_c and reduced P_{if}), together with elevated CFC and reduced σ may greatly imbalance

the fluid flux (Jv). Wiig and co-workers have studied the tracheal interstitium under normal and inflammatory conditions, acknowledging an unexpected high COP_{if} compared to COP_p (85% of plasma COP) during inflammation, implying a robust oedema buffer ability[104]. This is probably due to low interstitial net fluid filtration and concomitant increased protein diffusion that leads to higher protein concentration in the IF. This is sustained during fluid overload and increased filtration (mimicking inflammatory conditions), because of removal of filtered fluid by the lymph.

Oedema is a space-occupying process, and during inflammation, oedema follows reduction of P_{if} [87], although initially increased under normal conditions when capillary filtration is elevated[8], preventing oedema formation. The rationale for a reduced hydrostatic IF pressure is demonstrated *in vivo,* with proinflammatory immune modulation of β1 integrins in mice dermis counteracting collagen and GAGs' tensional strength and hence reducing P_{if} with increased fluid uptake[105].

1.9 Biochemical markers of inflammation and therapeutic hypothermia

Hypoxic ischemia (HI) of nearly every organ causes initial tissue damage due to oxygen deficiency or reduced blood flow, and extends through the phase during which the organ is reperfused[106]. Local activation of microglia, the innate immune cells of the brain, induces production and synthesis of proinflammatory cytokines and chemokines from hypoxia-influenced tissue. An increase in white blood cell (WBC) production follows, amplifying the inflammatory cascade as well as further systemic activation of proinflammatory actions and other immune regulatory tasks[107]. Inflammation during injury is normally a beneficial physiologic response. In the course of reperfusion in hypoxic ischemic brain tissue, formation of reactive oxygen species (ROS), apoptosis, and inflammation intensifies the inflammation, stimulating unfavourable effects. The neuroprotective effect of TH is linked to the reduction of hyperaemia during reperfusion[108]. Expression of ROS during haemorrhagic shock is probably influenced by the indirect effect of temperature on microvascular

permeability in animal models, in which enhanced endothelial barrier function improves during mild hypothermia[109].

Morkos and co-workers found that elevated WBC count and actual neutrophil count (ANC) are related to adverse neurological outcome for non-cooled asphyxiated babies[107], whereas Chakkarapani et al. linked low WBC count to the beneficial effects of TH[110]. Its effect on function, however, is unknown[110]. Complicating this finding is the link between severe cerebral injury and persistent low WBC when normal temperature is re-established[111]. Although studied in rats, inflammatory cytokines and chemokines seem to be expressed differently under hypothermic conditions, primarily as a consequence of reduced microglial and astrocytic activation; this reduces proinflammatory tumor necrosis factor-alpha (TNF_{α}) and interleukin (IL)-6[112]. Down-modulation of IL-6, IL-8, and the anti-inflammatory cytokine IL-10 during TH produces a better outcome[113, 114], suggesting a predominantly suppressive effect on inflammation of these cytokines.

1.10 Neonatal asphyxia

Asphyxia during birth affects 1 to 10 out of 1000 live births globally[115], and can be caused by several conditions depending on the timing of a pathologic event[116]. A newborn suffering from ante-, peri-, or postpartum hypoxia with ischemia; hypercapnia; and metabolic acidosis may develop hypoxic ischemic encephalopathy (HIE). HIE manifests in the first days of life with symptoms reflective of a 'disorganised' brain, such as reduced consciousness level, seizures, decreased tone and reflexes, and often reduced ability to maintain adequate ventilation[117]. Moderate or severe HIE is a major source of long-term disabilities such as cerebral palsy, mental retardation, and epilepsy[118, 119], and accounts for over 700,000 deaths worldwide[120].

The sustained failure of brain tissue oxygenation during hypoxia induces a switch from aerobic metabolism, with maintained respiratory chain activity and ATPase formation, to less effective anaerobic metabolism of glycolysis, with lactate production, causing acidosis. Unlike the adult brain, the immature brain of the

neonate is particularly vulnerable to this realignment of metabolic fuel use and is thus more susceptible to glutamate-associated neuronal damage and generation of ROS[121]. During the first phase of primary energy failure, a limited number of neurons are expected to die[122], although the impaired cell function with cytotoxic oedema, accumulation of neurotoxic substances, and impaired aerobic metabolism recover partially during re-oxygenation[123]. This is followed by a latent phase of 1 to 6 hours, during which recovery of oxidative metabolism, residual mitochondrial injury, inflammation, and receptor hyperactivity occur. Due to different pathways of cellular mechanisms being activated or modulated, a secondary phase of energy failure, occurs from the first 6 to 72 hours of life[124] in moderate to severe HIE. This phase is characterised by deteriorating mitochondrial function, accumulation of excitotoxins, and cytotoxic oedema.

HIE in neonates is classified as mild, moderate, or severe, based on consciousness level, autonomic function, need for respiratory support, and blood acid-base balance. When these criteria are met, the neonate is considered for TH. Standard care and treatment for HIE changed dramatically in 2010, when TH was introduced to neonatal resuscitation guidelines[125]. TH consists of induction (less than 6 hours after birth) and maintenance of a lowered core temperature (to 33.5°C or mild hypothermia) for 72 hours, and is the only single treatment modality for HIE with proven reduced mortality and morbidity[126]. Although TH may improve outcome, many treated children still suffer from neurological disability, suggesting that neonates in this group are quite diverse and thus may require further stratification by physiologic parameters in order to determine what their optimal treatment might be. Treatment by whole body cooling is usually commenced in a tertiary Neonatal intensive care unit (NICU), although it is generally initiated in smaller units before transport for definitive care. Criteria for TH eligibility are shown in Table 1. By lowering the core temperature, all organs, and particularly the brain, are forced into a state of virtual ‘hibernation’, wherein metabolic demands are decreased, thus limiting the ongoing cellular damage[127]. Other hypothermia-induced physiologic changes include cardiovascular and respiratory changes, which are important to acknowledge and need to be addressed in the context of altered physiology after perinatal asphyxia.

Although TH is standard care in industrialised countries and although it suppresses many potentially deleterious mechanisms, it is still difficult in its application to differentiate between cooling-induced physiological beneficial changes and changes that are either ineffective, or even deleterious. Also, managing primary cell death before initiation of cooling and subsequent rewarming is still unresolved and may partially explain the poor outcome for neonates with severe HIE. Hence, including adjunct therapy is of great importance, as it might enhance TH's efficacy in reducing inflammation and cellular dysregulation.

Inclusion criteria	**Exclusion Criteria**
A: Gestational age ≥ 36 weeks	Expected need for surgical treatment (before 3 days of age)
B: At least one of the following: 1. Apgar score ≤ 5 at 10 minutes after birth 2. Requires positive pressure ventilation10 minutes after birth 3. pH < 7.00 in umbilical arterial blood or arterial blood within 60 min after birth 4. Base excess ≤ −16 mmol/L in umbilical arterial blood or arterial blood within 60 min after birth	Severe birth defects with expected poor prognosis
C: Signs of moderate to severe encephalopathy with at least one of the following: 1. Hypotonia 2. Abnormal reflexes or constricted/deviated, dilated, nonreactive to light pupils 3. Weak or absent sucking reflex 4. Presence of seizures	Age > 6 hours before hypothermia could be initiated

Table 1. Inclusion and exclusion criteria for using therapeutic hypothermia in neonates[128].

1.11 Hypothermia and oedema generation

Mild hypothermia will normally decrease heart rate by 10 beats per minute for each 1°C decrease from a starting temperature of 37°C[129]. This results in decreased cardiac output[130], but less rarely, decreased blood pressure needing inotropic support[126]. Since blood pressure is the product of cardiac output and total peripheral resistance, the latter must increase by means of peripheral vasoconstriction to maintain acceptable body temperature (BT), given the small fluctuations in stroke volume that occurs during TH. During hypoxia, the initial vascular response is vascular dilatation, which serves to increase blood flow to prioritised organs, like the brain.

