

Bachelor Thesis Technical Medicine

An optimal therapeutic window in a transdermal treatment against circulating tumor cells (CTCs), in terms of CTC behavior, perfusion limits and time frame.

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Preface & Acknowledgment

This thesis was written as a final assignment for the Bachelor Technical Medicine at the University of Twente. The students who worked on this assignment for 11 weeks were Manon Kloosterman, Martine Schulten, David Tanis and Lisa Vulders. David has a bachelor's degree in Medicine and is finishing the pre-master Technical Medicine. Manon, Martine and Lisa are finishing their Bachelor Technical Medicine by performing this bachelor thesis.

On this page we would like to express our gratitude to our supervisors and the people who helped us during this bachelor thesis. With their help we were able to obtain a profound understanding of the matter and to combine the vision of different disciplines in our paper.

Enjoy reading.

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Abstract

Introduction: In the Netherlands, cancer is the number one cause of death, often induced by metastasis. Metastasis is induced by circulating tumor cells (CTCs), individual cells that are shed from the primary tumor and are distributed through the lymphatic and hematogenous system. Many methods for the elimination of CTCs have been developed, but most of them are invasive and damaging. Therefore, a non-invasive and more accurate therapy should be developed. This research is a continuation on the peppered skin project of the Biomedical Photonic Imaging (BMPI) research group. The aim is to enhance the perfusion of the skin, ultimately in order to collect and destroy CTCs transdermally. First, the BMPI group has focused on a model to link temperature change to perfusion, in order to determine the absolute amount of skin blood flow. To validate this model, more test subjects are required, for which accordance of the medical ethics review (METC) is needed. Before detection and elimination methods can be assessed, an optimal therapeutic window should be found. This involves finding the highest achievable perfusion to clear the blood from CTCs in an optimal time frame. Besides that, the behavior of CTCs in the microcirculation can influence the transdermal treatment possibilities of CTCs. These matters combined give rise to the following research question: 'How can the therapeutic window in a transdermal treatment against CTCs be optimized, in terms of CTC behavior, perfusion limits and timeframe?'

Method: A proof of concept study has been performed, in which six different rubefacients are compared with each other: Midalgan (*methyl-nicotinate, glycol-salicylate*), Tigerbalm (*Menthol*), Tatum (*capsaicin, benzyl-nicotinate*) and Capsaicin cream with 0,025%, 0,075% and 0,1% capsaicin concentrations. In this experiment three parameters were determined for each rubefacient. The parameters are the relative increase in perfusion, the effect duration and the effect of reapplication. Furthermore, a literature study was performed on the behavior of CTCs in the microcirculation, the possibilities of detection and elimination and on the requirements for an METC request.

Results: Midalgan, Capsaicin 0,075% and Tatum were found to be the most promising and were therefore further investigated. Reapplication had little effect for Midalgan but did cause a repeated increase of perfusion for Capsaicin 0,075% and Tatum. After correcting the arbitrary units of perfusion for their baseline, it was seen that Tatum causes the highest relative increase in perfusion (factor 10-25) for the longest period of time. Literature study showed that CTCs appear in clusters and as individual cells, but clusters are more likely to create metastasis and might need a different therapeutic approach. Furthermore, CTCs not only appear in the microcirculation but also in the lymphatic system, which is important to take into consideration when developing an elimination method.

Conclusion: Tatum shows the highest perfusion increase and has the longest effect duration and would therefore be the most valuable with a view to a future treatment. From the literature research can be concluded that the presence of CTCs in the lymphatic system cannot be ignored in a novel treatment method. Secondly, the penetration depth of the therapy is paramount to the therapeutic window given the vessel plexuses. Furthermore, CTCs can enter the smallest capillaries and therefore reach the entire microcirculation. Finally, clusters of CTCs might need a different therapeutic approach than single cells, because of their size and higher risk of metastasis compared to single cells. An METC approach is needed for further research with test subjects in order to validate the model of the BMPI group.

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Abbreviations

ACh	Acetylcholine
AS	Average Signal
a.u.	Arbitrary units
BM	Baseline Measurement
BMPI	Biomedical photonic imaging
Ca 0,025 %	Capsaicin cream with 0,025 % capsaicin
Ca 0,075 %	Capsaicin cream with 0,075 % capsaicin
Ca 0,1 %	Capsaicin cream with 0,1 % capsaicin
CCMO	Central Committee on Research Involving Human Subjects ('Centrale Commissie Mensgebonden Onderzoek')
CGRP	Calcitonin Gene-Related Peptide
CTC	Circulating Tumor Cell
EDHF	Endothelium-Derived Hyperpolarizing Factor
EMT	Epithelial-Mesenchymal Transition
EpCAM	Epithelial Cell-Adhesion Molecule
FSMW	Functionalized Structured Medical Seldinger Guidewire
IR	Infrared
M ₁	Measurement 1
M ₂	Measurement 2
MAF	Moving Average Filter
METC	Medical Ethics Review Committee ('Medisch Etische Toetsingscommissie')
MHC-I	Major histocompatibility complex 1
Mi	Midalgan Xtra warm
MN	Methyl Nicotinate
NK-cell	Natural Killer cell (large granular lymphocyte)
NO	Nitric Oxide
NSAID	Non-Steroidal Anti-Inflammatory Disease
PAFC	Photoacoustic Flow Cytometry
PU	Perfusion Unit
SLN	Sentinel Lymph Node
SNP	Sodium NitroPrusside
Spaser	Surface Plasmon Amplification by Stimulated Emission of Radiation
TA	Topical Analgesics
TAN	Tantum warmte extra warm
TGF- β	Transforming growth factor beta
TR	Tigerbalm Red
TRPM8	Transient Receptor Potential Melastatin 8
TRPV1	Transient Receptor Potential Vanilloid 1-receptor
VEGF-C	Vascular Endothelial Growth Factor C
VEGFR-3 Ig	Vascular Endothelial Growth Factor Receptor 3 - Immunoglobulin
VSMC	Vascular Smooth Muscle Cells
WMO	Medical Research Involving Human Subjects Act ('Wet medisch-wetenschappelijk onderzoek met mensen')

1. Introduction

In the Netherlands, cancer is the number one cause of death, both in men and in women.[1] Ninety percent of all cancer-related deaths are induced by metastasis.[2][3][4] Metastasis is caused when heterogeneous cells of the primary tumor exit their source and reach distant organs by travelling through the bloodstream and the lymphatic system. These heterogeneous cells are called circulating tumor cells (CTCs) and are the main cause of metastasis. They have the ability to form a secondary tumor and adapt to the new environment.[5][6] To prevent the growth of a secondary tumor, it is important to remove the primary tumor and eliminate CTCs from the bloodstream.[3] Currently, adjuvant chemotherapy is the most common method to clear the circulation of CTCs. However, it has many drawbacks, including damage to bone marrow stem cells and repeated perforation of the skin for venous access.[7] Therefore, a non-invasive and more accurate therapy able to eliminate the CTCs should be developed.

