

**Linköping University Medical Dissertations No. 1125**

**Assessment of microvascular effects of vasoactive drugs**

**Methodological *in vivo* studies in humans  
based on iontophoresis**

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“I keep six honest serving men  
(They taught me all I knew)  
I call them What and Where and When  
And How and Why and Who”

Just So Stories For Little Children  
**Rudyard Kipling** (1865-1936)

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## Abstract

Cardiovascular disease is the leading cause of death in western societies, and endothelial dysfunction is one of the earliest signs seen in the development of such conditions. The development of prognostic tools to aid in the prediction of micro- and macrovascular disease, based on assessment of vascular reactivity, is therefore of paramount importance.

Transdermal iontophoresis offers a quick, non-invasive, and relatively straightforward way to deliver vasoactive substances in order to provoke a vascular response in man. When combined with either laser Doppler flowmetry (LDF), or tissue viability imaging (TiVi), for quantification of these responses, the methodology offers a potentially powerful tool for vascular investigations. The technique has, however, not been established in clinical practice yet and is mostly used in experimental settings. The lack of consensus on what data analysis technique to use, uncertainty concerning the actual drug dose applied, and the difficulties associated with the assessment of responses to vasoconstrictors, may have contributed to this. The aim of this thesis is therefore to address these issues, and thus facilitate the use, and improve the applicability of transdermal iontophoresis, for assessment of cutaneous microvascular function.

More specifically, a non-linear dose-response model ( $E_{\max}$ -model), which is commonly used in *in vitro* investigations of vascular function, was applied to the iontophoresis data. The results show that the  $E_{\max}$ -model accurately describes the cutaneous vascular responses to transdermally iontophored acetylcholine (ACh) and sodium nitroprusside (SNP). The  $E_{\max}$ -model generates variables that can be used for quantitative statistical analysis of data, and enables a more powerful analysis in comparison with the methods presently used. It is further demonstrated that the maximal dose effect and vascular responses vary between different protocols with the same total iontophoretic charge but with different current strengths and durations. This finding implies that the assumption that the local drug dose is linearly proportional to the iontophoretic charge (used for estimation of delivered drug dose to the microvascular bed) may be inaccurate for *in vivo* investigations and that there is need for a more refined model.

It is also demonstrated that in a vasoconstrictive setting (iontophoresis of noradrenaline and phenylephrine) TiVi is the favourable technique for measuring vascular responses as it is sensitive enough to generate data that can be fitted to the  $E_{\max}$ -model even without pre-dilatation of the vessels.

The results from this thesis may contribute to eventually establish transdermal iontophoresis as a widespread clinical tool for detection of vascular function.



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## Abbreviations and Terminology

A	Ampere
A.U.	Arbitrary units
ACh	Acetylcholine
ANOVA	Analysis of variance
ASA	Acetylsalicylic acid
Atr	Atropine
AUC	Area under the curve
CI	Confidence interval
CV	Coefficient of variation
EC <sub>50</sub>	Half maximal effective concentration
ED <sub>50</sub>	Half maximal effective dose
E <sub>max</sub>	Maximum effect
<i>In vitro</i>	Latin: within the glass. Experimental testing outside an organism.
<i>In vivo</i>	Latin: within the living. Experimental testing inside an organism.
LDF	Laser Doppler flowmetry
LDPI	Laser Doppler perfusion imager
LDPM	Laser Doppler perfusion monitor
mC	millicoulomb
NA	Noradrenaline
NaCl	Sodium chloride
NO	Nitric oxide
NOS	NO-synthase
P.U.	Perfusion units
Phe	Phenylephrine
RBC	Red blood cell
RBC <sub>conc</sub>	Red blood cell concentration
ROI	Region of interest
SEM	Standard error of the mean
SNP	Sodium nitroprusside
TiVi	Tissue viability imager
VOP	Venous occlusion plethysmography

## List of Articles

This thesis is based on the following four articles, which are referred to in the text as “Study” followed by their Roman numerals.

- I. **Henricson J**, Tesselaar E, Persson K, Nilsson G, Sjöberg F  
Assessment of microvascular function by study of the dose-response effects of iontophoretically applied drugs (acetylcholine and sodium nitroprusside) – Methods and comparison with *in vitro* studies  
Microvascular Research, 2007, Mar;73(2):143-9
  
- II. O’Doherty J, **Henricson J**, Anderson C, Leahy MJ, Nilsson GE, Sjöberg F  
Sub-epidermal imaging using polarized light spectroscopy for assessment of skin microcirculation  
Skin research and technology, 2007, Nov;13(4):472-84
  
- III. **Henricson J**, Tesselaar E, Baiat Y, Nilsson G, Sjöberg F  
Assessment of microvascular response to iontophoresis of noradrenaline and phenylephrine using local heating and laser Doppler flowmetry  
Submitted
  
- IV. **Henricson J**, Nilsson A, Tesselaar E, Nilsson G, Sjöberg F.  
Tissue viability imaging: microvascular response to vasoactive drugs induced by iontophoresis  
Microvascular Research, 2009 Sept;78(2):199-205

## Sammanfattning

Kardiovaskulära sjukdomar är idag den vanligaste dödsorsaken i västvärlden och ett av de tidigaste tecknen på sjukdom är förändringar av funktionen hos endotelet. Utvecklingen av en enkel och pålitlig metod, avsedd för kliniskt bruk, som kan upptäcka och storleksbestämma sådana endotelförändringar är därför av mycket stort intresse.

Jontofores genom huden erbjuder ett snabbt, oblodigt och relativt enkelt sätt att tillföra kärlen olika substanser för att undersöka tillståndet hos endotelet. I kombination med en mätmetod, antingen baserad på blodflödesmätningar med laser Doppler (LDF) eller på fotografering med polariserat ljus (TiVi), så är jontofores ett mycket användbart verktyg i undersökningar av kärl- och endotelfunktion. Jontofores är trots detta inte en metod som används i någon större utsträckning i kliniska sammanhang idag utan främst som en experimentell metod. Några bidragande orsaker till detta kan vara bristen på standardiserade metoder för att analysera de kärlsvar man får, osäkerhet kring hur mycket läkemedel som verkligen passerar in genom huden, samt svårigheter att använda och mäta effekterna av vasokonstriktorer.

Det övergripande målet med denna avhandling är därför att vidare undersöka dessa problemområden för att förbättra och förenkla användningen av jontofores som metod vid undersökningar av kärlfunktion.

Ett mer specifikt mål är att pröva om data från kärlförsök med jontofores går att analysera med hjälp av en olinjär dosresponsmodell,  $E_{\max}$ -modellen, som ofta används vid undersökningar av kärlfunktion utförda i *in vitro*-modeller. Resultaten visar att  $E_{\max}$ -modellen går att använda för att analysera kärlfunktion vid jontofores av acetylkolin (ACh) och natriumkväveprussid (SNP). Detta stärker jontofores som undersökningsmetod eftersom  $E_{\max}$ -modellen genererar variabler som går att använda för att statistiskt säkerställa skillnader i effekt och verkan mellan grupper av patienter och läkemedel på ett effektivare sätt än vad idag rådande metoder tillåter. Vidare så visar resultat från försök med olika tider och strömstyrkor, men med samma totala elektriska laddning, att den maximala kärlresponsen varierar beroende på vilket protokoll som använts. Denna variation tyder på att den tillförda dosen läkemedel och den elektriska dosen inte är den samma vid jontofores *in vivo* samt att det krävs en mer komplex modell för att beräkna den tillförda dosen läkemedel.

Det konstateras också att under försök med vasokonstriktorer (noradrenalin och fenylefrin) så är TiVi att föredra som mätmetod av de inducerade kärlsvaren då metoden är nog känslig för att kunna användas på kärlbäddar i normaltillstånd, utan fördilatation, och de kärlresponsdata som registreras kan analyseras med  $E_{\max}$ -modellen.

Resultaten från den här avhandlingen kan förhoppningsvis leda till att jontofores blir en mer vanligt förekommande metod i klinisk verksamhet för att upptäcka förändringar i kärlens funktion.



# 1. Introduction

Of the many functions of the human cardiovascular system the most important task is the steady delivery of nutrients, gases and water to, as well as the removal of by-products from, each cell in the human body. This task is achieved by a complex network of vessels that reaches into every part of the human body. The web of fine blood vessels and the flow of blood within this system are referred to as the microcirculatory system and the microcirculation, respectively, and play a pivotal role in the fulfilment of this task.

Blood flow to different tissues and organs is controlled by changes in the diameter of the local blood vessels. When the smooth muscle cells that surround a vessel contracts the diameter of the vessel is reduced, this leads to a reduced flow of blood into the area that is downstream of the contracted vessel. The opposite happens when the smooth muscles cells relax. Under normal conditions the rate of blood flow to each tissue and organ throughout the body is carefully regulated to ensure proper delivery and function. The flow is controlled by a series of complex mechanisms that can be divided into two major control systems; the extrinsic or external control and the intrinsic or local control. The local mechanisms can be thought of as a basic layer of control that adjusts distribution and flow of blood to a single organ; and the extrinsic mechanisms as a higher level of control that serve the interest of the entire organism. Ultimately, the balance between extrinsic factors and local regulatory mechanisms in humans *in vivo* determines vascular function and, therefore, the blood flow within the tissue.

Many acute and chronic medical conditions such as diabetes, hypertension, heart failure, and sepsis are associated with impaired or lost vascular regulatory function that upsets this balance, eventually leading to impaired functionality, or even loss of, an organ or limb (Haisjackl, M. et al. 1990; Hinshaw, L.B. 1996; Hutchins, P.M. et al. 1996; Tooke, J.E. 1996).

One of the earliest signs seen in these conditions is changes in the endothelial function. The endothelium is a single layer of cells that lines the inner walls of each vessel in the body. These cells produce a series of substances, including nitric oxide (NO), prostacyclin and endothelin that all have an effect (they are said to be vasoactive) on the vessel diameter and blood flow. Nitric oxide, although it has a very short lifespan and is quickly degraded, is perhaps the most important of these three substances. It is a powerful vasodilator and plays a major role in causing local vasodilatation (Furchgott, R.F. and Zawadzki, J.V. 1980).

Loss or impairment of the production of NO by the endothelium contributes to development of arterial disease.

The endothelial functionality of the microcirculation often reflects the status of the entire vascular system and changes in regulation often appear in an early state of disease, not seldom before any effects on the systemic regulation are noticed (Quyumi, A.A. 2003; Verma, S. et al. 2003; Hadi, H.A. et al. 2005). However, such changes may be difficult to assess, especially in a clinical setting. It may be stated that few, if any techniques, are used clinically today for this purpose. More work is therefore aimed to refine existing methods and develop new and reliable techniques that can be used *in vivo* for accurate assessment of microvascular flow regulatory function. Such techniques may be valuable in terms of both diagnosis and prognosis, and may also prove useful in guiding treatment (Haisjackl, M. et al. 1990; Beed, M. et al. 2009) and may be used to better understand the underlying mechanisms of common cardiovascular diseases.

Of all the various microvascular beds in the body, the cutaneous is the most accessible and readily investigated. The condition of the cutaneous microvascular bed is also considered to be representative for the condition of that of the entire vascular system, which makes the skin a highly interesting organ for assessment of vascular function (Holowatz, L.A. et al. 2008).

Transdermal iontophoresis of various vasoactive substances in combination with laser Doppler flowmetry techniques (LDF) as well as the newly developed tissue viability imaging system (TiVi) offers a non-invasive, effective, and relatively simple way to investigate microvascular function in the skin. The combination of these techniques is often used in experimental settings but has not yet become common in clinical use. There may be several reasons to this: for example there are presently a vast number of protocols (duration, current strength and drug concentration) in use and also various opinions on how to best analyse the obtained vascular response data. This lack of consensus on what protocol and analysis method to use complicates the interpretation and comparison of the vascular response data obtained from iontophoresis of vasoactive substances.