The pathophysiological vascular adaptation to TH is less functionally effective in neonates with severe brain injury, in whom cerebral blood flow is maintained despite markedly reduced cardiac output accompanied by downregulated flow to other vital organs[130]. Whether this is induced by hypoxia-related alterations in local vessel resistance or changed perfusion pressure due to hypothermia is not known. Therefore, these alterations in local vascular pressure during TH would increase the possibility of fluid shifts occurring, leading to the development of fluid retention and subsequently to oedema. Also, inflammation preceding a ischemic-reperfusion injury is likely to trigger a cascade of local mediators that increase microcirculatory permeability, an effect which seems to be reduced by hypothermia in a rat model (due to reduced ROS production) after haemorrhagic shock[109]. Although most of our current understanding of TH and its cardiovascular effects originate from studies of adult humans and animal models, clinical experience and available data from neonates with HIE indicate that oedema formation is not appreciable[131].

1.12 A paediatric perspective

The first line in Nelson's Textbook of Pediatrics — one of the world's most trusted paediatrics textbooks — states: "Children are the world's most important resource"[132], implying that children are regarded as representing the future and that investing in their health endorses the present values of our society and the future.

There are clearly huge differences throughout the world in advocating the well-being of children, where even in developed countries, allocation of resources for this purpose is sometimes vague.

Today, in the field of paediatrics, knowledge and evidence are often generated from data extrapolated from adults, whose medication use and dosages are based on the clinician's experience and common empirical evidence. Children continuously mature over time, and this process is accompanied by changes in physiology and pathophysiology and corresponding changes in pharmacokinetics and pharmacodynamics. Obviously then, children need special attention and must not be regarded as small adults. The safety profile, efficacy, and indication for medications or treatment modalities for adults are different than that for children. So, using the adult parameters for children's safety profiles may result in possible unintended harmful, or even deleterious effects in the absence of adequate research. Body fluid composition and therapy, for example, is one of several paediatric subfields in which therapeutic intervention has been much debated due to old recommendations based on healthy children[133] and scarce evidence from a significant amount of hospitalized children[2].

Our present knowledge on fluid mechanics has been, to a large extent, based on empirical studies and extrapolation of data from animal models. The work done for this thesis addresses age-specific differences in some of the forces that affect fluid balance, variations that have not, to our knowledge, been elucidated before and thus may influence future liquid treatment recommendations for infants and children. In many senses, fluid therapy must be viewed as a drug prescription, one tailored for a specific need and demographic.

These concepts and data discussed in this work are based on literature search that was concluded 30 November 2015.

2. Aims of present study

The overall aim of the present study was to evaluate a simple method of sampling IF from subcutaneous tissue in adults under conditions aimed to reduce the procedural pain experience, and to determine some of the forces directing transcapillary fluid shift in both healthy children and sick neonates suffering from asphyxia.

The specific aims of the study were as follows:

1. To determine the COP and protein distribution in fluid from subcutaneous tissue in adults where interstitial fluid was extracted from dry and wet (soaked in isotonic water) nylon wicks inserted into subjects at different implantation times (Paper I).
2. To evaluate whether topical application of local anaesthetics influenced COP measurements of IF, and to assess whether topical application of local anaesthetics prior to implantation of wicks lessened experienced pain (Paper I).
3. To determine the relationship between COP in subcutaneous skin and plasma of healthy children aged 2-10 years, and to determine age-related values for this specific population at different implantation times (Paper II).
4. To assess the effect of gravity on changed interstitial COP in children by sampling IF at increasing distances from the heart (Paper II) and to evaluate interstitial COP as a function of optimal implantation time of wicks in adults and children (Paper I and II).
5. To determine plasma and subcutaneous COP and its relation to cytokine concentrations in skin and blood serum of asphyxiated neonates treated with TH (Paper III).

3. Study populations and methods

3.1 Study populations and study design

Paper I

Healthy volunteers with no chronic medical history issue and who were working at the Children's Clinic, Haukeland University Hospital, were invited to participate in this non-blinded, sequential descriptive study. Thirty-six males and 14 females were enrolled. To compare equilibrium time for saline-soaked wicks and dry wicks, 20 subjects had four dry wicks implanted in one upper arm location and four wet nylon wicks implanted in the contralateral upper arm. One dry and one wet wick were withdrawn simultaneously after 30, 60, 90, and 120 minutes of implantation; thus, each subject served as his/her own control subject. In 10 supplementary subjects, three wicks were withdrawn after 60, 75, and 90 minutes in the same manner to gain a better understanding of how implantation time affects COP. Twenty additional subjects had one wet wick implanted in each upper arm where the skin on one arm was preconditioned with a eutectic mixture of local anaesthetic (EMLA) cream. Experienced pain was assessed using a visual analogue scale. After insertion of wicks and achieving light venous stasis, a 5 ml venous blood sample was drawn from all participants.

Paper II

The relationship between COP_{if} and COP_p using wet wicks was investigated in paper II. Eighty-seven healthy paediatric patients, ranging in age from 2 to 10 years were included if they met criteria for tonsillectomy and/or adenotomy and/or tympanic paracentesis, as judged by examination of a physician at the Department of Ear, Nose, and Throat, Haukeland University Hospital. Twelve additional patients were recruited from the Department of Ear, Nose, and Throat, Akershus University Hospital, to boost the number of participants. Forty-five patients were females. Children were excluded if they had a medical history of acute illness, chronic disease, or were presently on medication(s) that might interfere with protein metabolism. According to

local policy, induction of anaesthesia and therefore implantation of wicks could not be performed without the subject fasting for at least 8 hours. Seventy-nine patients had one wick implanted in the upper arm and one wick in the medial part of the leg, with an implantation time of 60 minutes. An additional 20 patients, of any age between 2-10 years, had one wick implanted in each leg, and wicks were withdrawn after 60 and 90 minutes to evaluate implantation time. For all participating patients, when a peripheral intravenous cannula was inserted, 0.5 ml venous blood was collected after light haemostasis was achieved.

Paper III

In paper III, we evaluated capillary leakage with changes in COP_{if} and COP_p in asphyxiated neonates treated with TH. Twenty-nine newborns met inclusion criteria for neonatal TH and 17 patients were enrolled for the study at the NICU, Children's Department, Haukeland University Hospital. One wick soaked in NaCl (9 mg/ml) was implanted in the arm or leg 6, 12, 24, 48, and 72 hours after birth. These wicks were withdrawn after 60 minutes of implantation time. Blood samples (0.5 ml) were drawn from an arterial line before implantation and withdrawal of the wick. The association between COP_p and COP_{if} and inflammatory processes was assessed by measuring a wide range of growth factors, cytokines, and chemokines, both in plasma and in IF during TH.

A summary of the three studies' parameters is presented in Table 2.

Paper	Paper I	Paper II	Paper III
Study group and N	50 healthy adults	99 healthy children, aged 2-10 years	17 asphyxiated neonates
Intervention	None	Tonsillectomy, adenotomy, tympanic paracentesis	Therapeutic hypothermia (TH)
Wicks	Total of 160 (wet and dry) wicks withdrawn at: 30, 60, 90, 120 min; total of 60 (wet and dry) wicks withdrawn at 60, 75, 90 min; 40 wet wicks (20 with local anaesthetic) withdrawn at 60 min	158 wet wicks withdrawn at 60 min; 40 wet wicks withdrawn at 60 and 90 min	68 wet wicks implanted at 6, 12, 24, 48, 72 hours after birth; withdrawn at 7, 13, 25, 49, 73 hours after birth
Blood sample	5 ml venous blood sample after wick insertion	0.5 ml venous blood sample after wick insertion	0.5 ml arterial blood sample before and after each wick insertion
Local anaesthesia	Lidocaine/ prilocaine	None	None
General anaesthesia	None	Weight-related doses of sodiumthiopental/ propofol, fentanyl/ remifentanil, morphine, atropine and mivacurium chloride. Sevoflurane gas induction if venous cannulation failed	Weight related doses of morphine/fentanyl and midazolam

Table 2. Protocols and interventions for achieving specific Aims.

3.2 Ethics

All studies were approved by the Regional Committee for Medical and Health Research Ethics, Western Norway, and the Norwegian Data Inspectorate. Written informed consent was obtained from all participating subjects in Paper I after explanation of the study and responding to patient's questions.

The Declaration of Helsinki, which governs the ethical principles for medical research involving human subjects, is particularly strict when it comes to vulnerable groups and individuals, like the paediatric population[4]. The Norwegian Health Research Act also regulates medical and health research in Norway. It states in Chapter 4, paragraph 18, that consent by parents or responsible guardians for persons younger than 16 years who lack the ability to approve their own participation in clinical research must be unambiguously documented. Furthermore, the following are compulsory for minors participating in clinical research: (**a**) any risk or inconvenience to the person be insignificant; (**b**) the person himself does not oppose it; and (**c**) there is reason to assume that the results of the research can be of benefit to the person, or to other persons with the same age-specific disorder, disease, injury, or condition[5]. It is also a prerequisite that the research question under consideration cannot be reasonably answered by similar research done on adults.