A way to exterminate CTCs could be a non-invasive therapy in the superficial skin. By increasing the skin perfusion within a therapeutic window, the CTCs will present themselves in the upper skin layer and can be destroyed. Therefore, an optimal therapeutic window should be found. This involves finding the highest achievable perfusion to clear the blood from CTCs in an acceptable time frame, where skin perfusion is defined as 'the amount of blood delivered to the capillary beds of a tissue area in a certain period of time'.[8]

The Biomedical Photonic Imaging (BMPI) research group of the University of Twente has done explorative research regarding skin perfusion. They increase the perfusion using a vasodilator rubefacient in a research called the 'peppered skin project'. More information about this project is given in their video on YouTube.[9] Using a specific cooling method, they are developing a model that connects temperature-gradients to an absolute quantity of perfusion. The research group attempts to determine this connection using COMSOL, which uses the bioheat, and data regarding the heating process of the skin. To validate this model, further research with more test subjects is required, for which a request to the medical ethics review committee (METC) is needed.

The ultimate goal of the peppered skin project is to eliminate all CTCs by increasing the perfusion of the skin in an optimal therapeutic window. To reach this goal two important aspects need further elaboration. Firstly, it is necessary to understand the behavior of CTCs in the capillaries. The interaction of these cells within the microcirculatory environment is paramount for developing a successful elimination method. Secondly, the absolute skin perfusion needs to be maximized. To create a therapeutic window where a sufficient amount of blood presents itself within an acceptable time frame, all blood in the body should pass the therapeutic window at least once. Physiological active vasodilation can increase total cutaneous blood flow up to 10-fold above the level at rest, for example when necessary for increased heat dissipation. Under these circumstances it takes less than 2 hours for all 5,5 liters of blood in the human body to pass a 20x20cm skin patch.[10][11][12][13] Possibly, artificial enhancement by a rubefacient can increase local perfusion even more than 10-fold, further decreasing the time needed. Treatment of 5,5 liters of blood through this skin patch does not ensure clearance of all blood, because CTCs will redistribute themselves in the circulation due to diffusion. However, it does indicate the feasibility of the persecuted timeframe. Maximizing perfusion would lead to a shorter timeframe in which the blood can be cleared from CTCs.

This thesis examines both the maximized skin perfusion and the behavior of CTCs, intended to develop a transdermal CTC elimination method. This leads to the following research question: 'How can the therapeutic window in a transdermal treatment against CTCs be optimized, in terms of CTC behavior, perfusion limits and timeframe?' To answer this question an extensive literature research regarding the behavior of CTCs within the microcirculation will be performed. A closer investigation into which rubefacient works optimal and to what extent skin perfusion can be artificially enhanced will be assessed. This will be done in an experimental proof of concept study design. Findings on the optimal setting for enhancing skin perfusion can guide further research on a transdermal elimination method of CTCs.

2. Objectives

This research will investigate the behavior of CTCs in the microcirculation and the effect of different rubefacients on the perfusion of the skin. The following main question and sub-questions have been composed.

2.1 Main question

How can the therapeutic window in a transdermal treatment against CTCs be optimized, in terms of CTC behavior, perfusion limits and timeframe?

2.2 Sub-questions

1. Circulating tumor cells

Sub-question 1.1: How do CTCs behave within the microcirculation of the skin?

Sub-question 1.2: What is the relative role of metastasis through the lymphatic system compared to metastasis through the blood circulation?

Sub-question 1.3: Which existing methods are used to detect and eliminate CTCs in vivo?

Sub-question 1.4: What is the evidence that removing CTCs will enhance the prognosis for the patient?

Sub-question 1.5: What method can be designed in order to eliminate CTCs in vivo?

2. Experiment

Sub-question 2.1: Which drugs might give a higher perfusion than the currently used Midalgan?

Sub-question 2.2: What is the mechanism of perfusion enhancement using Midalgan and other drugs?

Sub-question 2.3: How can an optimal therapeutic window with maximal perfusion and optimal time frame be ensured?

Sub-question 2.4: What are the risks of maintaining an increased perfusion for a longer amount of time using Midalgan and other drugs?

Sub-question 2.5: What is the maximal perfusion that can be achieved?

3. METC request

Sub-question 3.1: What steps need to be taken in order to apply an METC request?

3. Literature research

For a thorough understanding of the relevance of this research, it is useful to get an idea of the physiology of skin perfusion and the current methods for detection and elimination of CTCs.