In vascular studies performed *in vitro*, data are often analysed using a dose-response model called the  $E_{max}$ -model. This model generates variables such as  $EC_{50}$  (half maximal effective concentration), Hill slope, maximum and minimum responses that can be used to compare the effects of various concentrations of drugs or the effect of a drug on different groups. Application of the  $E_{max}$ -model on data from iontophoretic drug delivery has the potential to improve the usefulness of the technique as a clinical tool for investigation of vascular function.

The conventional way of calculating the administered drug dose during iontophoresis *in vivo* is by calculating the electrical charge by multiplying the current strength by the duration of the pulse. It is assumed that the drug molecules make up the current and that the applied drug dose therefore is equal to the electrical charge. The actual drug dose delivered to the tissue during iontophoresis *in vivo* is, however, inherently unknown due to the competition between the drug molecules and other charged particles that are present in the vehicle solution or in the body. The drug dose that reaches the target organ or receptors is further influenced by factors such as local blood flow and diffusion. Therefore, the common way to calculate drug dose during iontophoresis may be inaccurate in *in vivo* settings. Analysis models that take these factors into consideration may therefore help establish iontophoresis as a pharmacological *in vivo* methodology for vascular investigations.

Until recently LDF has been the most common technique to assess vascular responses to iontophoretically administered substances. Due to technical shortcomings of the LDF technique (low sensitivity to low perfusion) assessment of vascular effects by iontophoretically administered vasoconstrictors has been cumbersome. These disadvantages may have contributed to further limit the usability of iontophoresis in a clinical setting. By using TiVi, a technique that utilises polarised spectroscopy and that is sensitive only to the concentration of red blood cells in the tissue, such investigations are facilitated.

## 2. Methods to Investigate Vascular Function

There are several methods for the investigation of vascular function, *in vitro* as well as *in vivo*, besides transdermal iontophoresis, which have contributed substantially to our current knowledge concerning basic vascular pharmacology and physiological processes of the microvasculature. In the following sections, some of the most commonly used and important ones will be outlined. These methods can be generally divided into vascular provocations and techniques to measure the response to these provocations.

### 2.1. Provocations

Studies of unprovoked vessels or cutaneous perfusion in its resting state generally do not contribute with a lot of useful information about physiological or pharmacological mechanisms. Also, such measurements on the cutaneous circulation usually generate results of a wide range as the blood flow in the skin is highly variable both over time and between localisations (Salerud, E.G. et al. 1983). Therefore, microvascular function is often studied in response to a stimuli – most commonly some sort of pharmacological or physiological provocation. Measurements on provoked vessels generate responses of less variation compared to baseline values and the results can be related to specific pharmacological and/or physiological mechanisms.

Two commonly used drugs for studying how vasodilatation is regulated, in *in vitro* vascular assays as well as in *in vivo* models, are acetylcholine (ACh) and sodium nitroprusside (SNP). Acetylcholine is dependent on a fully functional endothelium to induce its vasodilatory effect (Furchgott, R.F. and Zawadzki, J.V. 1980). When ACh binds to the muscarinic receptors on the endothelial cells it triggers the synthesis and release of NO from the endothelium. The NO then diffuses to the surrounding smooth muscle cells causing them to relax.

Whether ACh-mediated vasodilatation is dependent solely on NO or if there are more mechanisms involved is, however, a matter of debate - especially for the ACh-induced cutaneous microvascular response. It has been suggested that prostaglandins may also contribute to the vascular response to ACh (Noon, J.P. et al. 1998), while others report no such measurable involvement (Morris, S.J. and Shore, A.C. 1996; Berghoff, M. et al. 2002). Despite these conflicting opinions concerning the exact vasodilatory mechanisms of ACh there is a general consensus on the usefulness of the drug in investigations of the endothelial function and it is therefore commonly used for this purpose, also in the iontophoretic vascular model.

Sodium nitroprusside, in contrast to ACh, is considered to be independent of the endothelium to exert its vasodilatory effect. When administrated to the tissue SNP dissolves and releases NO that acts directly on the smooth muscle cells, thus by-passing the endothelium. Sodium nitroprusside is therefore commonly used as an endothelium-independent control in vascular assays (Turner, J. et al. 2008).

Atropine (Atr), is a competitive antagonist to ACh (Arunlakshana, O. and Schild, H.O. 1959), and can be used to further investigate the receptor physiology of the iontophoretic model and is also used in *in vitro* vascular assays.

The degree of vasodilatation induced by local warming of the skin has been proposed as a clinical tool for evaluation of vasomotor dysfunction in diabetes and other disease states (Sandeman, D.D. et al. 1991; Carberry, P.A. et al. 1992). Human skin responds to increases in local temperature with a biphasic increase in skin blood flow. The initial response is rapid and transient while the second is slower and more sustained and typically rises above the initial peak (Kellogg, D.L., Jr. et al. 1999; Charkoudian, N. 2003). When a local anesthetic is used to block sensory input, the first phase of the biphasic flow response is abolished, suggesting sensory neural-mediated microvascular dilatation. The second phase is diminished with the addition of NO-synthase (NOS) inhibitors (Kellogg, D.L., Jr. et al. 1999; Charkoudian, N. 2003), suggesting NO-dependent microvascular dilatation.

Constriction of the cutaneous vessels may be induced by either noradrenaline (NA) or phenylephrine (Phe). Noradrenaline acts by activating  $\alpha$ -adrenergic receptors (both  $\alpha_1$  and  $\alpha_2$ ) while Phe is a selective  $\alpha_1$ -receptor agonist.

## **2.2. Measurement of Vascular Responses**

Venous occlusion plethysmography (VOP) is a non-invasive method for measurement of total blood flow to an organ (Yvonne-Tee, G.B. et al. 2006). The most common procedure is to perform the test on the forearms. Although there are variations in the instruments used, the basic principle is similar for all. The venous outflow of blood is interrupted by inflation of a pressure cuff while the arterial inflow is unaltered. Blood can flow into the forearm but cannot flow out which causes the forearm blood volume to rise. The initial linear increase in forearm volume over time is proportional to arterial blood inflow. By provoking the circulation, pharmacologically or mechanically, the flow pattern of healthy subjects and patients can be studied and compared. The method has contributed to the current knowledge of how the autonomic nervous system controls blood flow in human limbs; how blood flow is affected by exercise; the effects of various drugs on the human blood vessels; the mechanisms involved with Raynaud's disease; the effects of cardiovascular risk factors on endothelial function and vascular changes in cardiovascular disease (Joyner, M.J. et al. 2001).

Microdialysis is a minimally invasive method for the *in vivo* measurement of fluid concentrations of tissues and organs of the body (Ungerstedt, U. 1991). The method can be used to test the functionality of the cutaneous microcirculation by the administration of substances that induce specific responses or to extract mediators released by the vessels in response to a provocation. The basic principle of the technique is to mimic the function of a capillary blood vessel.

A microdialysis probe is a small tube made of a semi-permeable membrane at its tip with pores that allow molecules of a certain size to pass through (Vinik, A.I. et al. 2001). Microdialysis in living human skin allows for a variety of applications such as: the measurement of inflammatory mediators in normal and diseased skin; studies of skin metabolism; and absorption of drugs by the skin (Schnetz, E. and Fartasch, M. 2001).

Capillaroscopy is the name of a series of methods used to study the capillary network in real time (Yvonne-Tee, G.B. et al. 2006; Cutolo, M. et al. 2008). In its simplest form this method utilises basic light microscopy, usually in the nail skin folds, by which it is possible to evaluate the morphology and capillary blood flow of a limited part of the skin. By the more sophisticated dynamic capillaroscopy it is also possible to study microvascular dynamics,

flow distribution, and permeability, and if combined with fluorescent dyes, transcapillary diffusion (Cutolo, M. et al. 2008).

The method that may have had the most impact on our current understanding of the basic vascular pharmacology and physiological functions of the microvasculature is perhaps *in vitro* preparations of strips or strings of isolated vessels (Angus, J.A. and Wright, C.E. 2000; Struijker-Boudier, H.A. et al. 2007). The vessels are usually harvested from experimental animals but human isolated vessels are sometimes also used. After removal the vessels are cut into 3-4 mm long segments and mounted on hooks that in turn are directly coupled to a force transducer. Such setups allow for precise studies of the effects of for example, various diseases, age, species, receptors, or structural change on the pharmacology of a vessel without the influence of for example circulating hormones or neural influences. The strength of this methodology in biology is clearly demonstrated by achievements such as the clarification of regulatory functions of the vascular endothelium (Furchgott, R.F. and Zawadzki, J.V. 1980) and the discovery of the pathways of nitric oxide.

An alternative methodology to strain gauge is the pressure-flow system where the vessel segment is connected to a tube at both ends so that fluid can be pumped through. In this assay, the transducers measure pressure and flow rather than force. Changes in lumen diameter, which correlates directly with vasoconstriction or relaxation, can be monitored using a microscope.

The pressure-flow system is about ten times more sensitive than strain gauge and mimics the *in vivo* conditions to a higher extent. However, the pressure-flow system is technically more difficult to perform and therefore limits the number of vessels that can be studied simultaneously (Hillier, C. and Bunton, D. 2007).

Regardless of what vascular assay methodology that is chosen for clinical purposes human vessels are to prefer in these kinds of investigations. However, vessels of human origin are generally hard to come by, often of poor quality and difficult to standardise (Hillier, C. and Bunton, D. 2007). Therefore, many investigators use vessels from animals instead. Unfortunately, animal tissue has often proved to be a poor predictor of human response to drugs (Olson, H. et al. 2000). *In vitro* experiments are further complicated by the fact that they take place outside the body albeit in a controlled but otherwise artificial environment which makes it difficult to apply the results in a straightforward way to clinical situations. Increasing efforts are therefore being directed towards the development of vascular models in which the vessels and vascular effects may be investigated in a tissue environment that incorporates the influences not only of the vessels themselves – as in the case of *in vitro* models – but also of nerve endings and mechanical and humoral factors (Angus, J.A. and Wright, C.E. 2000).



## 3. Transdermal Iontophoresis

### 3.1. Introduction

Compared to other methods (such as needle injections, oral distribution or topical application) used to deliver substances for vascular provocations *in vivo*, transdermal iontophoresis have several advantages. As the technique is non-invasive, there is no contribution of injection trauma to the vascular response. The minute and highly localised dose administered reduces the risk of systemic effects to near null and compared to topically application the delivery rate is usually much higher and easier to control during transdermal iontophoresis. Together these qualities make it an excellent technique for the delivery of various vasoactive substances and highly suitable for pharmacological studies *in vivo*.

The iontophoretic technique is based on the general principle stating that identical charges repel each other. Basically iontophoresis is the application of an electrical potential that maintains a constant electric current across the skin and enhances the delivery of ionized, as well as non-ionised, substances (Wang, Y. et al. 2005). The general procedure (Figure 3.1.1.) is that two electrode chambers of opposite charge are placed on the skin by double adhesive tape. The positive electrode is called anode and the negative electrode is called cathode. The drug that is to be delivered is usually dissolved in a vehicle solution, commonly saline or water, and then placed in the electrode chamber. Positively charged drugs are administered to the skin via the anode and negatively charged drugs via the cathode. When an electrical voltage is applied, the drug is driven into the skin by repulsive electromotive force. The process of the drug ions moving from one area to another is called electromigration.