Therefore, after suitable research protocols were approved for the present thesis research, all patients contributing data for papers II and III were included in the studies only after obtaining written informed consent from either a parent or guardian after carefully explaining the studies to them and satisfying any questions they had. All three studies were done in accordance with good clinical practice and are registered at the website, ClinicalTrials.gov.

3.3 Monitoring and measurements

3.3.1 Isolation of IF

To gain access to the interstitium, we used the wick method to isolate IF for the studies reported in papers I, II, and III. The method is based on the assumption that the time-limited, subcutaneous implantation of a wick absorbs fluid that reasonably represents the actual innate IF *in situ*. This assumption is supported by the idea that the content of the implanted wick, dry or preloaded with saline, equilibrates with the local interstitial environment and that the wick does not appreciably interfere with subcutaneous homeostasis. Traditionally, wicks are implanted and harvested in proximity to the heart, with the assumption that this location is where the average capillary pressure close to the level of the heart is found [134].

The concentration of IF proteins promotes transcapillary fluid flux and can be measured as COP_{if}. After Scholander described the wick method in 1968[135], Aukland and Fadnes developed and described a modified wick method in 1973, in which saline-soaked wicks were introduced in the subcutis of a rat model[136]. The method was later evaluated and further developed in different animal models[137, 89, 91]. This modified method was developed, because traumatic implantation of a wick may 'pollute' the IF with cellular proteins and may falsely increase capillary permeability with extravasation of plasma proteins, confounding true measurements of transcapillary fluid flux. With further refining of the method[93, 138], sampling of IF from humans in both healthy[139, 140]and disease[94] states by subcutaneous implantation of wicks is now recognised as an acceptable and representative method for sampling native IF, given use of optimal implantation times between 60 and 90 minutes. Several other techniques for isolation of subcutaneous IF are possible and are summarised in Table 3 (modified from Wiig and Swartz[46]). For human research purposes, wick implantation, prenodal lymphatic cannulation and suction blisters are of clinical relevance.

Method	Technique	Sampled material	Advantages	Disadvantages
Implanted wicks	Absorbed fluid in wick	Fluid absorbed from implantation in subcutis	Easy applicable, relative atraumatic	Implantation trauma, inflammation, bleeding
Wick catheter	Absorbed fluid by suction	Subcutaneous fluid	Versatile, relative atraumatic	Implantation trauma
Lymph sampling	Cannulation of prenodal lymphatics	Prenodal lymph	Relative atraumatic	Less versatile
Micropipettes	Suction of fluid	Fluid from subcutaneous fascia	Relative atraumatic	Small volume
Suction blisters	Suction by negative pressure	Fluid between epidermis and dermis	Non-invasive	Inflammation, time consuming

Table 3. Techniques for isolation of native subcutaneous IF (modified from Wiig and Swartz[46]).

3.3.2 The wick method

Double-threaded, gamma-irradiation sterilized (Institute for Energy Technology, Kjeller, Norway), multifilamentous nylon wicks (~0.8 mm in diameter; Polyamid no. 8; Norsk Fletteri AS, Bergen, Norway) were used as wicks. These were introduced subcutaneously by straight suture needles (Acufirm, 210/3, Dreieich, Germany) in lengths of 4 to 5 cm. Wicks were either dry or soaked in isotonic saline. Evaporation of sampled IF from skin and wick was minimised by application of adhesive plastic film (Tegaderm, 3M, Ontario, Canada) around the injection site after implantation. Implantation times ranged from 30 to 120 minutes, and withdrawn wicks were placed into 1.5 ml Eppendorf centrifuge tubes with funnel (Sarstedt, Nümbrecht, Germany)

containing mineral oil. IF was separated from the mineral oil by centrifugation, and then the IF was aspirated and stored frozen in plastic tubes at -20°C until analysis.

For analysis, room-tempered IF samples were transferred to non-heparinized glass capillary tubes by pipette and then centrifuged in a haematocrit centrifuge (Haematocrit 20; Hettich, Tuttlingen, Germany) to separate the mineral oil from the wick fluid. The effect of topical anaesthetics on the COP_{if} measured from saline-soaked wicks was evaluated by preconditioning of skin with 2.5 g of EMLA cream, covered with an occlusive dressing for 60 minutes prior to insertion of the wicks (1 g equals a narrow strip that is 38 mm x 5 mm wide containing 2.5% lidocaine/2.5% prilocaine).

3.3.3 Analysing colloid osmotic pressure

A colloid osmometer measures a pressure difference between a sample and a reference solution separated by a semipermeable membrane. If the sample contains large particles (e.g., particles with a molecular weight over 30 kDalton), a shift of water from the reference chamber to the sample chamber will create negative pressure, which is detected by a transducer in the reference chamber. The disadvantage of extracting IF from wicks is the small amount of sampled liquid obtained and the minimum volume needed for analysing the COP. A typical commercial colloid osmometer often requires a sample size of > 100 µL, and although the measurement volume is < 10 µL, a wick of 5 cm in length will produce only 5 µL of fluid.

In 1974 Aukland and Johnsen designed an alternative colloid osmometer for small samples, requiring > 5 µL of fluid[141]. With additional technical refinement and the use of a low compliant industrial transducer, Wiig et al. made a reliable and accurate osmometer for sample volumes as little as 0.1-0.2 µL[142]. This type of colloid osmometer was used in all three papers for this thesis. The Wiig et al. device is made of pellucid polymethylacrylate (Plexiglas®) and acrylnitrilmethylacrylate (Plexidur plus) plastic (Röhm Chemische Fabrik, Darmstadt, Germany). It contains a transducer (SensoNor A/S, Horten, Norway) connected to an amplifier and a recorder

(Easy Graph P930, Gould Inc., Ohio, USA). It is separated from the sample chamber by a semipermeable membrane that is impermeable to molecules > 30 kDalton (PM-30 Amicron, Lexington, MA, USA). Figure 4 schematically illustrates the details of this colloid osmometer. Operation of the colloid osmometer is thoroughly explained in paper I.

In addition to wick fluid, serum was isolated for COP analysis. COP determined from either plasma or serum was reported to be equal by Noddeland[93].