3.1 Anatomy of the skin

The skin is the body's largest organ and consists of two layers. The most superficial layer is the epidermis, which is a thin cellular layer of approximately 0,1mm. It consists of a keratinized, horny epithelium and provides protection to its lower regenerative basal layer. Because the epidermis itself does not contain blood vessels or lymphatics, it relies on the underlying vascularized layer of deep connective tissue, called the dermis. As seen in figure 1, the dermis provides the blood supply in the form of a network of anastomosing arteries.[10] These are called papillary loops, which are located in the most upper layer of the dermis, the papillary layer, see figure 4. The dermis contains most nerve endings in order to sense touch, pain or temperature, although some reach the epidermis. The interlacing network of collagen and elastic fibers found lower in the dermis provide the strength and toughness of the skin. When describing the skin as an organ, also known as the integumentary system, the subcutaneous tissue is often included, although it is officially not part of the skin. This subcutaneous layer not only contains sweat glands, superficial tissue blood vessels, lymphatic vessels and cutaneous nerves, but also provides for most of the body's fat storage, see figure 1. Subcutaneous tissue participates in thermoregulation as it functions as insulation and retains heat in the body's core.[11]

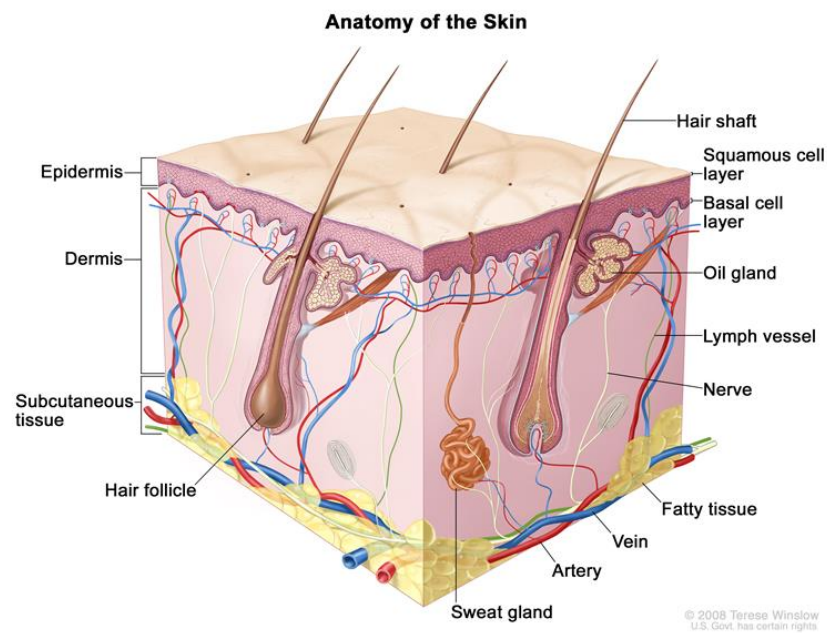


Figure 1: Anatomy of the skin.[10]

The skin has several functions, including protection of the body from environmental factors, containment for the body's structures (e.g. tissues and organs), sensation and synthesis and storage of vitamin D.[11] Besides that, the skin perfusion plays a major role in the process of heat regulation. When more blood flows through the skin instead of through the deeper layers of tissue, more heat is lost through convection. In cold situations, vasoconstriction causes a reversed effect, where the scarcely perfused skin acts as an isolation layer surrounding the core. This mechanism relates perfusion to thermoregulation.[12]

Total resting skin blood flow is approximately 250 mL/min, although depending on many different factors.[12][13] In a patch of skin of 20x20cm this results in 5,5 ml/min, assuming equally distributed skin perfusion over the entire body.[14]

3.2 Physiology of perfusion

Vasodilation and -constriction are regulated by contractility of vascular smooth muscle cells (VSMC). Precapillary vessels changing their contractility is the primary principle of modulating perfusion of a particular tissue. Vasodilation occurs when the muscular walls that surround blood vessels are relaxed, causing the interior of the blood vessel to widen. This allows more blood flow to get through, hence increasing perfusion. The structure of such an arterial wall with smooth muscle cells can be seen in figure 2 below.[15] The true capillaries do not have VSMCs and consist only of endothelium. Control of this vasodilation happens through nervous or chemical stimulation.[16]

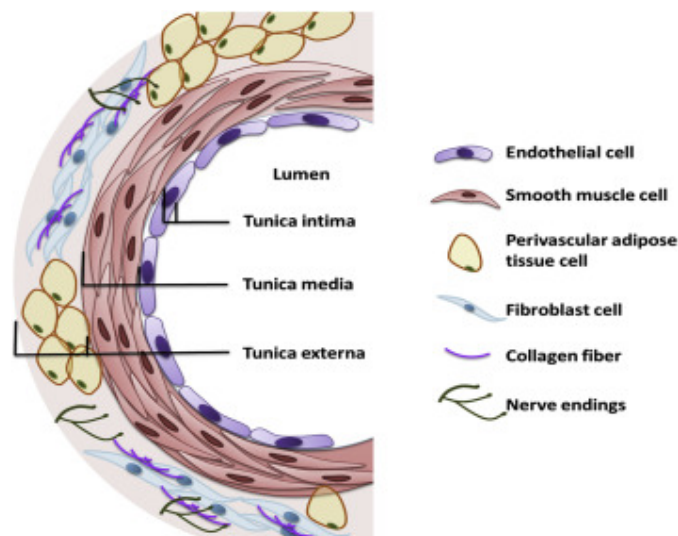


Figure 2: Structure of an arterial wall with its different components.[15]

In the central nervous system, the preoptic anterior hypothalamus takes the role of a thermostat, trying to regulate the internal (core) and surface (skin) temperature. All skin areas are provided with sympathetic vasoconstrictor fibers that secrete norepinephrine at their nerve endings. This ensures at rest a tonic vasoconstrictor influence on skin blood vessels. This tonus at rest is caused by a continuous nerve discharge of one to three pulses per second and can be varied, inducing vasodilation or vasoconstriction. An increased discharge on the sympathetic system will cause further vasoconstriction under cold conditions. On the other hand, the sympathetic vasoconstrictor system is reduced under warm conditions.[16] As a vasodilator neural control mechanism, the skin receives cholinergic nerve fibers. The most probable neurotransmitter is Acetylcholine (ACh). After ACh stimulation, a co-transmitter will provoke relaxation of smooth muscle cells in the vascular wall. In conjunction with ACh, several modulators influence the neural effects on vascular tone. For example the menstrual cycle in women modulates the neural control, as progesterone affects the threshold for cutaneous vasodilation.[16] Regular physical exercise also enhances endothelium-dependent vasodilation.[16][17]