At a physiological pH (pH~5.5) the skin is negatively charged which favours the transport of positively charged species, such as sodium ( $\text{Na}^+$ ). The “resistance” of the skin to the transfer of positive ions from the anode to the cathode is therefore less than that of negative ions in the opposite direction. For every positive ion (cation) that moves from the positive electrode a negative ion (anion) moves in the opposite direction to maintain electroneutrality (Burnette, R.R. and Ongpipattanakul, B. 1987; Wang, Y. et al. 2005). How much of the current that is carried by the respective type of ions is determined by how easily they move – termed the transport number. A high transport number indicates an easier transport. For saline (NaCl), which is the most commonly used vehicle to dissolve drugs during iontophoresis, Na has a greater transport number compared to Cl. This difference results in a net increase of NaCl at the cathodal side creating an electrochemical gradient. To even out the imbalance water starts to migrate towards the cathode and on its way collides with drug molecules, or any other molecules, dragging or ferrying them towards the cathode. This process, termed electroosmosis therefore influences the transport of drugs into the skin, depending on their polarity.

Different types of electrodes have been used throughout the history of iontophoresis. Today the preferred type is silver/silver chloride electrodes as they do not change the pH of the solution during iontophoresis (Singh, P. and Maibach, H.I. 1994; Wang, Y. et al. 2005).

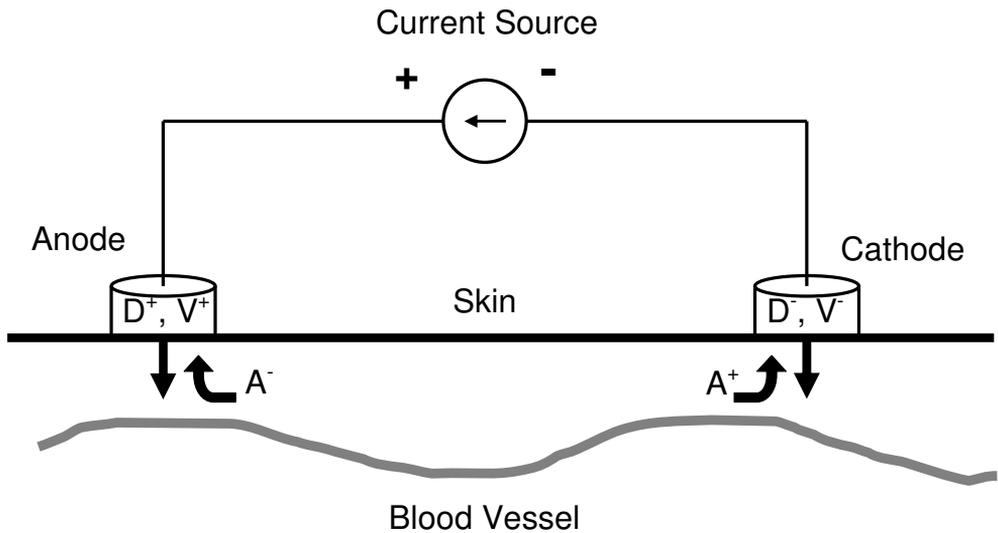


Figure 3.1.1. Schematic drawing of the transdermal iontophoresis technique.  $D^+$  and  $D^-$  represents positively and negatively charged drug ions, respectively.  $V^+$  and  $V^-$  represents positively and negatively charged ions of the drug vehicle solution and  $A^+$  and  $A^-$  represents counter-ions in the skin that travels in the opposite direction of the drug ions to maintain skin electroneutrality.

### 3.2. Applications

The applications of transdermal iontophoresis can be divided into three groups: therapeutic, diagnostic and experimental.

As iontophoresis enables a fast way to deliver a well controlled amount of substance to a localised area of the body with minimal risk of systemic effects, it is a well suited technique for treating conditions such as hyperhidrosis (tap water iontophoresis) (Sloan, J.B. and Soltani, K. 1986), rheumatic disorders (non-steroidal anti-inflammatory drugs) (Garagiola, U. et al. 1988) and cancer pains (opioids) (Mercadante, S. and Fulfarò, F. 1999). Another therapeutic application of transdermal iontophoresis is the administration of lidocaine for dermal anaesthesia (Greenbaum, S.S. 2001).

Transdermal iontophoresis can also be used for diagnostic purposes, for example for diagnosis of cystic fibrosis (Blythe, S.A. and Farrell, P.M. 1984) and to monitor blood glucose levels in diabetic patients (Potts, R.O. et al. 2002).

Iontophoresis has also become a well established method in various experimental settings. Since first used in 1986 by Lindblad and Ekenvall for investigating  $\alpha_1$ -receptor effects in the skin vessels of fingers (Lindblad, L.E. and Ekenvall, L. 1986) the technique has been applied in investigations of various diseases such as diabetes (Morris, S.J. et al. 1995), Raynaud's

phenomenon (Anderson, M.E. et al. 1996), and in cardiovascular disease (Turner, J. et al. 2008).

An experimental application of iontophoresis that is increasingly being adopted for clinical use is the delivery of vasoactive drugs to the cutaneous microcirculation for assessment of endothelial function (Turner, J. et al. 2008). As mentioned in the introduction, the endothelium plays a pivotal role in the regulation of vessel tone and diameter by the release of various vasoactive substances. A change in this function of the endothelium is often seen in the early development of most cardiovascular diseases (Joannides, R. et al. 2006). Iontophoresis of vasoactive drugs has in this setting been used to demonstrate that an impaired endothelial function of the skin resistance vessels correlate to an increased risk of coronary heart disease (RG, I.J. et al. 2003). Further, the method has been used to demonstrate the reduced endothelium-dependent vasodilatation of skin resistance arteries seen in hypertension (Farkas, K. et al. 2004) and to investigate the effects of aging on the vessels (Tao, J. et al. 2004).

### **3.3. Methodological Issues**

#### **3.3.1. Iontophoresis Protocols**

There are almost as many protocols for iontophoresis as there are research groups working with the method and this diversity makes it difficult to compare results between studies. Despite this variation, all protocols can be divided into two main categories: multiple pulse and single pulse protocols. In multiple pulse protocols each iontophoretic charge (current strength x duration) is separated by a “resting” period to allow for the maximum response to develop. From that perspective these protocols therefore resemble the *in vitro* vascular assay methodology of cumulative doses. However, it may be questioned how valid this comparison is as in the iontophoretic model clearance of drug out of the skin by for example tissue blood flow most likely will affect the dose as well, something that is not relevant in an *in vitro* model. Another drawback with pulsed protocols is that they are time consuming as time is lost after each pulse for the maximum response to develop.

In single pulse protocols the iontophoretic charge is given continuously during one period without a break. These protocols tend to be shorter and measurements of the blood flow can be done either at the end of the pulse or continuously during the administration of the drug. A further advantage compared to pulsed protocols is that the blood flow response at a certain point in time is related to the iontophoretic charge at the very same instant. However, because the iontophoretic charge is given as one long pulse without a time delay to allow for a vascular response, the response seen at the end of the pulse may not necessarily represent the maximum response to that dose. Dose-response curves from single pulse protocols can therefore, at least in theory, be expected to be right-shifted compared to dose-response curves from pulsed protocols.

The protocols used in the investigations (study I to IV) included in this thesis were single pulse protocols optimised for maximum drug responses and at the same time eliciting minimal non-specific responses (Droog, E.J. et al. 2004).

### 3.3.2. Non-specific Vasodilatation

A well known and complicating phenomena observed during iontophoresis is the non-specific dilatation of vessels in response to the method itself. Substances without known vasoactive effects in man such as tap water, deionised water, and sodium chloride have been demonstrated to increase local blood perfusion (Thysman, S. et al. 1995; Berliner, M.N. 1997). The mechanisms behind this reaction are yet not fully known but it has been suggested that it is caused by the current directly (Grossmann, M. et al. 1995).

The first attempt to deal with the effect of non-specific responses was to subtract the effect induced by the vehicle solution to the effect caused by the drug (Morris, S.J. et al. 1995; Morris, S.J. and Shore, A.C. 1996). Another strategy has been to add high concentrations of sodium chloride to the drug solution as it was noted that it resulted in a lower voltage over the skin and reduced the effects (Asberg, A. et al. 1999). Application of topical anaesthetics (EMLA) have also been found to attenuate the non-specific response and has been used rather frequently during iontophoretic investigations (Kubli, S. et al. 2000; Hannemann, M.M. et al. 2002; Pellaton, C. et al. 2002; Christen, S. et al. 2004).

The perhaps least complicated method for dealing with this problem is to reduce the current density and total iontophoretic charge. This approach has been successfully used by a number of investigators (Hamdy, O. et al. 2001; Newton, D.J. et al. 2001; Khan, F. et al. 2004) and seems to have fewer disadvantages compared to using topical anaesthetics or subtraction of non-specific response from the total response. However, by lowering the current density and the iontophoretic charge there is a risk that the vascular response may not be maximised as less drug will be delivered. Recent studies, however, have shown that by using drug solutions of physiological ionic strength vascular response plateaus can be reached using ACh and SNP even if the current density and total electric charge is lowered (Droog, E.J. et al. 2004).

### 3.3.3. Analysis of Data

Just as for the protocols there is no general consensus about how the blood flow responses should be examined and analysed. Some researchers have used the absolute change in perfusion after one or multiple iontophoretic pulses (with or without subtraction of baseline perfusion values) as a measure of microvascular responsiveness. Others have expressed the responses in terms of relative change with respect to baseline. Yet another method used is to calculate the area under the blood flow response curve (AUC) (Asberg, A. et al. 1999; Anderson, M.E. et al. 2004). The problem of analysing blood flow responses by these methods is that they suffer from two major limitations. Firstly, the measured maximum response may not necessarily reflect the physiological maximum response. Secondly, just looking at the maximum response may lead to that important physiological characteristics are overlooked. Many researchers therefore use a third alternative by looking at the responses to increasing doses or iontophoretic charges (Morris, S.J. et al. 1995; Christen, S. et al. 2004; Khan, F. et al. 2004). Results from such investigations contain more information about the vascular physiology than does analysis of the response to a given dose or a maximum response alone. The obtained data are then often compared using multiple-way analysis of variance (ANOVA).

### 3.3.4. Dose-response Analysis

Vascular response data from *in vitro* studies using vascular assays are generally analysed using a pharmacodynamic dose-response model called the  $E_{\max}$ -model. The model can be described by the following equation:

$$E(C) = E_{\min} + \frac{(E_{\max} - E_{\min})C^n}{C^n + EC_{50}^n}$$

where E is the response elicited by the drug,  $E_{\min}$  is the minimum response,  $E_{\max}$  is the maximum response,  $EC_{50}$  is the concentration that is needed to elicit half of the maximum response and n is the Hill slope, which defines the steepness of the response. C is the concentration of the drug.

When adapted to iontophoretic dose-response analysis, the equation looks like:

$$E(t) = E_{\min} + \frac{(E_{\max} - E_{\min})D(t)^n}{D(t)^n + ED_{50}^n}$$

where D is the electrical (or iontophoretic) charge applied to the skin and  $ED_{50}$  is the electrical charge needed to elicit half the maximum response. In the case of iontophoresis, D depends on the current strength and the time during which the current is applied:  $D(t) = I \times t$ .

The  $ED_{50}$ , Hill slope and maximum/minimum response can be used for comparison between drug effects or vascular responses between patients groups in a quantitative way.

The shape of the curves formed from raw vascular response data obtained during iontophoresis in the experiments made prior this thesis were found to resemble response data curves from *in vitro* studies. Thus it was speculated that the  $E_{\max}$ -model could be applicable also to the iontophoretic data. As mentioned earlier a major concern with the iontophoretic model, however, is that the administrated absolute drug dose is unknown. Several ways to determine the dose have been proposed, but the most commonly accepted model, validated by *in vitro* experiments by Phipps and colleagues (Phipps, J.B. et al. 1989), is that the administrated drug dose during iontophoresis correlates linearly to the iontophoretic charge. This assumption is based on Coulomb's law that states that an electrical charge is equal to the strength of the applied electrical current, in ions per second, multiplied by the time the ions are transported. Results from a recent investigation by Tesselaar and co-workers, however, question this assumption and present evidence for a far more complicated reality that include competition between drug ions and ambient ions as well as significant clearance of drug from the local area of the iontophoresis by both active and passive transport routes involving the blood flow of the microvascular bed (Tesselaar, E. et al. 2008).