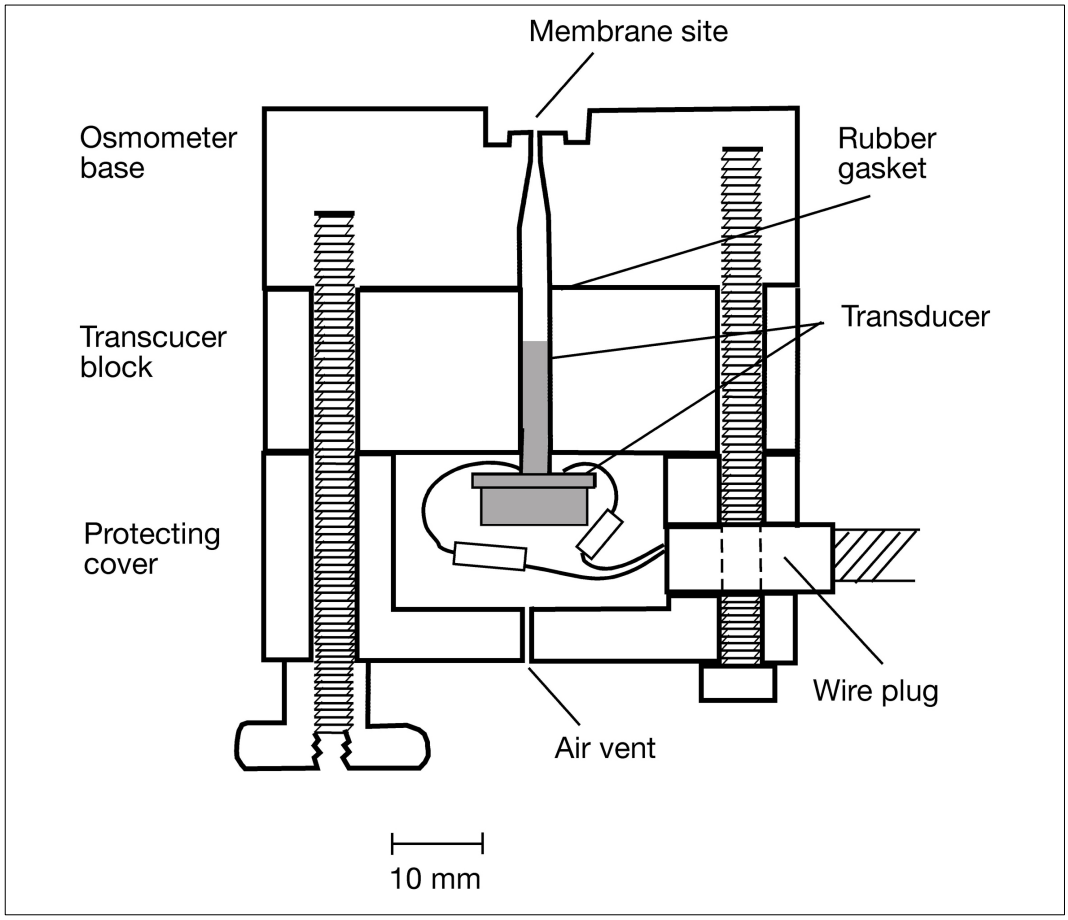

Figure 4. Schematic drawing of the colloid osmometer used in the studies of all three papers (modified from Wiig et al.[142]).

3.3.4 Biochemical monitoring

Patients in the studies reported in papers II and III were monitored and cared for by the attending physician. Additional blood tests, such as measurement of serum albumin and haemoglobin concentration, were analysed by an automatic colorimetric analyser (Cobas 8000 c702, Roche Diagnostics, USA; and CELL-DYN Sapphire, Abbott Diagnostics, USA, respectively) at the Laboratory of Clinical Biochemistry, Haukeland University Hospital.

3.3.5 High-performance liquid chromatography by size exclusion chromatography

A mixed sample of unknown contents can be analysed with high-performance liquid chromatography (HPLC). This method separates the sample's constituents, quantifies them, and identifies them by size. The sample of interest is injected in a mobile phase under high-pressure through a solvent-filled column that has discriminating adsorption patterns for various solute components. The sorbent that divides the sample components is made of solid silica granulate, separating molecules by their size and molecular weight. Several detectors record the components as the sample elutes from the column. The time elapsed as the solute travels through the column and is eluted or detected is called the retention time. Different solutes in the sample will have different retention times, which provide data for analysis. Small particles will elute more slowly and thus have longer retention times than large particles, data of which can be quantified in a chromatogram. Distribution of macromolecules was determined in the IF obtained from the wick method and in plasma by high-resolution size exclusion chromatography in paper I. Size distribution of proteins from IF (obtained from dry and wet wicks) and plasma were compared to determine whether implantation of wicks caused enough bleeding to sufficiently contaminate IF.

3.3.6 Determination of inflammatory markers in IF and serum

In paper III, a multiplex, magnetic bead immunoassay (Milliplex HCYTOMAG-60K; Merck Millipore, Damstadt, Germany) was used to determine and simultaneously

analyse the levels of 15 different cytokines present in IF and in serum. All procedures were performed using the manufacturer's recommendations. IF and serum were analysed at a 1:12.5 dilution using a Luminex 100™ (Luminex Corp.) instrument with StarStation software (Applied Cytometry Systems, Dinnington, UK); samples were analysed at the Broegelmann Research Laboratory, Department of Clinical Sciences, University of Bergen. Arterial blood was allowed to clot for approximately 30 minutes, and then was centrifuged to separate serum; serum was aspirated and then stored in plastic tubes at -20°C until analysis.

For analysis, samples were subject to two freeze-thaw cycles. The following 15 cytokines were assessed: interferon gamma (IFN-γ), interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-1 receptor antagonist (IL-1RA), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-12p40 (IL-12p40), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), transforming growth factor-α (TGF-α), tumour necrosis factor-α (TNF-α), tumour necrosis factor-β (TNF-β), and vascular endothelial growth factor (VEGF). All values were reported as picograms per ml (pg/ml). Results were not verified by parallel tests due to small amounts of source material.

3.3.7 Neonatal TH and ventilation

Patients in the study reported in paper III were all admitted to our NICU and were treated with TH to temper or completely prevent the deleterious effects of neonatal asphyxia. Lowering the core temperature of an organism decreases metabolism, and all body functions are virtually in a neuroprotective state of hibernation.

According to protocol, the patient was placed in an open incubator (Dräger Babytherm 8010, Dräger Medical, Lübeck, Germany), and TH induction (decreased body temperature and maintenance of mild hypothermia at 33.5°C) was managed by servo-controlled, whole-body cooling equipment (Criticool, Mtre, Pennsylvania, USA). All babies born from January 2011 onward were ventilated during TH with a Dräger Babylog® VN500 (Dräger Medical, Lübeck, Germany), and before 2011,

they were ventilated with a Stephanie Neonatal and Paediatric ventilator, (Stephan, Gackenback, Germany). Neonates were maintained hypothermic for 72 hours after birth, and then they were gradually rewarmed to 37°C, a process that took approximately 12 hours.

3.3.8 Cerebral monitoring

Brain function was assessed during TH by monitoring the child's electroencephalogram (EEG) and amplitude-integrated electroencephalogram (aEEG), (both Viasys HealthCare, NicoletOne, www.carefusion.com). This is a relatively non-invasive way to monitor brain function over time, recording voltage gradients originating from the brain tissue using needle electrodes on the scalp. Both short-term and long-term EEG monitoring provide information on possible brain dysfunction[143], and is a valuable tool together with magnetic resonance imaging (MRI). MRI was performed on patients with the expected poorest prognosis during cooling (second or third day of TH). Otherwise, patients underwent cerebral ultrasound scanning, conducted during the first 3 days and before discharge.

3.4 Statistics

SigmaPlot 11 (SyStat Software, Inc., Germany) was used for statistical analyses. Two-tailed paired t-tests were used for comparison of different groups in paper I and all values were presented as means ± one standard deviation (SD). A P value < 0.05 was considered significant in all papers

Results in paper II are presented as numbers with proportions (% of total) and means and plus/minus one SD. One-way ANOVA was used for evaluating the COP_p and COP_{if} for the different age groups, followed by an all-pairwise Holm-Sidak multiple comparison procedure, if a factor was significant in the one-way ANOVA. When comparing smaller groups with non-normally distributed data, we used a non-parametric test (Mann-Whitney).

In paper III, one-way ANOVA was used to evaluate COP_p, COP_{if}, cytokines, MAP, and fluid balance across different treatment time points. If there was a significant difference between the groups, an all-pairwise multiple comparison procedure (Holm-Sidak) was used to assess reliable differences in specific groups. Nonparametric tests for correlation calculations of continuous variables were performed by Spearman's rank correlation test, and categorical variables were analysed by the Mann-Whitney U test. Continuous variables were expressed as means ± SD, and categorical variables as counts and percentages.

4. Summary of results

Paper I: Effect of topical anaesthetics on interstitial colloid osmotic pressure in human subcutaneous tissue sampled by wick technique.

Background: The Starling equation describes the different forces that determine fluid flux over the microvascular wall, where the transcapillary difference in COP is important for capillary exchange of water. Measurement of COP in IF can be done by subcutaneous implantation of nylon wicks. However, wick implantation time and the use of dry versus wet wicks remain controversial issues. Additionally, the use of topical anaesthetics in this context may reduce the experienced pain from implantation of the wicks, but it may also alter microcirculation in the skin, indirectly confounding true COP values.

Methods: COP was measured in plasma and IF from skin of healthy adults using dry or wet wicks. Wick implantation times ranged from 30 to 120 minutes, and the skin was partial pretreated with topical anaesthetics (EMLA cream®).

Results/Conclusions: The use of topical anaesthetics did not influence interstitial COP values, and this modified procedure was subjectively experienced as being less painful. The water content of wicks before implantation also did not affect interstitial COP when using implantation times between 60 and 90 minutes. The COP data obtained in the present study were similar to reference data obtained using other sampling methods. Moreover, our COP results indicate that a wick implantation time between 75-90 minutes is optimal.

Paper II: Interstitial colloid osmotic pressure in healthy children.

Background: Normal and specific pathological values of both hydrostatic pressure and COP measured in plasma and interstitial fluids are known for adults and to some extent in newborns. However, an information gap still remains for the paediatric population in understanding microvascular permeability. The link between interstitial and plasma COP as a function of age and implantation time for children is unknown.