The humoral control consists of circulating chemicals in the bloodstream and affects the wall contractility as well. Systemic humoral agents (e.g. angiotensin II) are distinguished from local agents (e.g. histamine), in which local mechanisms can override any of the neural or systemic humoral influences. In capillary beds the endothelium of the vessel wall releases these locally vasoactive compounds. Nitric Oxide (NO) is one of the most important ones. It is a lipophilic gas with a short half-life, that diffuses locally outside the endothelial cell. After binding to a receptor inside the VSMC, NO relaxes the cell, facilitating a potent mechanism for vasodilation. Endothelium-derived hyperpolarizing factor (EDHF) and Prostacyclin are other examples of a vasodilator agent.[18]

There are several stimuli that induce vasodilation. Physical stimuli include shear stress exerted on the vascular wall by the flowing blood, and pulsatility to the vessel wall. Pharmacological stimuli include a variety of endothelial agonists, such as ACh, bradykinin, trombin, serotonin, estrogens, etc.[16]

3.3 Laser Doppler flowmetry

The perfusion has been measured with a laser doppler monitoring device which allows a measurement of capillary perfusion in the skin in arbitrary units (a.u.). The technique is based on the emission of a beam of low power laser light carried by a fiber-optic probe.[19][20]

The probe sends a coherent light source towards the tissue, where photons are scattered and partially absorbed by both static and moving objects. Light hitting static objects is unchanged and will either be absorbed or reflected via the same pathway as incoming light. When light hits moving particles, e.g. blood cells, it undergoes a change in wavelength, called the Doppler shift. Since red blood cells move relatively slow, the frequency shift of the photons is approximately 10^{-11} of the original frequency of the used light. Therefore, a monochromatic light source is recommended, in this case a laser. The magnitude and frequency distribution of changes in wavelength are directly related to the velocity of the blood cells and the amount in the sample. A photodetector will pick up on the reflected light and calculate the concentration of moving red blood cells through a power spectrum. This process is shown in figure 3.[20]

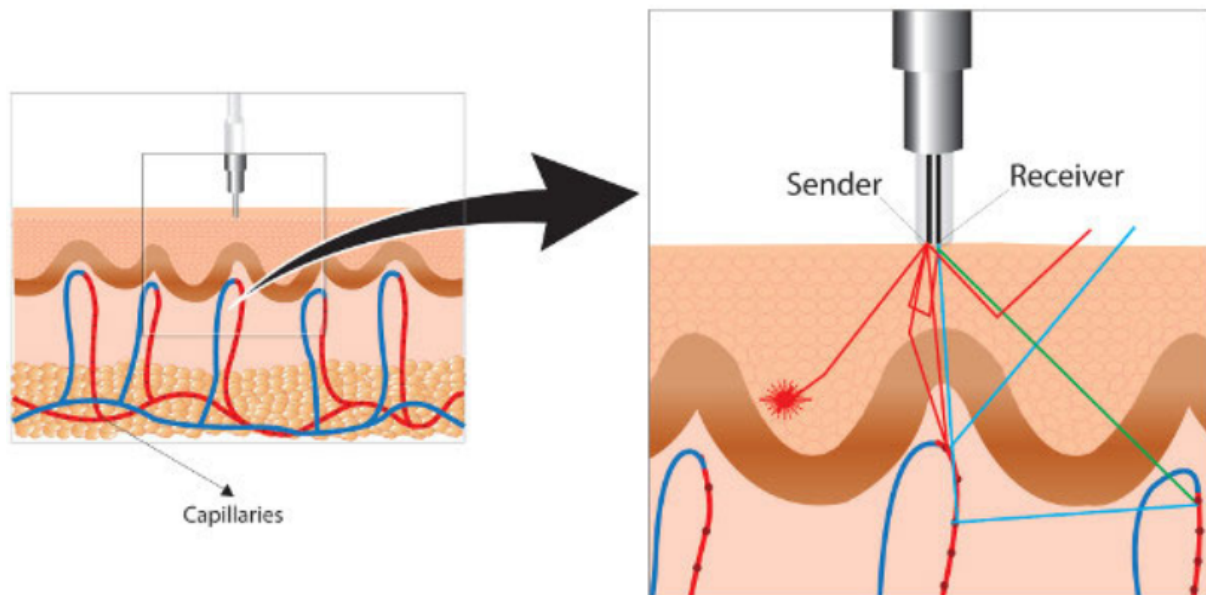


Figure 3: Example of a probe using laser Doppler monitoring on skin surface. The red-light beams represent scattered and absorbed light. The green/blue light beam represents the light reflected by the red blood cells.[19]

In this research a probe with standard fiber separation (0,25mm) and a 780 nm wavelength laser was used. According to the given specifications, the measuring depth will be in the order of 0,5-1 mm. Factors influencing measuring depth include structure and density of the capillary beds, pigmentation and oxygenation of the tissue. Blood content itself causes a decrease in measuring depth, because the passage of light is partially blocked upon scattering.[19]

Measuring blood flow with the laser Doppler monitoring is possible on the skin and many different organs. For an even distribution of the rubefacient a hairless surface is preferred. Therefore, the volar forearm seems an appropriate location. Other areas of skin have a better baseline blood flow, e.g. the buttocks or the shoulder.[21] However, in this research only relative changes in blood perfusion will be assessed and they should be of comparable level in the different body parts.

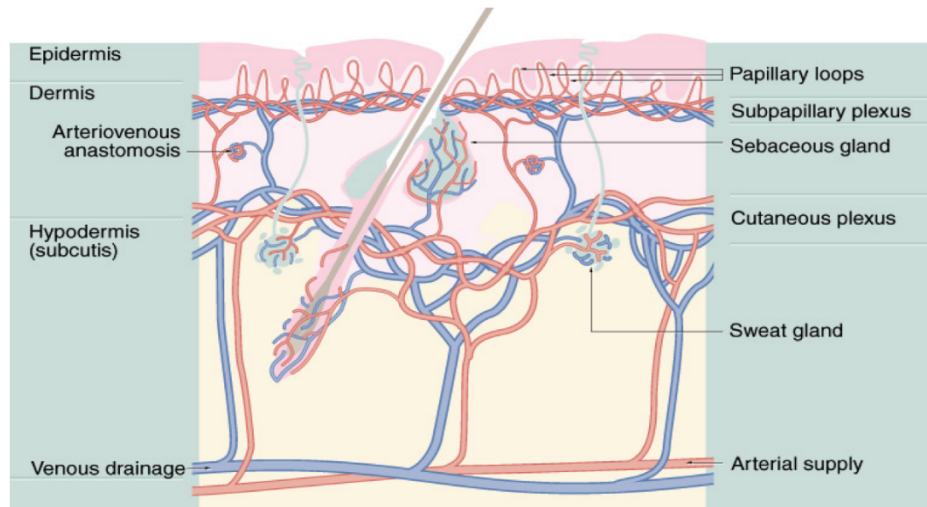


Figure 4: Vascular system of the skin.[23]