## 4. Laser Doppler Flowmetry

### 4.1. Introduction

The most common method for measurements of vascular response to iontophoretically administered vasoactive substances has been laser Doppler flowmetry (LDF). This technique offers a non-invasive way to monitor the microcirculation in various tissues and organs continuously and in real time. There are several detailed reviews written on the theoretical background of LDF (Holloway, G.A., Jr. and Watkins, D.W. 1977; Nilsson, G.E. et al. 1980; Bonner, R., Nossal, R. 1981; Gush, R.J. et al. 1984; Nilsson, G.E. 1984; Duteil, L. et al. 1985; Johansson, K. et al. 1991) and it is not within the scope of this thesis to further discuss the details of LDF. A brief introduction to the technique will, however, follow below.

In LDF a low power laser beam is directed to the surface of the tissue that is under investigation. As the beam hits the surface a fraction of the light will penetrate into the tissue and interact with both static and moving cells (red blood cells, RBCs). When light that has been scattered by static objects is directed onto a remote surface a static speckle pattern (black and bright spots) can be distinguished. If the reflecting object includes appropriately sized particles in motion, such as RBCs, this speckle pattern fluctuates at a rate dependent on the speed of the RBCs due to the Doppler effect. If the light is lead onto a photo detector instead, the speckle pattern can be transformed into a photocurrent signal that is related to the RBC average speed and number of moving RBCs. By using a dedicated algorithm (Bonner, R., Nossal, R. 1981), common in most modern LDF equipment, the output signal delivered scales linearly with tissue blood perfusion, which in turn is defined as the product of the mean velocity and concentration of the moving RBCs within the sampling volume (Leahy, M.J. et al. 1999).

Laser Doppler systems can be separated into two categories, namely the laser Doppler perfusion monitors (LDPM) and the laser Doppler perfusion imagers (LDPI).

Laser Doppler perfusion monitors usually consists of a probe containing an illuminating fibre and one or several detecting fibres (Yvonne-Tee, G.B. et al. 2006). The probe is attached directly to the skin by double adhesive tape and continuously collects perfusion data from a depth of about 0.5-1 mm. As a result of this the LDPMs have a high temporal resolution but measurements can only be made at a single point on a restricted area of the skin. As the skin microcirculation is highly heterogeneously distributed this could be a problem as different perfusion values could be generated from two adjacent areas of the skin (Tenland, T. et al. 1983; Fullerton, A. et al. 2002).

Laser Doppler perfusion imagers on the other hand are non-contact devices that can assess perfusion across a region rather than at a single point by scanning the surface of the skin with the laser beam. As this scanning procedure takes some time, dependent on the area scanned, LDPIs have lower temporal resolution. Whether this poses a problem or not depends on the blood flow dynamics during the measurement and the focus of interest. The spatial resolution of the LDPI depends on the number of measurement points, the surface area that is being scanned and the diameter of the laser beam.

Laser Doppler flowmetry has been used in several clinical applications and has proven to be a powerful tool in combination with iontophoresis for assessment of cutaneous vascular regulatory function. However, the technique suffers from some limitations such as susceptibility to movement artefacts and low resolution in the perfusion range of, for example, normal skin during decreased perfusion such as during vasoconstriction.

## **4.2. Vasoconstriction**

A majority of the investigations made using iontophoresis and laser Doppler flowmetry have been performed with vasodilators, particularly ACh and SNP, and only a few include the effects of vasoconstrictors. This over-representation is most likely due to the low resolution of the laser Doppler technique to detect low or decreased perfusion values (Lipnicki, D.M. and Drummond, P.D. 2001). In its resting state the cutaneous microcirculation has low relative perfusion which poses a challenge to the laser Doppler techniques as the perfusion values registered are typically close to the biological zero (the contribution of the flow signal caused by the natural movement of molecules in the tissue). Assessment of changes in the circulation caused by vasoconstrictors without first enhancing the contrast is therefore difficult to perform using laser Doppler based perfusion detectors (Brown, H. et al. 2003).

## 5. Tissue Viability Imaging

In response to the need of a method that can measure responses to vasoconstrictors (as well as vasodilators) in a simple way, a new measurement technology called Tissue Viability Imaging (TiVi) has emerged (Figure 5.1.1.). The system provides information about back-scattered light from the tissue and its microcirculation by using subsurface polarised light spectroscopy. The TiVi system consists of a standard off-the-shelf digital camera equipped with polarisation filters that are perpendicularly placed in front of the flash and detector respectively. When the flash fires it emits a broad spectrum of white light and as this light passes the filter in front of the flash it becomes linearly polarised. When the light reaches the surface of the skin a portion of it is directly reflected while the rest enters the tissue. The directly reflected light retains its original polarisation state and can because of that not pass the second filter which is fitted in front of the detector. A greater part of the light, however, continues into the tissue and this “sub-surface” light then successively becomes randomly scattered. When re-emitted to the surface it has become depolarised and part of it can pass the polarisation filter that is located in front of the lens.

The RBCs present in the microcirculation are prone to absorb light in the green wave-length region (about 500-600 nm) to a much higher extent than light in the red wave-length region (about 600-700 nm). The surrounding tissue components of the dermis, in comparison, absorb green and red light to approximately the same amount. The TiVi-technology takes advantage of this difference in absorption by separating the images into their three different colour planes of red, green, and blue. Each photograph is then subjected to a dedicated algorithm that subtracts the value of each picture element in the green colour matrix from the corresponding value in the red colour matrix. The result is divided by a signal proportional to the total light intensity within the actual wave-length region. The obtained values for the resulting matrix are referred to as TiVi values. The TiVi value scales linearly with the momentary concentration of red blood cells ( $RBC_{conc}$ ) in the actual tissue volume (O'Doherty, J. et al. 2007).

Investigation of vascular function is typically performed as the assessment of the development of a response over time to a certain provocation. To gain reliable information from such investigations it is crucial that the chosen measurement technique performs as accurately as possible. Recently, a series of performance tests have been undertaken in order to evaluate the qualifications of the TiVi system and technology. It is not within the scope of this thesis to go into detail of those investigations but a summary of the different findings is however of relevance to this thesis and will follow below.

Nilsson and colleagues (Nilsson, G.E. et al. 2009) have tested the TiVi system regarding its performance of critical instrumental parameters including short- and long-term drift, variation in sensitivity between instruments, dependence of the distance between camera and object on sensitivity, influence of ambient light on image quality, and image distortion caused by the curvature of the object.

The instability of a single TiVi unit during short- (20 min) and long-term (2 months) use were found to be limited to about 1%. Evaluation of the drift of critical parameters over longer periods of time has not been performed yet and therefore a possible effect on the stability due to degradation of the polarisation filters and the spectral signature of the flash cannot be

excluded. Cameras from different batches were also tested and a variation in sensitivity of about 4% was found.

The distance between the object and the camera (within the range of 13-28 cm) was reported to have no effect on the sensitivity of the technique. However, a difference in sensitivity was seen within a single picture with the largest variation between the centre and the boundaries of the picture. This disadvantage can be compensated for by choosing a ROI (region of interest) in the centre of the image thus cutting off the border areas. As the distance between the camera and the object was found to have no effect on the sensitivity a sufficient field of view can still be achieved by moving the camera further away from the object.

The effect of ambient light on the results was tested by positioning a 40 W light bulb at a distance of 50 cm above the object. This reduced the sensitivity to 10% which means that the intensity of the flash is high enough to ensure use of the device under ordinary daylight conditions without distortion of image quality.

A minor distortion of the TiVi value can be seen during measurements of curved objects, such as the human forearm. This distortion is more noticeable at the boundaries of the image and this limitation has to be considered when photographs are captured from a curved object.

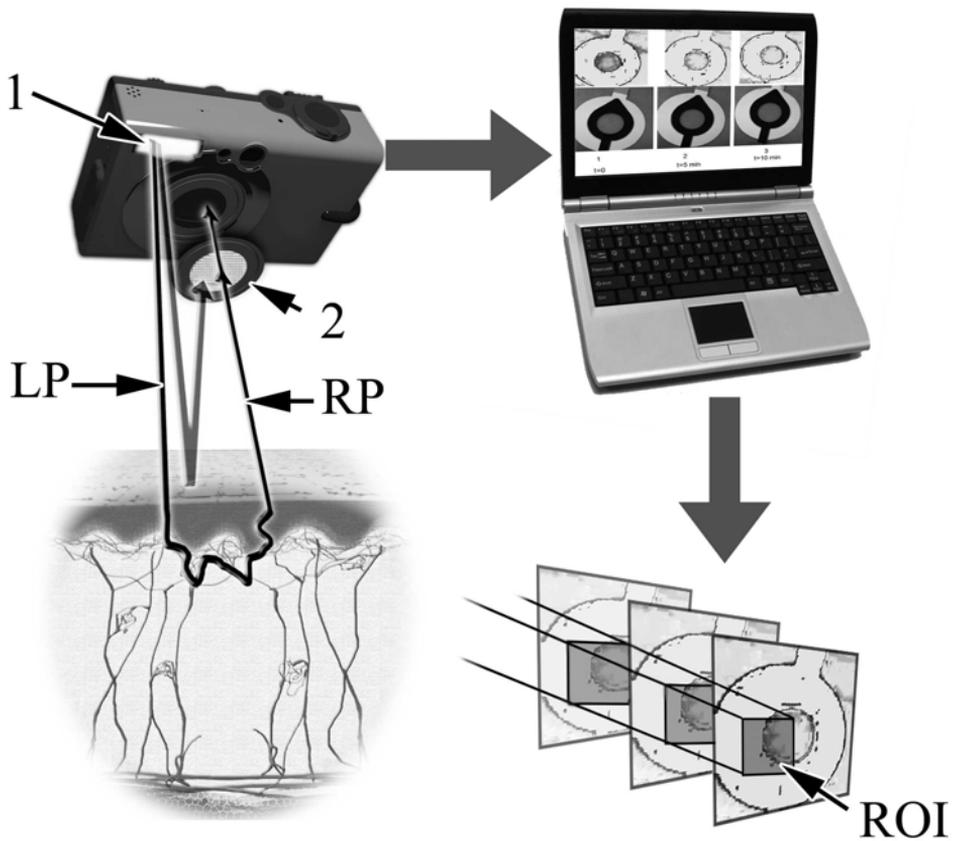
Veins and melanin-rich rich brown spots in the in the skin also have an influence on the resulting image. Veins appear as blue objects in the image and the brown dots as areas of high  $RBC_{conc}$ . Such effects that are not related to the  $RBC_{conc}$  in the tissue could lead to misinterpretation of data but are easily avoided by routinely displaying a photograph of the object next to the TiVi image in the user interface.

Based on these findings it was therefore concluded by Nilsson and colleagues that the TiVi system is robust and reliable and that there are only minor effects on the critical performance parameters in the present of ambient noise sources. The differences in response between areas and/or changes over time within a specific region measured by the TiVi in the cutaneous bed can therefore be attributed to physiological variability in  $RBC_{conc}$  and not to instability of the technique itself.

Results from tests performed on healthy test subjects can be useful as reference values for comparison and evaluation of stimuli-response tests on patients with cardiovascular disease. It is therefore important to know how much the  $RBC_{conc}$  varies in healthy unprovoked skin and how well the TiVi technique is able to quantify these changes. This variation has been investigated by Zhai and colleagues (Zhai, H. et al. 2009) and the day-to-day variation in skin erythema was found to be of the order of about 10%. As the day-to-day instability of a TiVi unit is limited to 0.27% on average (Nilsson, G.E. et al. 2009) Zhai and colleagues concluded that the variations seen in fact were the result of alterations in human skin  $RBC_{conc}$  caused by capillary pulse, respiration and occasional vasomotion and not by variations in the TiVi technique.