Moreover, whether gravity influences COP at distances peripheral to central circulation remains a question.

Methods: IF was harvested at different time points below and at the heart level in 99 presumably healthy children between 2 and 10 years old. The children were sedated and intubated during a minor surgical procedure.

Results/Conclusions: Plasma COP values for children were similar to those of adults. Increased plasma COP and interstitial COP with advancing age resulted in a raised transcapillary gradient for COP. Neither implantation time for 60 or 90 minutes nor harvesting of IF close or distant to the heart affected COP.

Paper III: Transcapillary fluid flux and inflammatory response during neonatal therapeutic hypothermia (submitted).

Background: Perinatal asphyxia leads to hypoxic ischemic encephalopathy. Treatment with TH may be neuroprotective by reducing metabolism and supressing the immune system. The physiological response to hypothermia and its effect on epithelial permeability is unclear. Moreover, few studies have addressed the mechanisms of fluid imbalance during TH, which can lead to fluid overload and oedema formation. If interstitial and plasma COP change during hypothermia, could a local inflammatory response be detected at the tissue level?

Methods: IF was harvested from nylon wicks at different times after implantation, and COP was measured in plasma and the interstitium, together with measurement of selected markers of inflammation.

Results/Conclusions: Asphyxiated neonates had reduced COP both in plasma and the interstitium, creating an unaltered COP gradient opposing fluid extravasation. No visible oedema was experienced in subjects. IL-1α in the interstitium was elevated during TH, and was modestly correlated with decreased mean arterial pressure. This potentially explains a lowered cardiac output in asphyxiated neonates.

5. Discussion

5.1 General discussion; methodological considerations

Understanding the mechanisms governing fluid flux across capillaries in children is based mostly on knowledge and experience gained from adults. One issue that still is a matter of debate is haemodynamic control using fluid-therapy management [144, 145]. When fluid leaves the capillaries and distributes throughout the interstitium, interacting with the local environment, the balance of forces regulate the amount of fluid remaining in the interstitium. Elevated fluid accumulation in the tissue and oedema may be the result of enhanced endothelial permeability, imbalance between the colloid osmotic and hydrostatic forces, or to reduced lymph drainage[40]. To understand these processes from a paediatric viewpoint, one must first understand the normal states in this population. The limited ability to include children in clinical research has, until recently, restricted our knowledge to a certain extent.

This thesis work focused on gaining a better understanding of fluid shifts between the capillaries and the interstitial space in adults, children, and neonates under both normal physiological conditions and severe asphyxia. Attention was especially focused on the methodology used to sample IF in humans and gaining new knowledge of interstitial inflammatory mechanisms that contribute to altered water balance under hypoxic circumstances. These could potentially favour fluid shift from the circulation to the interstitial compartment.

5.1.1 Reliability of the wick method

To accurately determine COP of the IF (i.e., the plasma proteins and ultrafiltrate of plasma, and possible bioactive compounds in IF), the following assumptions about the fluid sample must be true: (1) the sample represents native IF in undisturbed tissue, and (2) the sample represents the ‘true COP’ acting outside of the capillary wall.

Therefore, the methodology of isolating IF from vascularised subcutaneous tissue has

been developed and refined over the last 40 years, and different techniques have been compared to assess the validity of different sampling methods[8]. The introduction of a device through the skin will certainly disrupt to a degree the natural *in situ* state of the interstitium; this is unavoidable. However, the practical questions are whether it samples the interstitium in a *sufficiently* natural state, and whether the method is applicable with only minor discomfort for the patient. Experience and studies suggest that the wick technique is the most appropriate for studies of human IF. The different methods for native IF isolation are summarised in Table 3. The functional mechanism of and issues using the wick technique will now be discussed.

Implantation of a wick in the IF for a specific period of time, equilibration with the interstitial surroundings, and subsequent removal of the wick, are prerequisites for analysing the wick content. It is obvious that the wick volume must equilibrate with the relatively greater volume of interstitium and preferably without any disrupting influence on measuring the true COP_{if}. When introduced by a needle into the IF, however, the relatively large volume of the wick, compared to that of the capillaries, causes some tissue trauma and local inflammatory reaction[146]. Inflammation may alter local endothelial permeability to some degree, intensifying the already on-going leakage of both plasma proteins and blood cells. This reaction is evident by increased uptake (in wicks) of labelled albumin from the blood after 30 minutes of implantation[93], and by a comparable albumin/globulin ratio in prenodal lymph after additional implantation time[146]. However, native IF, free from inflammatory and dilutional influence, can be obtained in a rat model. In the model, anaesthetised rats are killed before implantation of wicks, and therefore native IF can be examined in the absence of circulation. Kramer et al. found only 1-3% of labelled albumin in wicks from such an experiment, indicating short-term implanted wicks have an insignificant influence on interstitial protein[137].

In an experimental rat model, it was shown that endothelial ‘leak’ was reduced by Non-steroidal anti-inflammatory drugs (NSAIDs), followed by a reduced wick COP and interstitial hydrostatic pressure[146]. This indicates that some protein leakage is required to compensate for the dilutional effect of wick implantation on the IF, and

hence the interstitial protein content. In paper III, we showed that throughout TH, the proinflammatory cytokine IL-6 was expressed at lower levels in IF compared to serum. This result supports the notion of negligible and transitory tissue damage and subsequent inflammation occurring when using the wick technique. Also, a saline-soaked wick may initially dilute the IF, and therefore the interstitial protein content. Then, because of a low attained protein content of the wick, a negative hydrostatic pressure follows. Because of its resistance to dehydration, an underestimate of COP_{if} occurs as a consequence[146]. A small influence of protein concentration is evident when comparing wick-fluid-to-protein ratio of dead and anaesthetised rats[137], and this is in agreement with the assumption of reduced inflammation and osmotic equilibrium after 60 minutes of implantation[146].

To achieve a measurement of 'true' IF, under conditions in which there is equilibrium between wick, plasma proteins, and IF and with suppressed inflammation, the 'crossover technique' can be employed[89, 91]. With this method, saline-soaked wicks or wicks soaked with systematically varying concentrations of protein are implanted in rats shortly after circulatory arrest. Different time points are used, and COP of the priming solution and COP from wick fluid is recorded and plotted in a linear fashion. The protein concentration that remains unchanged throughout implantation represents the 'true' COP_{if}. Kramer[137], Wiig[89], and Heltne[91] all found a higher COP_{if} when using the crossover method and one-hour implantation times compared to COP_{if} obtained with the saline-soaked wicks. This outcome was probably due to known issue that wet wicks tend to dilute IF *ex vivo*, with an expected lower COP_{if}. Additionally, Kramer found a significant change in protein content but not COP_{if}, suggesting that the wick fluid represents osmotic equilibrium but not necessarily protein compositional equilibrium. Unfortunately, human *in vivo* experiments using the crossover technique are, at best, very difficult to conduct.

Because of the lack of a true 'gold standard' or reference method for IF sampling, exploration of other possible methods have led to an even better understanding of COP_{if}. In anaesthetised rabbits (both control settings and volume expansion), COP in sampled prenodal lymph and wick fluid are correlated[147]. Calculated COP in

matching fluid from implanted capsules are also correlated[148]. The suction blister technique, is a third way to harvest IF, although with it, hyperfiltration of fluid and lowered protein content are known to occur[46], which lead to a slightly under estimated COP_{if} of approximately 25% compared to the wick method. However, it is in good methodological agreement with the wick technique[138]. A rather new method to isolate native IF is tissue centrifugation, in which excised tissue is exposed to centrifugal G-force, leading to precipitation of IF[149, 150]. Brekke et al. compared tissue centrifugation to wicks in a swine model with increased fluid load, and found that the wick technique underestimates COP_{if}[151]. It should be understood, however, that in this head-to-head comparison, it is not unreasonable to expect a higher COP_{if} with centrifugation due to additional cell debris and proteins present in this method. Interestingly, the measured COP_{if} from wet wicks was close to that measured previously using crossover techniques[91] and similar wet wick experiments[152, 153]. The centrifugation method is not suitable for human *in vivo* studies, although use of this method in isolation and proteomic studies of tumour IF may be important for detection and monitoring oncological disease[154].

For the purpose of human experiments, the wick method is likely the most convenient and clinically applicable approach, since it is less traumatic and produces less inflammation when allowing for sufficient time for the wick to equilibrate.