A patch of skin on the volar forearm has an average epidermal thickness of 0,075 mm, causing the papillary loops to commence at this depth. Right below these papillary loops, the papillary plexus can be found. This is a plexus of vessels immediately below the dermis.[21] A standardized skin model developed by Fredriksson et al. in 2008 considers, among other parameters, the thickness of the skin layers, as seen in table 1.[22] Figure 4 [23] visualizes the anatomy at these different layers.

Layer	t [mm]
Epidermis	0,075
Papillary dermis	0,15
Superior blood net	0,15
Reticular dermis	0,80
Inferior blood net	0,40
Subcutis	10

Table 1: Layers of the skin with their thickness.[22]

Considering the maximum measuring depth of 1 mm, the possibility of measuring deeper than the reticular dermis is deemed neglectable. However, the subpapillary plexus of the superior blood net is likely included in the measurement. Another research investigated the measurement penetration depth with laser Doppler specifically at the volar forearm, using the same specifics as this research. Here a penetration depth of 0,53/0,56 mm for the forearm skin and 0,66/0,67 mm for heat provoked forearm skin were estimated.[24]

3.4 The behavior of CTCs in the microcirculation

CTCs have proved to be of prognostic value in the diagnosis of cancer as liquid biopsies in blood sample analysis. These samples can be used for early detection to estimate the severity of the illness and to keep track of the efficacy of a treatment.[5][25] However, the number of CTCs in the blood of a patient who suffers from metastatic cancer is much smaller than the amount of other blood cells. In a 10mL sample of blood, around a hundred individual CTCs and an additional five clusters of CTCs can be found. As a comparison, this amount of blood contains around fifty billion red blood cells. This big scalar difference and great variety in size and biology can make it more difficult to detect CTCs in blood.[3][5]

As stated above, CTCs can be found as individual cells or in clusters. Clusters of CTCs mostly derive from the primary or secondary tumor directly. It is less likely that individual CTCs bind together and form a cluster inside the bloodstream.[3][26] Clusters of CTCs are up to 100 times more likely to create metastatic tumors compared to individual cells.[3][27] Cells can detach from a tumor in two different ways. CTCs can undergo epithelial-mesenchymal transition (EMT) or they can leave the tumor without this mechanism. EMT is a process of multiple steps in which epithelial cancer cells undergo a transformation. They lose their epithelial specific characteristics and develop properties of mesenchymal cells. While epithelial cancer cells are stiffer and less

invasive, mesenchymal cells have a more invasive behavior and are very likely to deform. With these new characteristics, the cancer cells can break down parts of the basement membrane of the vessel and migrate through the extracellular matrix into the blood. Without EMT, cells can leave the tumor, for example through passive infiltration into the bloodstream.[5][28][29] This process in which cancer cells derive from the primary tumor and become CTCs by entering the bloodstream is called 'intravasation'.

CTCs in the microcirculation

Originally, it was believed that only individual tumor cells are small enough to reach distant organs through the bloodstream. However, several recent studies show that it is possible for clusters of CTCs to reach distant organs and therefore create metastasis.[4] The average diameter of a capillary is around 7 μm , where a single CTC can be up to 20 μm and CTC clusters can have a diameter around 100 μm . [30] This would suggest that CTCs are not able to pass through the capillaries. However, CTCs are considered to be viscoelastic and to have little stiffness. This gives them the ability to change shape in order to pass the capillaries.[30] Nevertheless, CTCs cannot undergo an unlimited amount of deformation events. After repeated deformation in the microcirculation, cell fatigue can appear, and the cell will reach its plastic deformation domain. Besides that, the cells are temporarily obstructed upon reaching the entrance of a capillary. The CTCs experience friction with the capillary walls and therefore have a lower velocity. Once they deform into their elongated shape, their shape is more suitable to fit through the capillary. The resulting decrease in friction forces causes the velocity to rise again.[31]

To investigate the behavior of CTCs in the microcirculation, several models and set ups have been investigated. One of them is a microfluidic device with small channels which simulates the smallest vessels, the capillaries, in the microcirculation. In this experiment, the focus was on the behavior of clusters of CTCs in the microcirculation. The researchers used clusters of CTCs isolated from the blood of a patient. They found that clusters of twenty or more cells are able to pass even through the smallest channels in the microfluidic device. Clusters of CTCs unfold into an array of individual cells, so they can pass the constriction. At the end of the constriction single cells refold back into a cluster. CTCs can have weak and strong cell-to-cell interaction in a cluster. During the unfolding phase the weak interactions are temporarily lost, and the strongest ones are retained. The largest cell of the cluster limits the speed with which it passes through the constriction.[4][30]

Most CTCs will die rapidly from lethal damage due to toxic conditions of the immune system in the microcirculation. Shear forces are one of the factors that can cause lethal damage to CTCs. For example, when B16 melanoma cells are exposed to the shear stress of a capillary flow simulation for one hour, 70 percent of them will be killed. Due to shear forces, loss of cell viability will appear by damaging the cell membrane of a CTC. In some phases of the cell cycle the sensitivity for this type of damage is increased compared to a cell in interphase (resting phase). In addition, adhesions between CTCs and the vascular wall can be broken by shear forces. For that reason, it is important that CTCs are able to stabilize adhesions to the vascular wall rapidly and thereby extravasate. Adherence and subsequent penetration of the vascular wall is an essential mechanism for CTCs to survive, by escaping the shear forces and host defense mechanisms in the microcirculation.[32][33]