The ability of the TiVi system compared to colorimetry to assess skin blanching has also been investigated by Zhai and colleagues (Zhai, H. et al. 2009). The results suggest that the TiVi is suitable for operator-independent and remote mapping of human skin blanching and that the relative uncertainty in the blanching estimate produced by the TiVi was about 5%. This uncertainty was in the same order as for a chromameter operated by a single user and the TiVi may therefore offer new opportunities in quantification of skin blanching as it has fewer disadvantages compared to methods based on hand-held probes.

In contrast to imaging techniques involving laser Doppler, where movement generates artefacts on the perfusion value, the TiVi-index is insensitive to movement of the tissue. The technique is also as easy to handle as a standard camera and requires no special training. Furthermore, as an image of the chosen test area is captured instantaneously and not over a period of time, as in LDPI, there is no risk of misinterpretation of temporal variability as spatial heterogeneity in the tissue  $RBC_{conc}$ . The upper limit of measurement points for a TiVi unit is determined only by the number of pixels recordable by the camera.



*Figure 5.1.1. Quantification of red blood cell concentration in the cutaneous microcirculation by use of a TiVi system. Light from the flash is linearly polarised by the first filter (1). A part of the light is directly reflected from the surface of the skin and retains its polarisation. The unpolarised light is stopped by the second filter in front of the lens (2). A part of the light penetrates into the tissue and is randomly scattered, when re-emitted to the surface it has become randomly polarised and some of it can pass through the second filter. The images created by this light is analysed using a specially dedicated algorithm and colour coded maps are generated. (LP = linearly polarised, RP = randomly polarised, ROI = region of interest.)*

## 6. Aims

The aim of this thesis is to improve the applicability and to facilitate the use of iontophoresis in investigations of cutaneous microvascular function. Focus lies on vascular response data analysis methods, the introduction of a new measurement technique and the development of new protocols for administration of vasoconstrictors. The specific aims of each study are:

1. To apply the  $E_{\max}$ -model to the blood flow responses obtained by iontophoretic application of acetylcholine and sodium nitroprusside measured with laser Doppler perfusion imaging and to compare the results to the corresponding dose–response curves obtained by using the same drugs in *in vitro* vascular assay experiments. To examine the applicability of this method and to assess within and between the experiment variability. To develop a protocol for iontophoresis of an antagonist (atropine) and to investigate the receptor physiology of the iontophoretic model. To investigate the possible involvement of prostaglandins in the vascular response induced by iontophoreted acetylcholine. (study I).
2. To describe and evaluate the ability of the tissue viability imaging system to non-invasively quantify changes in the amount of red blood cell concentration both in an *in vitro* model and *in vivo* in the papillary dermis, as a response to a direct stimulus or physiological reaction (study II).
3. To develop a protocol for iontophoretic administration of noradrenaline and phenylephrine using local preheating in combination with laser Doppler flowmetry and to elucidate the effect of duration and current strength of the iontophoretic pulse on drug response (study III).
4. To investigate the ability of the tissue viability imaging system to detect and quantify changes in red blood cell concentration within the cutaneous microcirculatory network during iontophoresis of both vasoconstricting and vasodilating substances (study IV).

## 7. Test Subjects and Technology

### 7.1. Healthy Volunteers

Healthy volunteers (for demographic data see Table 7.1.1.) free from any medication (with the exception of oral contraceptives) and without ongoing or previous history of vascular or skin disease were recruited to participate in the investigations after having given informed consent. All volunteers refrained from drinking coffee or tea, use of tobacco or exercise for at least 2 hours prior the experiments. All studies were approved by the regional ethics committee at the Faculty of Health Sciences, Linköping University, Sweden, and procedures followed were in accordance with institutional guidelines.

*Table 7.1.1. Demographic data for the subjects that participated in the studies and the pharmacological provocations used.*

<b>Study</b>	<b>Drug</b>	<b>N</b>	<b>Mean age</b>	<b>Men</b>	<b>Women</b>
<b>I</b>	ACh	10	24	3	7
	SNP	10	26	7	3
	ACh/Atr	5	26	4	1
	SNP/Atr	2	27	1	1
	ACh/ASA	10	28	6	4
<b>II</b>	ACh	8	23	4	4
	Clobetasol 10µl	4	31	4	-
	Clobetasol 20µl	4	31	4	-
	Methylnicotinate 20µl	4	31	4	-
	Methylnicotinate 20µl	4	31	4	-
<b>III</b>	NA	8	26	4	4
	Phe	8	26	4	4
<b>IV</b>	NA	14	33	8	6
	Phe	14	33	8	6
	ACh	8	28	8	-

## 7.2. Experimental Settings

Room temperature was controlled during all experiments. Ambient light was dimmed during investigations involving laser Doppler measurements to avoid interference with the laser light. All investigations were performed on the volar side of the test subject's forearms with the arms at, or slightly below, heart level. Test sites were gently wiped with 70% ethanol before each experiment involving iontophoresis to improve conductivity. Test subjects rested for at least 10 minutes before the start of an experiment and all tests were performed while test subjects rested comfortably in a half-upright position.

## 7.3. Iontophoresis

Two different types of circular silver-silver chloride electrode chambers, LI 611 and PF 383, (Perimed AB, Järfälla, Sweden) were used to deliver the vasoactive substances. The first type (LI 611, Perimed AB, Järfälla, Sweden) was used together with LDPI and TiVi (study I, II and IV). The LI 611 electrode has an area of 1.8 cm<sup>2</sup> and is attached to the skin by double adhesive tape. To prevent leakage and evaporation, the chambers have a transparent plastic lid. The second type of drug delivery electrode (PF 383, Perimed AB, Järfälla, Sweden) was used with the LDPM (study III) and has an area of 1.1 cm<sup>2</sup>. This type of electrode is also attached to the skin by double adhesive tape.

A battery-powered iontophoresis device (PeriIont, Perimed AB, Järfälla, Sweden) was used to deliver a constant direct current to the skin. Depending on the polarity of the drug used the positive or negative lead was connected to the drug electrode. With the PeriIont device current strength can be set between 0.02 mA and 1 mA in steps of 0.02 mA. Iontophoresis protocols and drugs are summarised in Table 7.3.1.

Table 7.3.1. The various drugs, durations and current strengths used in the studies.

Iontophoresis protocols and drugs					
Study	ACh	SNP	NA	Phe	Atr
I	0.02 mA x 600 s	0.02 mA x 600 s	-	-	0.6 mA x 20 s
II	0.02 mA x 600 s	-	-	-	-
III	-	-	0.02 mA x 600 s	0.02 mA x 600 s	-
IV	0.02 mA x 600 s	-	0.02 mA x 600 s	0.02 mA x 600 s	-

## 7.4. Laser Doppler Flowmetry

Two different types of laser Doppler devices were used in these studies. In study I and IV a laser Doppler perfusion imager (PIM 2.0, Lisca Development AB, Linköping, Sweden) was used to measure skin perfusion during iontophoresis. In study I the imager was set to duplex scanning mode giving a continuous scan of 4 x 4 adjacent points at a scan rate of about 1 scan/s.

In study IV it was set to imaging mode and generated images of the size 10 x 10 measurement points at a scan rate of one scan every 7<sup>th</sup> s.

In study III a single point LDPM was used (Periflux 5010, Perimed AB, Järfälla, Sweden) with a sampling rate of 33 samplings/s.

For further information about the various laser Doppler flowmetry settings used see Table 7.4.1.

Table 7.4.1. Laser Doppler settings used (LDPM = laser Doppler perfusion monitor, LDPI = laser Doppler perfusion imager).

Study	Mode	Image size (No. data points)	Scan time (s)	Scan area (cm <sup>2</sup> )	Distance (cm)
I	LDPI (duplex)	4 x 4	1	1.5	15
II	-	-	-	-	-
III	LDPM	1	0.03	-	-
IV	LDPI (imaging)	10 x 10	7	1.5	15

## 7.5. Tissue Viability Imaging

Tissue RBC<sub>conc</sub> was quantified using a commercial TiVi system (TiVi600, WheelsBridge AB, Linköping, Sweden). The camera was placed perpendicular to the measurement areas at a distance of 7-10 cm. Image size was set to 640 x 480 pixels (setting: "small fine") and the flash and macro setting were used in all tests. The camera was controlled remotely from a laptop and photographs were continuously stored on the computer's hard drive. The specific settings used in study II and IV are listed in Table 7.5.1.

Table 7.5.1. TiVi settings and drugs used in studies II and IV.

Study	Drug (iontophoresis)	Size (pixels)	Macro	Flash	Interval
II	ACh	640 x 480	On	On	1 image / 10 s
IV	ACh, SNP, NA, Phe	640 x 480	On	On	1 image / 6 s
IV	ACh	640 x 480	On	On	1 image / 10 s

## 8. Review of Articles

### 8.1. Study I

The main aim of study I was to investigate the possibilities of using a sigmoidal  $E_{\max}$ -model to construct dose-response curves from blood perfusion data induced by iontophoresis of ACh and SNP and measured by LDPI, as the  $E_{\max}$ -model is commonly used in pharmacodynamic *in vitro* studies of vessels. A further aim was to compare the obtained results to responses from isolated bovine mesenteric arteries in organ baths. A protocol for iontophoresis of atropine (Atr) was also developed to further investigate the receptor physiology of the iontophoretic model. Also, the effect of acetylsalicylic acid (ASA) as a prostaglandin antagonist on the dose-responses was investigated.

The vasodilating drugs (ACh and SNP) were both administered using a single 10 minute current pulse (0.02 mA). Acetylcholine was given by anodal iontophoresis and sodium nitroprusside by cathodal iontophoresis. Atropine of increasing concentrations was given by anodal iontophoresis using a single 20 second current pulse (0.06 mA). Acetylsalicylic acid (ASA) was given orally as tablets (500 mg), one 24 h and two more 2 h before the start of the tests.

The results showed that the suggested  $E_{\max}$ -model could be accurately fitted to the perfusion data from the iontophoresis tests as well as to the response data of the bovine arteries (Figure 8.1.1.). Coefficients of determination,  $r^2$ , were generally high, typically  $>0.9$  indicating a high goodness of fit. Dose-response parameters generated by the model showed that the  $ED_{50}$  values (the effective dose of drug that elicit a 50% response of maximum) for SNP was significantly larger than for ACh. This difference was found both in the *in vivo* and *in vitro* experiments. Hill slopes were different for ACh and SNP in the iontophoretic experiments but not for the results from the organ baths.

Administration of atropine prior to iontophoresis of ACh attenuated blood flow responses in a dose-response fashion. Blood flow responses to SNP in sites pre-treated with Atr were unaffected.

The intake of ASA before iontophoresis of ACh and SNP had no measurable effects on the vascular responses.