5.1.2 Wick results are not affected by haemoglobin contamination

It is essential for the wick method that the extracted liquid be comparable to the native IF of the *relatively* undisturbed subcutaneous environment. It is understood, however, that the introduction of a wick via surgical needle may cause some trauma with different grades of bleeding and associated leakage of products from the bloodstream. Aukland and Fadnes demonstrated that an admixture of blood or erythrocytes might lead to a markedly elevated COP_{if}, if this represents more than 5% of the total protein concentration. Their studies recommend rejection of withdrawn wicks that are clearly smeared with blood, as these contain more haemoglobin than 0.2 g/dl[136]. However, in a related study, Noddeland compared duplicate samples of

pink and clear wick fluids and showed that these had similar COP_{if} values, providing evidence that measurement of COP_{if} from withdrawn wicks that are pink-coloured can be valid[134].

A low fraction of haemoglobin was confirmed in Paper I where we performed HPLC on wet and dry wicks of visual clear and light pink character[140]. We found virtually identical protein peaks in plasma and IF, as well as traces of haemoglobin in the latter, all in line with similar findings in animal models using the wick technique[151], indicating minimal admixture of haemoglobin or protein contaminants. An increased representation of haemoglobin or protein would lead to an overestimate of COP_{if}.

Previous human studies using wicks have reported that bleeding is a minor problem, with 7-15% of wicks having to be discarded because of protein contamination[93, 155, 156]. For wick implantation in these experiments, all subjects received intradermal injections of local anaesthetic lacking adrenaline (vasoconstrictor). In our experiments, we observed more frequent blood contamination of wicks, reaching 22% of wicks in paper I, 19% in paper II, and 32% in paper III. One probable explanation may be that when the interstitium lacks the small liquid volume introduced by the anaesthetic injection, less bleeding occurs, since there is no mass effect on local haemostasis. This possibility is in line with Rein et al., who used no local anaesthetic for wick implantation and recorded a bleeding frequency of 20%[157]. Comparing the activity of patients contributing data for papers I and II, those in paper I commenced daily activity after wick implantation, likely having limb movements that could induce microscopic bleeding near the wick. Evidence from other domains speaks to this issue. For example, patients suffering from hypothermia have inhibited coagulation enzymatic reactions[127, 158], and neonates that bleed during TH treatment are reported to show lowered platelet counts at the beginning of TH[159]. This is all in line with the rather increased proportion of blood-stained wicks we observed in the studies reported in paper III. We were concerned by this but were also constrained in our attempts to increase the number of observations when the implantation of wick produced bleeding. Due to our approved protocol and to the physically limited area for wick implantation in neonates, we saw no option for inserting additional wicks.

5.1.3 Acceptable wick implantation time ranges between 60 to 90 minutes

Another, and maybe more important, inherent problem with *in vivo* implantation is the inflammatory reaction that results from tissue being traumatized, which leads to the above-mentioned extravasation of plasma proteins and fluid. Earlier work with sampling IF in animals has revealed differences in this outcome in relation to type of tissue and species studies[89, 136]. Noddeland was first to systematically study implantation times ranging between 30 and 180 minutes and their effects on osmotic equilibrium in human subjects[93]. The time-dependent equilibrium for water and protein associated with less severe inflammation for COP_{if} measurement is close to 60 minutes. This is due to the fact that 80% of labelled albumin was wick-bound at 30 minutes, resulting in COP_{if} increasing slowly after 60 to 120 minutes[93]. This result agrees with the COP_{if} measured from both wet and dry wicks used in paper I.

The initially high COP_{if} measured from dry wicks and then decreasing COP_{if} measured from 30 to 60 minutes later (paper I) probably reflects leakage of plasma proteins into the wick, which subsequently decrease as implantation proceeds. This is a tendency that was also observed with wet wicks, and is in agreement with previous observations[146]. Kramer et al. reached the same conclusion, in which inflammation marginally influenced protein concentration in the wick after one hour of implantation[137]. In a related study, Wiig et al. found the 'true' COP_{if} using the crossover technique, showing that it slightly increases compared to COP_{if} measured in wet wicks after 60 and 90 minutes of implantation. His studies sampled IF from rat tissue that had functioning circulation, and they reported that the dry wick fluid was closer to the 'true' COP_{if}[89]. If the same situation is applicable in humans, the observation we reported in paper I that a slow but statistically non-significant increase in COP_{if} occurs for all wicks between 75 and 120 minutes after implantation, can be ascribed to local inflammation related to mechanical tissue trauma. Assuming that the inflammation due to wick implantation is transitory and that an osmotic equilibrium in the normal interstitium is achieved in approximately 60 minutes[89, 137], it is reasonable to accept 60 to 90 minutes as the preferred implantation time. Sixty minutes was selected as the standard implantation time in all the present thesis

experiments, although absolute conclusions about optimal implantation time are still not firmly established and need further study.

5.1.4 Local anaesthetics, microcirculatory changes and visual pain score

When harvesting IF from dry wicks, small volumes of fluid are obtained, which precludes multiple analyses[137]. The saline in wet wicks act as a medium for diffusion, and these wicks demonstrate from our pilot studies to cause less pain during implantation. Implanted dry wicks measure a significantly higher COP_{if} after 60 and 90 minutes in rats[89]. Saline-soaked wicks are primarily used in human studies [160, 161, 162, 163, 164, 138, 165, 94, 68, 166], as was used in the studies reported in papers I, II, and III. Less fluid was extracted from wet wicks in neonates (paper III) than in children (paper II) and less than in adults (paper I) due to the length of the implanted wick. However, the procedure still harvested enough IF for COP measurements and some additional tests.

The vasoconstrictive properties of topical skin anaesthetics (EMLA) primarily affect small vessels[167], is time-limited, producing delayed vasodilatation[168], and is rapidly reversed after wick removal from the skin. The finding of a non-significant difference in COP_{if} measured from wet wicks with and without EMLA application with increasing implantation time indicates that minor changes in local microcirculation occurred (paper I). Interestingly, the lower COP_{if} measured from skin treated with EMLA increased gradually, corresponded with COP_{if} measured at 90 minutes from skin lacking EMLA application, and peaked at 120 minutes of implantation. One explanation may be that pre- to post-capillary resistance ratio is reduced, with slightly raised filtration pressure and reduced protein concentration. One can speculate whether the vasoconstrictive effect, known to diminish over time, restores filtration before increased vasodilatation and possibly local inflammation takes over, and hence protein leakage into the IF.

Pain assessment during introduction of the needle for wick implantation and suturing with nylon thread showed that pain, as measured on a visual analogue scale (VAS), was significantly lower, which is consistent with the dermal and intramuscular

analgesic effect of local anaesthetics[169]. Lessening of experienced pain and the minor effect on COP_{if}, may contribute to the efficacy of the wick method in subjects who dislike medical procedures, especially those involving needles. This may be particularly important when studying children in future studies.

5.1.5 General anaesthetics' influence on the microvasculature

General anaesthesia and short-term sedation with propofol (2,6-diisopropylphenol) and sodium thiopental are commonly used in humans as intravenous anaesthetic agents to facilitate smooth transition to unconsciousness. Both agents are known to produce a dose response decrease on blood pressure, and propofol is known to lower vascular resistance accompanied by a diminished baroreceptor reflex and reflex tachycardia[170]. De Blasi and co-workers, for example, observed a propofol-induced increased muscle blood flow and decreased resistance in muscle microcirculation compared to sevoflurane[171], which facilitated a possible fluid shift to the interstitium. In a related study, Bruegger et al. measured CFC in the lower limbs with a non-invasive, computer-assisted venous plethysmograph and found that sevoflurane decreases CFC with less perioperative fluid substitution in contrast to propofol in women undergoing breast surgery[172]. By contrast in a pig model, propofol administered during cardiopulmonary bypass decreased fluid extravasation by almost 30% compared to isoflurane, and the pigs receiving propofol had significantly higher COP_{if} and less tissue oedema[92]. These findings are relevant to the results discussed in this thesis.

Patients in the studies reported in paper II primarily received propofol during anaesthesia maintenance during surgery (some were sedated with the volatile gas sevoflurane), and although nearly 80% of patients received more than their basic fluid requirement, no clinical oedema was observed. Also, the mean COP_{if} for all participants was 13.9±3.5 mmHg, which is comparable to normal adult values. It is reasonable to believe, then, that the use of propofol in surgical anaesthesia maintenance is associated with negligible fluid extravasation.