The flow of blood in the microcirculation can cause several changes in CTCs and endothelium, e.g. the activation of signaling cascades and modifications in gene expression. Mechanical forces caused by blood flow can also affect other cellular functions. CTCs in the blood flow are even able to induce damage to endothelial cells, followed by exposure of the underlying basement membrane. Another interesting outcome is the change in surface expression of adhesion molecules in endothelial cells as a result of variations in shear stress. This change may influence the adhesive properties of endothelial cells.[32][34]

The interaction of CTCs with other blood cells

Within the primary tumor, tumor cells benefit from an immunosuppressive microenvironment. When becoming a CTC, they leave the primary tumor and enter the blood circulation, exposing themselves to the immune system of the human body and allowing for interaction with other blood cells. The majority of the research on the interaction of CTCs with other blood cells focusses on platelets and immune cells, since these are believed to be the most important interactors. Most CTCs are killed by the native and adaptive immune system within a few hours, limiting interaction time with other blood cells. However, CTCs have a defense system which gives them the ability to survive and create metastasis.[35][36][37][38]

Platelets can be activated by the interaction with CTCs, particularly via a tumor cell induced platelet antigen. These activated platelets can attach to the surface of CTCs and stimulate them to undergo EMT.[34] Moreover,

platelets will secrete transforming growth factor beta (TGF- β) which activates the TGF- β pathway of the CTCs. This pathway will induce EMT by increasing the expression of mesenchymal markers and decreasing the expression of epithelial markers.[38] The aggregated platelets on CTCs have a protection purpose as well. They form a physical barrier between the CTCs and the other blood cells, such as macrophages, and protect the CTCs from death caused by shear stress. Both single and clustered CTCs can take advantage of this mechanism. In addition, they also risk being trapped in micro vessels, which increases the chance of extravasation of cancer cells.[37][38][39]

Under normal circumstances, natural killer (NK) cells will bind to foreign body cells with specific major histocompatibility complex 1 (MHC-I) receptors. CTCs have the ability to lose or downscale their MHC-I protein expression, with the consequence that they will not be attacked by NK cells.[36] Another mechanism that supports the development of metastasis is the CTC induced downregulation of Toll-like receptors on macrophages and NK cells. Through this mechanism, macrophages can also play an important role in creating a pre-metastatic niche, which will be described later.[40]

CTC attachment

CTC attachment to the vascular wall mostly occurs at the venular part of the microcirculation. This implies that CTC adhesion is facilitated by lower flow rates.[41][42] Cell adhesion is an important factor in metastasis, since CTCs can only extravasate after adhesion. CTCs act the same way as leukocytes, which is why models for leukocytes are suitable. Leukocyte adhesion is a process consisting of multiple steps, including tethering and rolling followed by firm adhesion, as shown in figure 5.[43] There are several models made both in vitro and in vivo for studying the behavior of leukocytes and therefore CTCs, a few of which are addressed in this paragraph.

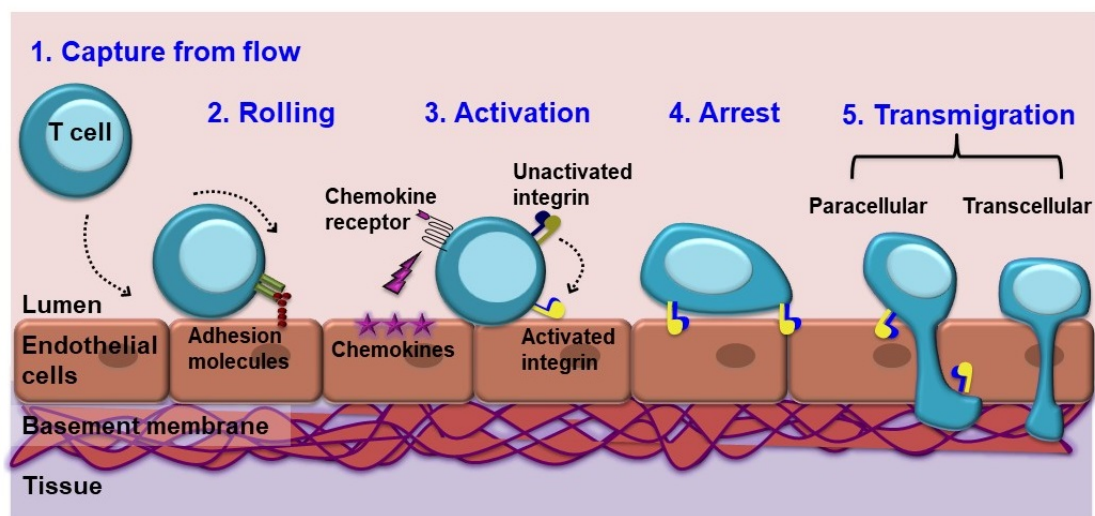


Figure 5: T-cells start with rolling on the lumen of endothelial cells. They bind to the lumen and will be converted into their activated state. Once they are arrested, they will either migrate between two cells or through one cell and end up in the basement membrane of the tissue.[43]

In these studies, it is shown that a weak ligand-receptor binding causes leukocyte rolling, while strong ligand-integrin binding induces a strong cell adhesion. Cell rolling isn't always possible, because the size of capillaries is often smaller than that of a CTC. In rolling, one side of the cell should undergo different forces than the other, which is not possible when the capillary is too tight. Without cell rolling, CTCs will arrest due to size restriction. Size restriction is therefore one of the factors responsible for extravasation.[44] Another model was developed by Hammer and Apte. It is an adhesive dynamics model for examining CTC adhesion. They found that CTC adhesion is depending on its immurement. In a small micro vessel, CTCs have a large number of receptor ligand bonds on their cell surface caused by a growing wall friction force. This is an important finding, which will be treated as a risk in chapter 3.9, risks of maintaining a high perfusion. In a bigger micro vessel, an enhanced lift force induced by the collision between red blood cells and CTCs inhibits CTC attachment. Another important investigation is that the blood flow resistance is increased by the presence of CTCs.[41]