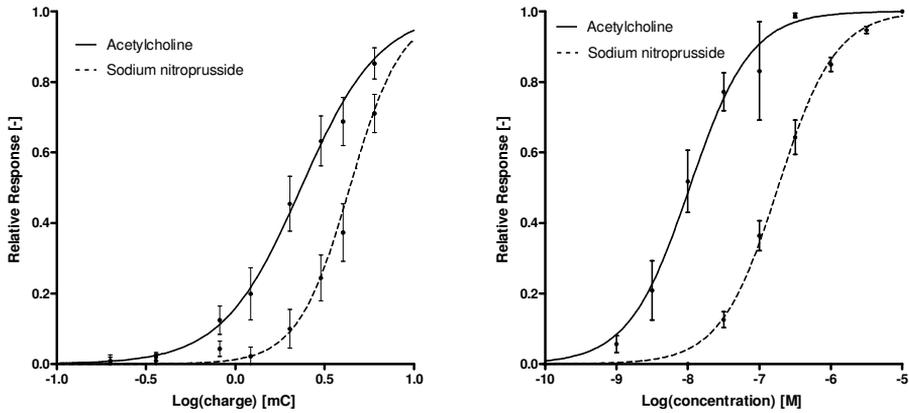


Figure 8.1.1 Pooled average cutaneous microvascular responses to iontophoresis of ACh and SNP (left,  $n=10$ ). Responses of isolated bovine mesenteric arteries in organ baths to increasing doses of ACh and SNP (right,  $n=7$ ).

## 8.2. Study II

Study II aimed to describe and evaluate the ability of the TiVi system to quantify changes in the amount of RBCs in the superficial dermal microcirculation. For this purpose the performance of the TiVi was evaluated in three separate experimental scenarios.

The first scenario aimed to investigate the response to a known increase in  $RBC_{conc}$  *in vitro*. For this a fluid model consisting of tightly wound latex tubing was developed (Figure 8.2.1). The tubing, simulating blood vessels was put on top of a background of skin-like bandage material painted with “bloodless skin” colour. Ten millilitre of fresh human blood mixed with saline was infused through the tubing by a syringe at blood concentrations ranging from 0% to 4% in steps of 0.2%. The results showed a high concentration-dependent sensitivity of the TiVi to these changes in  $RBC_{conc}$  (Figure 8.2.2.).

The effects of oxygenation on the TiVi output were tested by using fully oxygenated blood and blood bubbled with nitrogen gas to achieve minimal oxygen saturation. A sequence of five photographs was taken for each blood concentration.

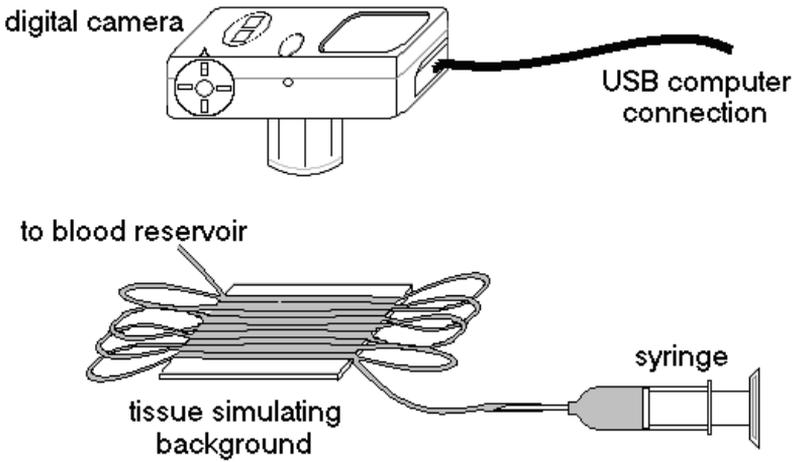


Figure 8.2.1. Setup of the fluid model used to investigate the TiVi-output to known changes in RBC concentration.

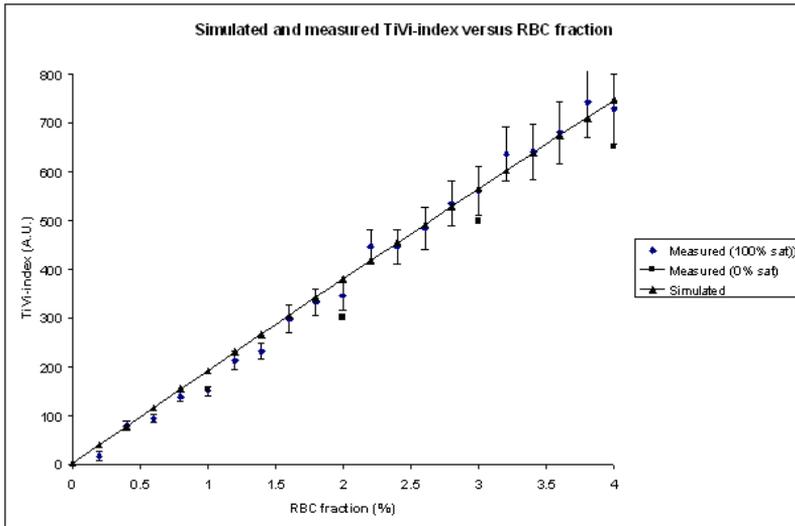


Figure 8.2.2. Simulated and measured linearised TiVi-index for RBC concentrations ranging from 0% to 4%. The correlation coefficient (measured TiVi-index vs.  $RBC_{conc}$ ,  $n=20$ ) was calculated to 0.997 for oxygen saturation = 100% and 0.998 for oxygen saturation = 0%, respectively.

A ROI was selected for each sequence of photographs and the average TiVi-index was calculated. The TiVi-index calculated for the minimal oxygen saturation was on the average 91.5% of that calculated for the maximal saturation. This corresponds to a deviation of less than -3.9% within the physiological range of oxygen saturation (100-70%)

The second scenario aimed to investigate the ability of the TiVi to quantify  $RBC_{conc}$  in living tissue by three separate experiments. In the first experimental situation the effects of iontophoresed acetylcholine on forearm microcirculation in eight healthy volunteers were investigated. The drug was delivered by a single pulse of 0.02 mA with a duration of 10 min (giving a total electrical charge of 12 mC). Photographs of the test area were taken with 5 second intervals. A ROI corresponding to the area of the electrode was marked in every sequence of photographs. The average relative increase in  $RBC_{conc}$  was calculated to 66% but in some spots the increase was up to 117% and in others virtually unaltered, thus demonstrating the spatial heterogeneity of the cutaneous microcirculation.

The second experimental situation was designed to investigate the ability of the TiVi to quantify a decrease in  $RBC_{conc}$  by topical application of clobetasol propionate. Clobetasol was applied to the forearm skin of healthy volunteers in two quantities – 10 and 20  $\mu$ l – at adjacent, round (20 mm diameter) sites under an occlusive cover. The cover was removed after 12 h and 20  $\mu$ l of methyl nicotinate (vasodilating) was then applied topically to a third adjacent skin site for comparison. A series of photographs was recorded for 10 minutes following the removal of the cover and the application of the methyl nicotinate. The average decrease of  $RBC_{conc}$  within the test sites treated with 20  $\mu$ l and 10  $\mu$ l clobetasol was calculated to 31% and 19% below normal skin control levels respectively. In the areas treated with methyl nicotinate the average increase of  $RBC_{conc}$  was calculated to 30% above normal skin control levels.

In the third experimental situation the ability of the TiVi system to quantify changes in  $RBC_{conc}$  after application of methyl nicotinate in areas with complicating factors such as nevi was investigated. The results showed that the effects of local static pigmentation (nevi) can be suppressed by subtracting the first image in a sequence from the following.

### **8.3. Study III**

In study III the primary aim was to develop a protocol for giving Na and Phe iontophoretically, and to verify that vasoconstriction induced by iontophoresis can be measured using LDF. The secondary aim was to examine the effects of duration and current strength of the iontophoretic pulse on the response to the drug.

The hypothesis was that LDF could be used to measure dose-dependent vasoconstriction caused by iontophoresed NA and Phe if the vessel bed is pre-dilatated. As a result of drug kinetics in the skin, the responses may vary for equal doses, depending on the protocol used.

The study was performed on the forearm skin of eight healthy volunteers. Local warming, iontophoresis of vasoconstrictors and measurement of the blood flow was accomplished by using a probe designed for this purpose. Stable background flow was reached after 20 min of pre-heating the test sites to 44°C. Once perfusion levels were stabilised NA and Phe were given using a single anodal iontophoretic pulse. Three different protocols, with the same total electrical charge (12 mC), were used; 0.04 mA x 300 s, 0.06 mA x 200 s, and 0.12 mA x 100 s.

All test subjects responded to the local warming with a significant increase in blood flow ( $p < 0.001$ ). The average increase after 20 minutes of warming was 40 perfusion units (1500% increase above baseline). The pooled responses are shown in Figure 8.3.1.

A total of 12 tests (out of 72) spread across 7 subjects were excluded because of lack of significant decrease in blood perfusion in response to the vasoconstrictors.

A significant effect of the protocol on the final perfusion response was found for both NA ( $p = 0.0032$ ) and Phe ( $p < 0.001$ , one-way ANOVA). The pooled perfusion response data for each protocol are shown in Figure 8.3.2. for both NA (A) and Phe (B).

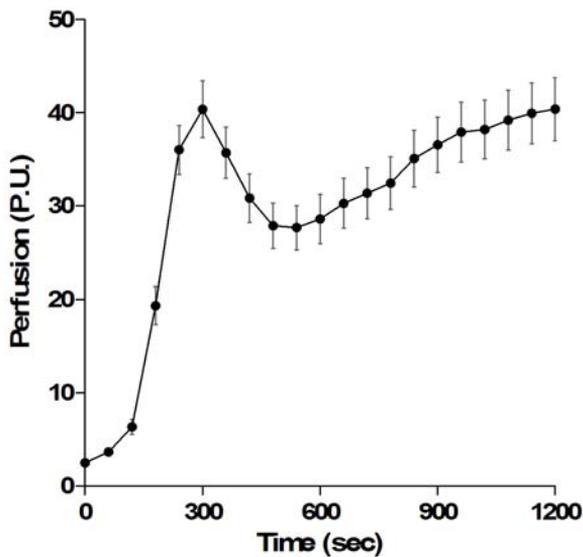


Figure 8.3.1. Skin perfusion response for all subjects (mean  $\pm$  SEM,  $N = 72$ ) to 20 minutes of local heating at  $44^{\circ}\text{C}$ . The typical biphasic blood flow response, with a transient sensorineural mediated peak and second NO-mediated response, is clearly depicted. The perfusion was measured by laser Doppler flowmetry.

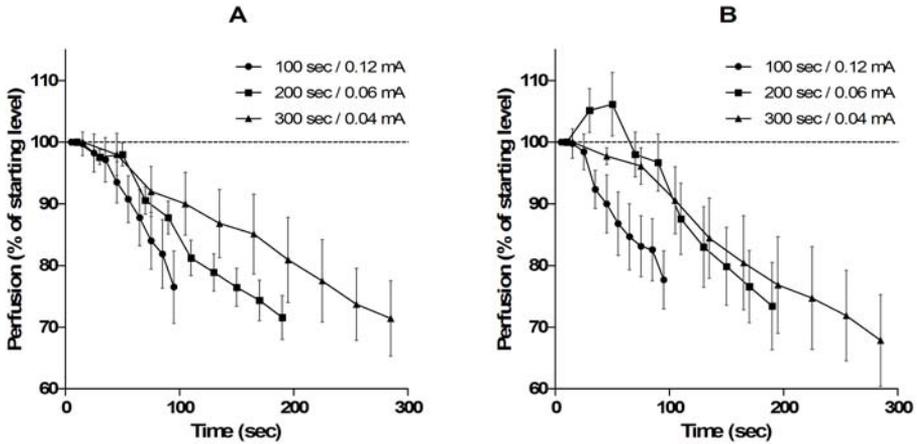


Figure 8.3.2. Skin perfusion responses to iontophoresis of noradrenaline (A) and phenylephrine (B) using three different protocols with the same total electrical charge, but with different pulse durations and current strengths (mean  $\pm$  SEM). A significant effect of the protocol on the final perfusion values was found for both noradrenaline ( $p = 0.0032$ ) and phenylephrine ( $p < 0.001$ ).