5.1.6 Analysing COP in serum

A marked fall in plasma COP observed after wick implantation in rats can be explained by the magnitude of insertion trauma and time after implantation for plasma sampling[89, 173]. Although decreased COP_p due to trauma is less likely in humans because of their greater skin-to-surface ratio, the plasma COP should optimally be sampled at the same time as harvesting the wicks. Optimally, it should not be done before implantation, because the blood loss potentially could alter COP. Although measuring a normal COP_p before wick implantation in patients with blood loss over 10% of circulating blood volume (CBV) (paper II), we observed that the mean COP_{if} in this group was significantly higher than the COP_{if} obtained from the other patients (16.0 ± 4.0 mmHg vs.14.0 ± 3.6 mmHg, P = 0.048, arm and leg values averaged). This is in agreement with increased sympathetic-mediated pre-capillary resistance causing a larger increase in COP_p compared to P_c accompanied by a corresponding fluid absorption to maintain circulatory balance. In patients who had no obvious bleeding in the studies of paper III, we measured plasma COP in 12 of 17 patients during wick insertion and immediately after withdrawing the wick. This comparison revealed practically the same COP_p values throughout TH.

5.2 Specific discussion

Wiig and Noddeland have thoroughly evaluated the working mechanism of implanted wicks in rat models and recommended implantation times between 90 and 120 minutes (dry and wet wicks)[89]; they recommend an implantation time of 60 minutes (wet wicks) in humans[93]. Results using dry wicks used in paper I support this view and the finding of an unchanged COP_{if} measured at 60 and 90 minutes. This is apparently inconsistent with findings from previous studies but can be understood by a consideration of technical issues. Increases in COP_{if} measured using both dry and wet wicks between 75 and 90 minutes after implantation[93] can be explained by local inflammation related to mechanical tissue trauma. An implantation time of 120 minutes seems to represent a plateau phase, producing practically equivalent COP values measured from dry and wet wicks.

To our knowledge, COP_{if} measured between 60 and 90 minutes has not been done before in humans, confirming our COP_{if} findings at 75 minutes. A small, non-significant increase in COP_{if} measured from the leg was observed between 60 and 90 minutes (11.3±2.8 mmHg to 12.9±3.1 mmHg) in the studies reported in paper II. This also indicated a lessening effect of interstitial saline diluting and transient local inflammation. We assumed that since the subjects participating in the studies of paper I were all healthy volunteers, the reflection coefficients and capillary filtration coefficients remained normal and unchanged, leaving only ΔP and ΔCOP as potential variables in the Starling equation (Eq. 1). Therefore, based upon previous experience and our own results, 60 minutes was confirmed as standard implantation time.

5.2.1 COP_{if} in adults and children

In humans, COP_{if} measured with wet wicks in normally hydrated subcutaneous tissue of the thorax region is reported to be in the range of 12.5-21 mmHg (mean ~16 mmHg at 60 minutes of implantation)[68, 134, 174]. This differs somewhat from our results reported in paper I. There, we reported a higher mean COP_{if} of 20.5±4.5 mmHg (range 14.8-29.9 mmHg) after 60 minutes of implantation. This difference may be due to higher wick protein content or to slight methodological differences. A higher mean COP_{if} was not associated with an elevated mean COP_p, which for subjects in the studies of paper I, was 27.6±1.8 mmHg (range 23.8-31.8). This would have decreased ΔCOP, favouring increased filtration from the capillaries and filtration by lymph, avoiding excessive interstitial fluid accumulation.

The experiments done for this thesis were done according to protocol and followed the methodological details of previous studies. Our previous pilot and similar studies from our group have produced comparable results to those reported by Bates[68] and Noddeland[93]. Although methodological errors or slight differences in our studies seem less likely, it should be noted that Noddeland injected local anaesthetics before implanting all wicks, potentially diluting the IF and lowering the measured COP_{if}. The dilution of local protein concentration introduced by the injection volume is still less likely, since Bates used EMLA cream as a local anaesthetic, yet he reported a

similar COP_{if}[68]. Also, the protein content in IF from wicks produced a similar protein peak and elution pattern as that obtained from plasma samples. One exception is a small peak in the low molecular weight range that is probably the peak for haemoglobin, indicating no interstitial proteins brought about a higher COP_{if}.

Noddeland observed an elevated COP_{if} measured in the morning and a lowered COP_p measured in the afternoon[93]. He also reported that COP_{if} sampled from the thorax showed non-significant body-posture–dependent variations[134]. All of our subjects in the studies of paper I were examined in the morning, and assuming both arm and thorax represent proximity to the heart, the higher observed mean interstitial COP probably reflects variation of normal physiology. The fluctuations of COP_p seen in all three papers are in accordance with other authors' results, and most likely reflect individual differences within a given study population. This is underscored by the findings of moderately low COP_p relating to low COP_{if}, the association being more pronounced after 90 and 120 minutes of implantation.

Due to a lack of previously unknown COP_{if} values in children, the age span of subjects should ideally include infants and children, from teenagers to adolescence. Our protocol used for the studies reported in paper II aimed to assess presumably healthy children, so did not involve patients covering this entire age range. This is because infants and older children are not admitted to the outpatient clinic. Moreover, the wick method is not pain-free, and the use of local anaesthetics does not entirely remove the presumed sensation of discomfort, precluding its use — for ethical reasons — in the healthy newborns and infants participating in the studies reported in paper II.

5.2.2 Effect of gravity and age on COP_{if}

Since the venous hydrostatic pressure increases with increasing caudal distance from the heart in an upright position (and therefore increased P_c), an expected change in P_{if}, COP_{if}, or COP_p from Eq.1 will prevent excessive fluid filtration in the lower extremities. Measuring the arterial, capillary, and venous hydrostatic pressure (i.e., P_a, P_c, and P_v) in the foot of a standing subject, Levick and Michel found increments of P_a and P_v to be higher than the corresponding increments in P_c due to the high ratio of pre-to-post-capillary resistance modulating P_c close to P_v[50]. In a study exploring body-posture–dependent fluctuations, Noddeland demonstrated a significant increase in calculated P_c measured in the ankle compared to that measured in the thorax, and a reduction in COP_p. There with only minor changes in both COP_{if} and P_{if} measured from an upright or horizontal position, but more importantly, Noddeland observed a significantly reduced COP_{if} measured in the ankle compared to that measured in the thorax[134]. These findings are somewhat at variance with ours.

In the studies reported in paper II, we found no significant difference in COP_{if} measured from the arm or leg. COP_{if} measured in the arm was just above COP_{if} measured in the leg of patients from 2-7 years of age (mean COP_{if} 14.2± 3.5 mmHg in ankle vs. 14.3± 3.4 mmHg in arm); there was an increased difference in patients 8-10 years of age. Subjects in the studies of paper II were positioned in the horizontal position for at least 1-2 hours before sampling of IF, which is 1 or 2 hours less than that in the Noddeland study. Prolonged horizontal positioning of patients in this latter study may have produced a greater difference. By contrast, our findings suggest that children do not experience the same orthostatic effects on COP_{if} as adults, a notion underscored by the lower COP_{if} measured in the leg than in the arm for patients closest in age to adulthood. This was not the case, however, for the younger children in the study. It remains uncertain whether there is a larger orthostatic effect with respect to height and poorer venous drainage correlated with age-specific characteristic changes in the veins or with less physical activity.

5.2.3 Adults and children have similar COP_p

The reported normal mean adult plasma COP of 25 to 27 mmHg[93, 95] is close to the mean COP_p of 27.6±1.8 mmHg for adults reported in paper I. These adult values are also close to the mean COP_p of 25.6 ± 3.3 mmHg we measured in children reported in paper II. The significant increase we observed in COP_p from 24.6 ± 3.2 mmHg at 2-3 years to 28 ± 4.2 mmHg at 8-10 years of age (P = 0.02) in paper II is also in continuum with the results from Sussmane et al. They reported that COP_p increased with age in the first postnatal year (mean 25.1±2.6 mm Hg[59]). The age-dependent increases in COP_p up to 12 months of age may be attributable to the coinciding reduction in TBW and extracellular fluid (see Figure 1), together with increased serum albumin during infancy[175]. An increased serum concentration of proteins (other than albumin) with advancing age[175] supports the corresponding observed elevation of both COP_p and COP_{if} in paper II.