Extravasation

Extravasation is the process in which a tumor cell exits the capillaries due to transendothelial migration. After extravasation, CTCs can either stay inactive or they can develop into a secondary tumor.[34] The characteristics of the microvasculature and the local hydrodynamic factors play an important role in the adhesion between the tumor cell and the endothelial cell and therefore in extravasation. Tumor cells are exposed to flow in the bloodstream during circulation in the primary tumor, adhesion on vascular endothelium and transmigration in a secondary organ. This flow affects the adhesion of tumor cells to the vascular endothelium, as seen in figure 6, and therefore the location of metastasis.[42] For this reason extravasation of a tumor cell will mostly occur in a blood vessel where shear forces are low, like post-capillary venules. For extravasation, structural and functional changes of both tumor cells and endothelial cells are required. Different cell surface receptors are activated and also the endothelial cells are remodeled. For instance, rearrangement of the cytoskeleton of both the tumor cell and endothelial cell are necessary, respectively during migration and during modification of the barrier function. Besides that, CTCs are able to attach themselves to the blood vessel wall and inject genetic material, which changes the lining of endothelial cells. This provides the cell hospitable conditions for the development of metastasized primary tumor cells.[45]

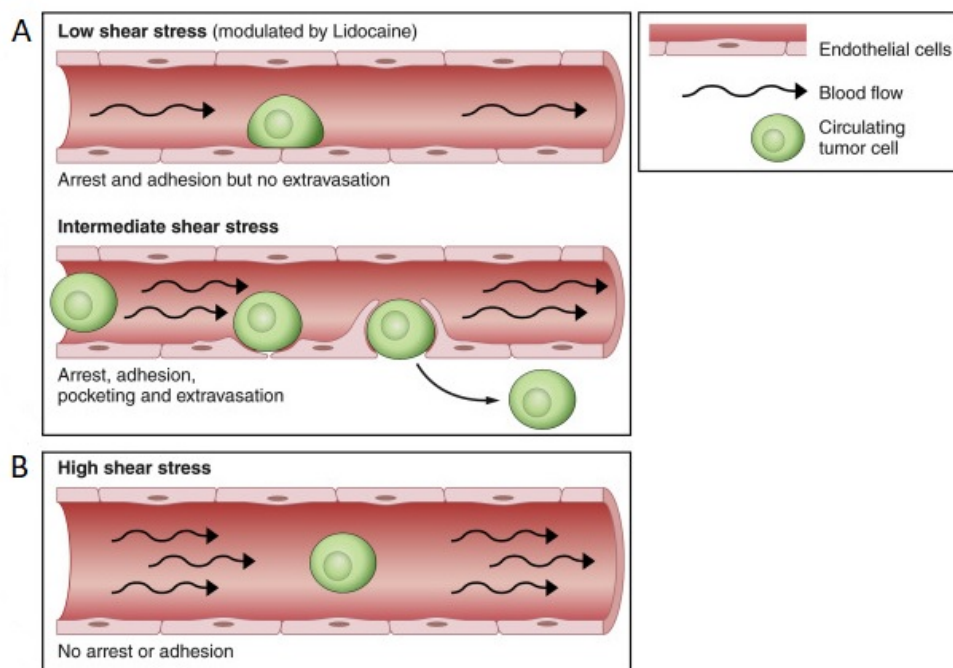


Figure 6: The arrest, adhesion and extravasation of CTCs depend on the blood flow velocity. Metastases are located in regions presenting lower blood flow rates, compatible with smaller vessel size. (A) Upper vessel: A Low flow rate permits CTCs to arrest and attach, but not to extravasate. (A) Lower vessel: Intermediate flow rates are required to allow endothelial remodeling (pocketing) and CTC extravasation without arrest and adhesion. (B) High blood flow in larger vessels avert cell arrest and extravasation.[42]

Metastatic niche

The anatomical routes of the vascular system may be important for the location of metastasis. The location of metastasis particularly occurs at the first capillary bed downstream of the primary tumor. However, some metastases are formed at other locations in the body. Therefore, organ tropism is an important mechanism discovered to play a role. Organ tropism is the attraction of e.g. chemical substances to appropriate organs.[46] Even though the primary tumor has not infiltrated the microcirculation, it is modifying the microenvironment by creating favorable conditions in distant organs for survival and growth by metastasis. It is creating its so called own pre-metastatic niche. The primary tumor does this by secreting soluble factors and extracellular vesicles to prepare its future soil. The first step in forming a pre-metastatic niche is vascular leakiness. Thereafter, the education of local resident cells like fibroblasts and the recruitment of non-resident cells follows. Finally, the survival and growing of CTCs upon extravasation will occur and thereby seeding has been achieved. This process is called the seed-and-soil hypothesis.[42][46][47]

3.5 Behavior of CTCs in the lymphatic system

It is well known that CTCs cause metastasis by travelling through the bloodstream. However, only 15-20% of all CTCs actually travel from the primary tumor to the bloodstream and end up in the target organs. The other 80-85% first migrates to the lymphatic system and passes the lymph nodes, as seen in figure 7.[48] The first lymph node downstream of the primary tumor is called the sentinel lymph node (SLN). Frequently, these lymph nodes serve as endpoints where CTCs die or cause metastasis, the most important endpoint being the SLN. Only a small amount of the CTCs reaches the hematogenous system again after entering the lymphatic system.[6][48][49] Therefore, the lymphatic system plays an important role in the prognosis of a patient. CTCs are a good predictor of the cancer progression in lymph node negative patients, where the lymph nodes appear clear and healthy. Almost 40% of these patients are still CTC positive, providing for an additional means of diagnosis.[50] To transport these CTCs into the lymphatic system, destabilization of the lymphatic wall is necessary. The intrusion of the lymph vessels takes place under the influence of the vascular endothelial growth factor C (VEGF-C), which is produced by the tumor cells. This causes destabilization of the thin, permeable wall of the lymph vessels, which allows the CTCs to diffuse into the vessel. The vascular endothelial growth factor receptor 3 - immunoglobulin (VEGFR-3 Ig) is able to inhibit this destabilization. However, when the CTCs are already in the vessels, VEGFR-3 Ig is no longer effective. Once CTCs reach the lymphatic vessel, they increase the diameter of the vessel wall using VEGF-C and allow cluster CTCs to move through the lymphatic and hematogenous system.[51][52]