#### 8.4. Study IV

The primary aim of study IV was to use the TiVi for investigation of vascular responses to iontophored vasoconstrictors (NA and Phe) as well as to vasodilators (ACh and SNP). One hypothesis was that the TiVi method is more sensitive at low skin blood flow values than LDPM, and hence that vasoconstriction tests can be performed without pre-dilatation of the vessels. Further, it was hypothesised that a sigmoidal  $E_{max}$ -model can be fitted to both individual, and pooled, TiVi response data to calculated dose-response variables. A secondary aim was to compare the performance of the TiVi and LDPI during iontophoresis of acetylcholine.

All drugs used in the study were delivered using a single anodal iontophoretic 10 minute pulse of 0.02 mA, with the exception of SNP, that was cathodally administered.

The  $E_{max}$ -model could be fitted to both individual and pooled vascular response data. The results from the individual and pooled data curve fits are shown in Table 8.4.1.

The best fit values for vascular response data for the TiVi and LDPI are shown in Table 8.4.2.

Table 8.4.1. Best fit variables of the individual and pooled curve fits of changes in RBC concentration to iontophoresis of NA, Phe, ACh and SNP measured by using the TiVi.

		TiVi								
		Individual data curve fits		Pooled data curve fits						
	Number	ED <sub>50</sub> (mean)	95% CI	r <sup>2</sup> (mean)	CV (%)	Number	ED <sub>50</sub>	95% CI	r <sup>2</sup>	CV (%)
NA	10	4.4	3.2 to 5.7	0.72	39.0	12	4.4	3.2 to 5.7	0.98	39.1
Phe	10	4.9	3.3 to 6.4	0.90	44.0	11	4.6	3.1 to 6.1	0.99	48.8
ACh	7	2.8	1.9 to 3.8	0.75	37.3	7	2.8	1.9 to 3.8	0.97	37.3
SNP	6	4.9	2.7 to 6.8	0.74	41.1	7	4.4	2.6 to 6.3	0.96	45.4

Table 8.4.2. Best fit variables of the individual and pooled curve fits of blood flow response to iontophoretically administrated ACh and SNP measured by TiVi and LDPI.

		TiVi				LDPI				
		Individual data curve fits		Pooled data curve fits		Individual data curve fits		Pooled data curve fits		
	ACh (n=7)	SNP (n=6)	ACh (n=7)	SNP (n=7)	ACh (n=7)	SNP (n=7)	ACh (n=8)	SNP (n=8)	ACh (n=10)	SNP (n=10)
ED <sub>50</sub>	2.8	4.9	2.8	4.4	1.9	3.4	1.9	3.4	2.3	4.4
95% CI	1.9 to 3.8	2.7 to 6.8	1.9 to 3.8	2.6 to 6.3	1.5 to 2.4	3.0 to 3.9	1.5 to 2.4	3.0 to 3.9	2.2 to 2.5	4.1 to 4.7
r <sup>2</sup>	0.75	0.74	0.97	0.96	0.95	0.97	0.95	0.97	0.61	0.74
CV (%)	37.3	41.4	37.3	45.4	36.4	19.2	36.4	19.2	25.0	26.0



## 9. Discussion

Cardiovascular disease is one of the leading causes of death in the western world, in Sweden it constituted for 42% of all deaths in 2005 (Socialstyrelsen 2009). Impaired endothelial vascular signalling, leading to endothelial dysfunction, is believed to be one of the earliest changes seen in the pathogenesis of cardiovascular disease (Quyyumi, A.A. 2003; Verma, S. et al. 2003; Hadi, H.A. et al. 2005). The rationale for finding a prognostic tool to aid in the prediction of micro- and macrovascular disease based on assessment of vascular reactivity is therefore quite clear.

There are, today, a number of non-invasive measurement techniques such as flow-mediated vasodilatation, pulse wave velocity and carotid intima thickness measurement that are used for assessment of vascular function. Many of these methods correlate highly with cardiovascular outcomes but as they primarily evaluate conduit artery function they offer little insight into the mechanisms of systemic microvascular pathology (Holowatz, L.A. et al. 2008).

Cutaneous (micro)vascular function can be clinically investigated using techniques such as venous occlusion plethysmography (VOP), microdialysis and capillaroscopy. The drawbacks of these techniques include complex and time consuming settings and analysis (VOP, capillaroscopy) (Yvonne-Tee, G.B. et al. 2006), and invasiveness (VOP, microdialysis) (Anderson, C. et al. 1994; Kreilgaard, M. 2002; Yvonne-Tee, G.B. et al. 2006).

### 9.1. The Skin and Transdermal Iontophoresis as a Model for Vascular Function

The skin is a highly interesting and potentially representative organ for studies of microcirculatory function and dysfunction as it is richly vascularised and easily accessible (RG, I.J. et al. 2003; Stewart, J. et al. 2004; Abularrage, C.J. et al. 2005; Holowatz, L.A. et al. 2008). Various investigations have shown that cardiovascular disease causes vascular dysfunction in the cutaneous circulation and that these changes may mirror the general functionality of the systemic circulation (RG, I.J. et al. 2003; Stewart, J. et al. 2004; Abularrage, C.J. et al. 2005; Rossi, M. et al. 2006).

*In vivo* vascular signalling is highly complex and investigations of skin vascular function such as seen in vasodilatation and/or vasoconstriction is mediated via several mechanisms. The elicited vascular response, therefore, is the sum of neural, endothelial, and vascular smooth muscle contributions and this makes the isolation of individual vascular signalling pathways difficult. This may be considered a limitation when using skin as a model for vascular function. At the same time this highlights the complexity of human *in vivo* integrative physiology of all vascular beds. Thus, skin microcirculation and skin-specific techniques could be argued to provide a relevant model representative for a global assessment of microvascular function (Holowatz, L.A. et al. 2008).

Transdermal iontophoresis offers a non-invasive method for delivery of various vasoactive substances across the skin into the underlying tissue. Since first used in 1986 by Lindblad and Ekenvall for investigation of  $\alpha_1$ -receptors in the fingers (Lindblad, L.E. and Ekenvall, L.

1986) the technique has been applied for investigation of various diseases such as diabetes (Morris, S.J. et al. 1995), Raynaud's phenomenon (Anderson, M.E. et al. 1996), and cardiovascular disease (Turner, J. et al. 2008).

The method is increasingly being adopted in clinical use for assessment of endothelial function (Turner, J. et al. 2008) and it has been used to demonstrate that an impaired endothelial function of the skin resistance vessels correlates to an increased risk of coronary heart disease (RG, I.J. et al. 2003). Transdermal iontophoresis has also been used to demonstrate the reducing effect of hypertension on the endothelium-dependent vasodilatation of skin resistance arteries (Farkas, K. et al. 2004) and to investigate the effects of aging on the vessels (Tao, J. et al. 2004). The potential power of iontophoresis as a prognostic tool for prediction of micro- and macrovascular disease is therefore rather undisputed.

## **9.2. Analysis and Presentation of Vascular Response Data**

The analysis of vascular dose-responses to transdermal iontophoresis is not straightforward. The translation of raw perfusion data into measures that are both clinically and statistically meaningful turns out to be challenging as a result of several issues.

Dose-response analysis, as the name implies, requires knowledge about how much of the drug that is administered to the target tissue. In iontophoresis, however, the absolute dose is inherently unknown. This problem is generally circumvented by assuming that the drug dose during iontophoresis is linearly proportional to the iontophoretic charge - if the iontophoretic charge is defined as the current strength multiplied by the duration of the current pulse (Phipps, J.B. et al. 1989).

When performing iontophoretic tests before and during study I it was found that a certain iontophoretic charge produced maximum responses of roughly the same magnitude every time. These findings seemed to suggest that there was a strong relationship between the charge applied and the drug dose delivered at the tissue level. It was also noted that when the vascular responses were plotted against the logarithm of the iontophoretic charge the appearance of the curve was similar to that of a dose-response curve generated from *in vitro* experiments using a vascular strip attached to a strain gauge meter. It is common to analyse such *in vitro* data by using the  $E_{max}$ -model as this model offers an effective and powerful way of vascular response analysis. The obtained  $EC_{50}$ , maximum or minimum responses, and Hill slope values can be compared in a quantitative, statistically supported way.

In Study I it was therefore hypothesised that the  $E_{max}$ -model could be used also on vascular response data elicited by iontophoresis of acetylcholine and sodium nitroprusside measured by LDPI.

The results showed that such curves could be accomplished repeatedly with a generally high goodness of fit. The spread of the  $ED_{50}$  values was also found to be low, which indicated that the model could be used to distinguish differences among separate drugs. Also, the reproducibility within and between experiments in the *in vivo* studies was found to be almost three times higher than that for the *in vitro* experiments. These results suggested that the *in vivo* model was working as well, and in some aspects, better than the *in vitro* model.

However, it has previously been suggested by others (Mudry, B. et al. 2006) that the assumed linear relationship between the iontophoretic charge and drug dose may be invalid in an *in*

*vivo* model. For the assumption to hold it is necessary that the current is made up entirely of drug ions. This is never the case in an *in vivo* situation as at least a part of the current will be made up of counter ions travelling from the body towards the electrodes to maintain electroneutrality and background ions present in the vehicle solution that compete with the drug ions.

In a study made by Tesselaar and co-workers using ACh and SNP and three different durations and current strengths, it was found that the resulting dose-response curves for the respective drug had different maximum responses and ED<sub>50</sub> values - despite the use of a constant iontophoretic charge of 12 mC (Tesselaar, E. et al. 2008). It was concluded that the absolute drug dose that reaches the target organ, or receptors, is affected by the local blood flow, diffusion of drug into other areas and the formation of local “drug depots” within the skin. This combination of active and passive transport routes thus forms an important consideration when estimating the locally administered drug dose applied during transdermal iontophoresis (Tesselaar, E. et al. 2008).

As the applied dose was unknown Tesselaar and co-workers developed and used a time-response model to analyse data and obtain valuable information about drug kinetics and pharmacodynamic characteristics (Gabrielsson, J. et al. 2000).

In study III included in the present thesis the test performed by Tesselaar and co-workers was repeated using the same protocol as before but replacing ACh and SNP with NA and Phe.

The hypothesis for study III were: 1) dose-dependent vasoconstriction in response to iontophoresis of NA and Phe could be measured using LDF if the vessel bed is predilatated by local heating; 2) as a result of drug kinetics in the skin, the responses may vary for equal doses, depending on the protocol used.

The slopes of the time-response curves generated in Study III were found to depend on the current strength used for both NA and Phe and the maximum responses were found to be dependent on the protocol used. However, the blood flow decreased to, at most, 70% of the maximum flow level induced by local warming and therefore none of the protocols resulted in stable maximum response plateaus.

The dependency of the slopes to current strength was anticipated as a higher current generates a higher ionic flow between the electrode and the skin and thus a higher infusion rate.

The finding that more vasoconstriction was observed for higher current strengths suggests a clearance of drugs out of the skin, similar to the findings of Tesselaar and co-workers. This result is interesting as it could be argued that with drugs that reduce blood flow, the clearance of these drugs would be less significant compared to when using vasodilators. It is likely, however, that the predilatation of the vascular bed by local warming actually results in a substantial clearance of the vasoconstricting drugs, especially in the beginning of the delivery period, when there still is maximal blood flow. The clearance of drugs is expected to decrease only gradually as the vascular bed constricts during the delivery period. This situation is different from delivery of vasodilators, where the effect of clearance by blood flow is expected to increase during the delivery period. Although it is difficult to predict the exact influence of clearance of drugs by blood flow, it may well explain why the flow only reduced to about 70% of the maximum flow in these experiments, given the maximum flow conditions at the start of the delivery period. The increased blood flow caused by the local warming may also be the reason to why none of the protocols resulted in stable maximum response plateaus.