The rise in ΔCOP from 2-7 years of age favours transport of fluid into the capillaries and accompanying reduced absorption of fluid by the lymphatic system in order to preserve homeostasis. Whether this is an oedema-preventing mechanism related to patients being immobilised and thus having less effective lymphatic drainage is unknown. On the other hand, ΔCOP decreasing in the 8-10-year-old group is probably a result of higher COP_{if} compared to a net increase in COP_p that occurs with age. If this is caused by a hydrostatic effect of patients being taller or by a result of too few observations in our study remains uncertain.

5.2.4 COP in the asphyxiated neonate

Several authors have analysed plasma COP in term- and pre-term neonates, reporting decreased COP with increasing gestational age (19.5 ± 2.2 mmHg for term[62] and 15.4 ± 1.3 mmHg for preterm babies[63]). Neonates with respiratory distress, regardless of maturity, have a further reduced COP[176]. COP_p also correlates with birth weight and gestational age in healthy neonates, in which term babies delivered by caesarean

section have equal to or reduced plasma COP compared with those born vaginally[177, 178]. Neonates suffering from asphyxia in the studies of paper III had the highest average value of COP_p (15.7±1.9 mmHg) 6 hours after birth, slowly decreasing with time and with no significant change during TH; this is in line with the above-mentioned studies. Also, these studies confirmed a reported correlation between COP and total plasma protein, which also is consistent with our findings of albumin concentration (represents approximately 80% of COP) within the reference range during TH. Ekblad and co-workers observed a higher plasma COP in neonates suffering from asphyxia compared to that of healthy babies[179], which is opposite to the finding of Wu et al.[180], possibly due to the fact that study groups were dissimilar.

The effect of global ischemia on reduced COP_p in neonates still remains uncertain. The lower but rather constant value of COP_p, along with a reduced haematocrit, partly reflects dilution of plasma proteins after resuscitation and associated intravenously administered fluid, followed stabilisation with fluid restriction (paper III). This is consistent with the initial increased fluid surplus compared to rest of the TH period. The persistent fluid excess may be partly due to hypothermic skin vasoconstriction, reduced urine output, and mechanical ventilation. Counteracting this, global ischemia with shedding of the endothelial glycocalyx, loss of barrier function, and increased vascular permeability (demonstrated during vascular surgery and global ischemia in humans[181]) will potentially promote a fluid shift towards the interstitium, concentrating plasma proteins and reducing COP_{if}. The latter view is supported by the finding reported in paper III of a nearly halved COP_{if} (9 mmHg) compared to infants and children (14 mmHg) throughout TH. The relatively unaltered COP gradient and decreased serum albumin raises several questions. Do other compensating plasma proteins maintain COP_p during TH? Or, are equal amounts of fluid and protein transported across the capillary to the interstitium? The latter is most likely, since ischemia triggers inflammation, which will additionally increase hydraulic conductance permeability to proteins and a decrease in the protein reflection coefficient. Additionally, the finding of decreased axillary microcirculatory blood flow (lowered MFI) during TH in asphyxiated neonates[32] can be interpreted as reduced microvascular pressure, reducing the ongoing leakage.

5.2.5 Asphyxia and markers of inflammation

Deep hypothermia and cardiopulmonary bypass (CPB) are known to elicit fluid extravasation, probably caused by liberation of inflammatory mediators[182, 183]. The finding of minimal loss of proteins in the interstitium with these procedures raises questions about whether CPB or hypothermia is the true cause of fluid extravasation. For example, surface cooling to 28°C in piglets results in a parallel shift of fluid and protein into the interstitium, suggesting that temperature-related induction of inflammation changes endothelial permeability, similar to what is observed in ischemia reperfusion injuries[152]. Heradstveit and co-workers observed that when both hypothermia and asphyxia are present in post-cardiac arrest survivors, treatment with mild hypothermia (TH) to a core temperature of 33°C results in a similar decreased plasma and interstitial COP[94], a finding consistent with our results reported in paper III. However, one difference is that these patients were administered fluids and vasoactive medication in contrast to the piglets[94]. This leads to a search for other possible mediators.

Experimental models of mild hypothermia imply that capillary perfusion is preserved and ischemia-triggered leucocyte-vessel activation is inhibited [184]. This is probably due to reduced ROS expression[109], a possibility illustrated by the report that systemic oxidative stress in asphyxiated neonates is attenuated when they are treated with TH[185]. A reduction of circulating WBCs during TH is common in neonatal animal models, and leukopenia is observed in neonates during TH[111]. A significant drop in WBCs with its subclasses during TH, with the nadir coming after rewarming, together with decreased IL-6, IL-10, and IL-8 was demonstrated in the studies reported in paper III. This drop is possibly associated with a better outcome after 12 months, as recently demonstrated by Jenkins et al.[113]. Although the Jenkins et al. paper employed TH for only 48 hours before rewarming, babies with a prolonged low WBC count had a worse outcome (death or severe neurodevelopmental problems), implying that an immune paresis occurred in the bone marrow.

The increasing body of evidence that TH has a dampening effect on WBCs and the immune response suggests that crosstalk occurs between released chemokines and leukocytes, although the purpose of this is not obvious presently. Although the patients in the studies reported in paper III did not have documented septicaemia or a significantly elevated C-reactive protein (CRP), a hypothermic-dampened immune system may bring about a worse outcome, as demonstrated in animal models and in TH applied in the presence of infection[186]. This justifies using prophylactic antibiotics during TH. A negative correlation between IL-1α and MAP could possibly explain a lower COP_{if} due to a TH-related increase in post-capillary resistance. This resistance could be altered by an inflammation-affected endothelium and a resulting elevated extravasation.

6. General conclusions

- Reproducible measurements of colloid osmotic pressure (COP) from interstitial fluid (IF) are achievable using the wick method (paper I).
- Water content of implanted wicks does not influence interstitial COP when using wick-implantation times between 60 and 120 minutes (paper I).
- Use of the wick method influences the tissue space minimally, causing negligible protein contamination of the wicks (paper I).
- Pretreatment of the skin with topical anaesthetics does not influence interstitial COP harvested from dry or wet wicks with different implantation times, and causes less discomfort during a procedure that some find distressing, like wick implantation (paper I).
- Plasma and interstitial COP values measured in children are within the range of reported values for adults (paper II).
- Increasing age from 2 to 10 years is associated with a rise in COP, assessed in both plasma and interstitial fluid (paper II).
- Wick implantation times between 60 and 90 minutes in children do not alter interstitial COP (paper II).
- An increased difference between plasma and interstitial COP is observed in children from 2 to 7 years of age, and possibly contributes to increased IF absorption (paper II).
- Resting in supine position for at least one hour before the wick procedure is performed does not change the measured interstitial COP in children. This observation is the opposite of what happens in adults and may be due to different orthostatic effects on IF filtration in adults that are absent in children (paper II).
- Optimal implantation time for wet wicks is between 60 and 90 minutes, in accordance with previous recommendations (papers I and II).
- Plasma and interstitial COP measured in neonates during therapeutic hypothermia (TH) is markedly reduced due to hypoxic ischemic

encephalopathy. This is in contrast to that observed in healthy neonates and children (paper III).

- An increased IL-1α in the interstitium of skin and a concomitantly reduced MAP during TH for neonatal asphyxia suggest that local endothelial tissue serves as a protection to prevent inflammatory oedema (paper III)

7. Implications and future perspectives

Although COP assessed in IF and plasma in healthy adults corresponds to that in children aged between 2 and 10 years, there is still a gap in information on characteristics of IF in children from birth to early childhood. The transition of TBW percentage from birth to the first months of infancy encompasses unknown alterations in hydrostatic adaptation, together with COP on both sides of the capillary. Although sampling of IF is less painful after application of topical EMLA cream in adults, the method needs validation in the paediatric population before further studies are conducted.

The growing body of knowledge about glycocalyx structure, function, and significance on COP highlights the importance of developing models for calculating and analysing IF for determining the COP of the glycocalyx.

Because small alterations in the pressure gradient over the capillary membrane may cause substantial fluid shifts, the implications of this shift, vis-à-vis COP, in both health and disease need to be addressed. Also, the influence of crystalloids, colloids, and diuretics need to be addressed, especially in the paediatric population.

Locally produced cytokines have not, to our knowledge, been studied before in neonates. Gaining a better understanding of tissue cytokine production may present an opportunity for modulating inflammation and suggest possible treatment approaches as an adjuvant therapy during TH in neonates.

8. References

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9. Original papers