Once the vascular response data have been collected two-way ANOVA can be used to compare two dose-response or time-course curves. The problem, however, with this approach is that ANOVA treats the different doses (or time points) the same way it treats different species or different drugs. The method does not take into consideration that the different doses (or times) are sequential or numerical and randomly distributed data will generate the same ANOVA results as for sequentially ordered data. Regression analysis of vascular response data using non-linear a dose-response model will generate values for ED<sub>50</sub>, Hill slope and maximum/minimum responses. These values can be used for comparison between drug effects or vascular responses between patients groups in a quantitative way (t-test or ANOVA). Curve fitting based on dose-response models may therefore be considered a superior technique compared to just comparing the curves using ANOVA (Gabrielsson, J. et al. 2000).

Whether the best way to analyse vascular response data to iontophoresed drugs is by pooling all the responses generated from several subjects or by considering the responses separately in each subject is a matter of debate. There are advantages and disadvantages with both methods and the decision to use either of them has to be based on the questions asked. The results from the studies included in this thesis were analysed using both options.

In the individual data analysis a curve and the best-fit parameters for each subject are constructed and calculated. This approach is favourable if the aim is to investigate the individual variation within a group.

In pooled data analysis a single curve is constructed for each group of subjects. This could, for example, be a single curve for each drug or one curve before and one after a treatment. In this case, the data points from each subject at every dose are considered simultaneously and weighted equally in the regression analysis. This type of analysis is suitable for studying a group effect. Pooled data analysis is easier to perform compared to individual curve fitting as the latter involves more statistical analyses. It can also be argued that pooled data analysis is more robust than individual data analysis, as some individual randomness due to artefacts or measurement errors is averaged out over the whole group. At the same time, some information about the variation within the group may be obscured by pooling data, such as the absence of defined response plateaus, or “non-ideal” responses. Care should therefore be taken not to overestimate the suitability of the chosen analysis model (in this case the E<sub>max</sub>-model) for describing the vascular responses

Normalisation of the data, that is converting the values on the Y-axis to a common scale, can be very useful for comparison of the shape or position (ED<sub>50</sub>) of two or more curves as it eliminates differences in maximum and minimum values. The drawback of normalising data this way is that quite some information about individual variation is lost. It also effectively produces stable bottom and maximum plateaus as the minimum and maximum values become defined. This type of normalisation could lead to the generation of “false” ED<sub>50</sub> values from curves that originally lack stable response plateaus, and incorrect goodness of fit. To avoid this in the present studies, data were initially plotted individually and curves that lacked stable maximum response plateaus were not included in the pooled data analysis.

The way of presenting the perfusion response as mean ± standard error of the mean (SEM) as it is done in the articles included in this thesis is motivated by the type of research question that has been formulated. Generally, what have been sought after are the differences in mean effects between treatments in the same group, rather than biological variation of individual

perfusion responses. Standard error of the mean, therefore, serves as an indication of how accurately determined the mean response values for a particular treatment are.

### **9.3. Methodological Issues When Using Vasoconstrictors and LDF**

Microvascular beds with few and slow-moving RBCs, such as the one in the upper dermis under normal conditions, generate LDF perfusion values that are typically close to the biological zero. It is therefore difficult in some applications, including blanching of the skin, to detect any alterations in tissue perfusion using LDF. To avoid the development of a floor effect in LDF during measurements with vasoconstrictors the vessel bed is usually predilatated. Predilatation is achieved either by pharmacological provocations or by local warming of the tissue. As local warming has been shown to induce significantly larger maximum responses compared to the administration of vasodilating drugs it is considered the preferred method (Christen, S. et al. 2004). However, as indicated by the results of Study III and other investigations (Wilson, T.E. et al. 2002) local warming may affect the outcome and complicate the analysis and interpretation of the obtained vascular responses.

Local warming has, in addition to the increased clearance caused by a higher blood flow, also been suggested to have a negative effect on  $\alpha$ -adrenergic vasoconstrictive responsiveness in humans. It has been reported that a dose of NA that under normothermic conditions would induce clear response plateaus could not overcome the vasodilatory effects of local warming (Wilson, T.E. et al. 2002).

Due to the lack of dose effect plateaus the obtained results in study III could not be analysed using the pharmacological  $E_{\max}$  dose-response model. Neither could the time-response model developed for analysis of vascular response data to ACh and SNP be applied.

The generated time response curves still gave valuable insight to the effects of transdermally iontophoresed vasoconstrictors as maximum responses and slopes could be calculated.

Further the experiment points out some of the problems involved using LDF and investigation of the effects of vasoconstrictors, especially the drawbacks of using local heating to improve the laser Doppler measurements.

The use of predilatation to enable the use of LDF, therefore, has to be weighed against the possible perturbations caused to the vessel bed and the involvement of further mechanisms into an already highly complex *in vivo* model.

### **9.4. TiVi for Investigation of Skin Microcirculation**

An alternative to using LDF for assessment of vascular responses is to measure the  $RBC_{\text{conc}}$  in the skin by using subsurface polarised light spectroscopy. The newly developed tissue viability imager, or TiVi, utilises this technique and is therefore sensitive only to the concentration of the RBCs within the measurement area. In that aspect, as the concentration of RBCs is primarily sensitive to the calibre and haematocrit of the vessels under investigation (Silverman, D.G. et al. 1994), assessment of vascular function using the TiVi resembles vessel studies performed *in vitro*, using vascular strips.

Moreover, the fact that TiVi does not rely on the movement of RBCs suggests that the method could be used for measurements in vascular beds with low or no perfusion at all.

The ability of the TiVi to quantify such reactions as blanching and vasoconstriction on unprovoked skin (i.e. no predilatation) was evaluated in Study II by topical application of Clobetasol propionate. The clobetasol was applied in two volumes, 20 and 10  $\mu\text{l}$  and the TiVi was able to discriminate between the sites with an average TiVi-index of 31% and 19% below normal skin control levels, respectively.

The sensitivity of the TiVi was also tested by adding blood with known RBC concentrations ranging from 0% to 4% to a fluid model simulating blood vessels. This concentration range was selected as it was considered to cover the physiological concentration of blood in tissue. The TiVi could discriminate between changes in concentration of 0.2% and measured values correlated well to simulated values. Non-linearity in absorption due to high RBC concentrations that would have otherwise had an effect on the TiVi-index was compensated for by inbuilt algorithms in the analysis software.

The level of oxygenation of the blood affects the amount of light that is absorbed by the RBCs and could therefore interfere with TiVi measurements. However, the wavelengths (ranging from 400 to 700 nm) used by the TiVi system makes it relatively insensitive to the oxygen state of the RBCs, which was confirmed by in-vitro experiments using blood saturated to 100 and 0%. The calculated TiVi-index for the deoxygenated blood was on the average 91.5% of that of the oxygenated blood, which correlates to a deviation of less than -3.9% within the physiological range of oxygen saturation (70-100%) for cutaneous blood.

The ability of the TiVi to quantify vascular responses *in vivo* was investigated by measuring the effects of iontophoresed ACh in healthy volunteers. The average relative increase in RBC concentration within the electrode area was calculated to 66%. Some areas, however, displayed an increase of up to 117% while other areas displayed a virtually unaltered TiVi-index value, thus demonstrating the spatial heterogeneity in the reaction pattern.

A high concentration of epidermal melanin, as well as the thickness of this layer, has a reducing effect on light transmission. Melanin distorts the relationship between the TiVi-index and the  $\text{RBC}_{\text{conc}}$ . The melanin layer also results in an off-set effect with baseline values higher compared to those of skin types with less pigmentation. This off-set may contribute to the relatively high variability seen in TiVi baseline values between subjects. Relative changes in  $\text{RBC}_{\text{conc}}$  can, however, still be investigated by subtracting a reference image recorded before the start of a stimuli-response experiment from all images in the sequence recorded during the experiment. This correction for the off-set is feasible as the concentration of melanin in the skin can be considered stable in comparison with the  $\text{RBC}_{\text{conc}}$ .

The TiVi-output is further affected by curvature with generally higher TiVi-index toward the periphery. This point out the importance of placing the camera directly above the area that is to be investigated in order to facilitate the collection and analysis of data. However, this anomaly can be balanced by multiplying the image with the normalized red plane values in the original photograph.

In Study IV the TiVi was used to investigate the effects of transdermally iontophoresed vasoconstrictors on the local tissue  $\text{RBC}_{\text{conc}}$ . It was hypothesised that vascular responses to iontophoresed NA and Phe (without the need of preheating), as well as to vasodilators (ACh, SNP), could be quantified and that the data could be analysed using the  $E_{\text{max}}$ -model.

The analysis showed that the TiVi raw values, in response to NA and Phe, decreased 20-25% on average compared to normal RBC<sub>conc</sub>. In comparison, the best case measured decrease using LDF and preheating was 30% of maximum blood flow.

The results look promising for future clinical applications for the TiVi. It could, for example, be used in conjunction with ACh and SNP to evaluate the effects of different diseases on the functions of the endothelium. It may also facilitate the investigation of the effects of vasoconstrictors, such as NA and Phe, on the cutaneous vessels. The relatively restricted confidence intervals for the ED<sub>50</sub> values generated both by the vasoconstrictors as well as by the vasodilators and the reproducibility between different experimental groups (in the present investigation healthy volunteers) further support the use of this technique in experimental medicine.



## 10. Conclusion

The general aim of the studies included in this thesis has been to facilitate the use of iontophoresis as a tool for investigation of cutaneous microvascular function.

A major part of the work has been directed towards dose-response analysis of vascular effects, measured by both LDF and TiVi, using the dose-response model ( $E_{\max}$ ) commonly used in *in vitro* vascular assays. The following conclusions were drawn from the different studies:

The application of the proposed dose-response model ( $E_{\max}$ ) accurately describes the vascular responses detected by LDF to iontophored ACh and SNP if the delivered drug dose is considered to be proportional to the applied electrical charge. Variables such as  $ED_{50}$ , maximum/minimum values and Hill slope can be estimated. An acceptable within (ACh = 36%, SNP = 19%) and between (ACh = 25%, SNP = 26%) experiment variability was found. An antagonistic effect, as anticipated, could be demonstrated for iontophored atropine in settings where antagonism is physiologically relevant.

No measurable involvement of prostaglandins was found using the present vascular model (Study I).

The tissue viability imaging system was found capable of accurately (high correlation) detect changes in  $RBC_{\text{conc}}$  in the *in vitro* setting at volume concentrations of 0% to 4%. A good resolution was also found for the detection of  $RBC_{\text{conc}}$  in the papillary dermis after provocations with topically applied clobetasol propionate and iontophored ACh (Study II).

A protocol based on preheating and LDF was developed in which the vasoconstrictive effects of both iontophored NA and Phe were readily detectable. However, the results of these investigations show that the dose-response analysis in a model of preheating is more complex than first anticipated. Firstly, the maximal dose effect was variable and secondly; vascular responses were found to be different when using protocols with the same total iontophoretic charge but with different current strengths and durations. These findings implies the need for a further refined analysis model, which also takes more variables into account, such as the effect of local blood flow and drug transport kinetics. The assumption that the local drug dose in the microvascular bed is linearly proportional to the iontophoretic charge may not be accurate in this model of preheating (Study III).

The TiVi system was found to adequately detect both iontophoretically induced vasodilatation and vasoconstriction. In the latter setting it appears a favourable technique as the model does not require predilatation of the vessels as in the case of LDF. As the TiVi methodology measures the RBC volume in the tissue, which depends mainly on vessel diameter and haematocrit, it resembles the *in vitro* vascular assay to a higher extent than the LDF methodology does (Study IV).

The analysis methods and the new equipment presented in this thesis enable new ways of presenting vascular response data and the assessment of vascular responses to vasoconstrictors. These findings improve the applicability of transdermal iontophoresis as a tool for investigation of vascular function and may contribute to the establishment of iontophoresis as a clinical tool in such investigations.

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