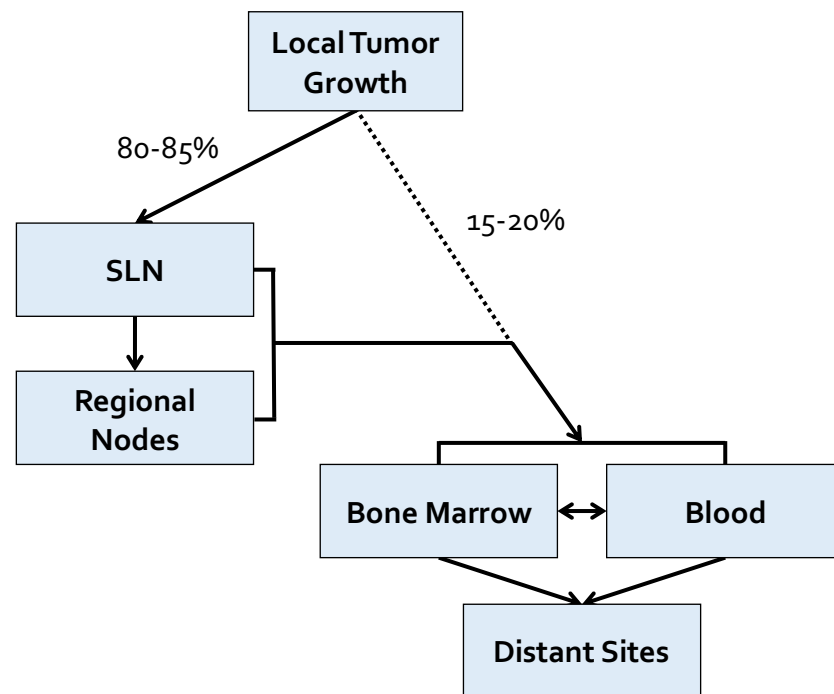


Figure 7: Pathways for CTCs to spread through the body. Occasionally (15%-20% of the time), micro metastatic cancer cells may bypass the lymph nodes and spread to distant sites directly.[48]

Since lymph vessels may not reach the target organs, CTCs have to diffuse from the lymph vessels into the blood. At the end of the lymphatic system, lymph vessels drain into blood vessels via the subclavian veins. From here CTCs can easily disseminate all over the body via the bloodstream and cause metastasis.[53][54][55]

3.6 Prognosis after removing CTCs

CTCs can be of good use as a prognostic marker in patients with cancer. Several studies have shown that the presence of CTCs is connected with a poor prognosis. Since CTCs cause metastasis, their concentration in the blood can be used to classify the progression of the cancer.[56][57] CTC count can also be used in the follow up assessment of cancer treatment or to contribute additional information to imaging. Even the presence of occult metastasis can be indicated by an increased number of CTCs in the blood.[58][59]

Since CTCs are the main cause of metastasis, one would expect that clearance of the blood from CTCs would have a positive influence on the patient's prognosis. However, since no methods to clear the blood from CTCs have been developed yet, this is difficult to prove. A condition for a curative treatment of cancer is that all CTCs should be eliminated, additional to removing the primary tumor. If a few CTCs are left in the circulation, metastasis can still arise.[60][61] Although the impact of eliminating CTCs from the blood on the prognosis should be further assessed, there is a proven correlation between a higher number of CTCs in the blood and a lower survival rate.[62][63] This strengthens the indication that eliminating CTCs from the blood is effective in increasing the overall survival rate.

3.7 Detection and elimination methods of CTCs

CTC detection is a difficult process, mainly due to the low amount of CTCs present. Therefore, adequate detection requires biological properties, chemical properties and physical characteristics to be taken into account when capturing the cells. With this in mind, several methods have been developed for the detection and removal of CTCs in blood. Most of them focus on filtration, sedimentation or immunomagnetic separation.[64][65] Other aspects that makes CTC detection difficult are heterogeneity and the EMT mechanism of CTCs, as was elaborated in chapter 3.4. Heterogeneity of tumor cells is a major problem, because most methods are based on their morphology and immunostaining pattern. As CTCs undergo EMT, they may become undetectable, since the expression of epithelial markers is downregulated.[2]

For CTC detection several methods exist, most of which require blood from the patient to be taken out of the circulation, filtered, and examined. CTCs can easily be removed from the blood by centrifugation or settling. The components of blood are then separated, and CTCs can be found in the white blood cell layer. Thereafter, a size-based filter can divide the cell types, because CTCs are larger than white blood cells. Conventional methods like this make use of physical properties to capture CTCs.[66]

The most recent techniques are based on antibodies against the epithelial cell-adhesion molecule (EpCAM), a protein that only sticks on the cell-surface of a CTC. The CellSearch System uses this EpCAM protein and it is the only Food and Drug Administration approved CTC detection technique. EpCAM antibodies are attached to magnetic beads and then removed from the blood sample using magnetic force.[66] There are a lot of other new methods like antibody-containing, 3D matrices using immunocapture and size-based separation, telomerase activity-based techniques, aptamer technology-based techniques, flow cytometry and dielectrophoretic methods.[2][64][67]

As a non-invasive and in vivo approach Photoacoustic Flow Cytometry (PAFC) can be used. This method uses the different light absorption rates of the cells and the vessel walls to detect the CTCs by sending a laser beam to the blood vessel. Ultrasonic waves with a high intensity will be sent back by the tumor cells and can be detected by an ultrasonic transducer.[68] Although PAFC is a sufficient technique to detect CTCs, PAFC is not able to eliminate them simultaneously.

Also a few CTC removal methods have been developed. Most of them overlap the detection methods and work ex vivo. Only in vivo methods are interesting for this research, so all other existing devices will not be taken into account. The existing in vivo methods that will be discussed are dialysis, the vein indwelling needle and the surface plasmon amplification by stimulated emission of radiation (Spaser). None of these methods are already used in clinical practice.[61][62][68][69]

An in vivo detection method with the opportunity to eliminate the CTCs is the intravenous approach. In this method, the tumor cells attach to EpCAM, which is present at the surface of the intravenous needle. Several methods have been developed that only differ from each other by small variations in the used material. The first intravenous approach, the functionalized structured medical Seldinger guidewire (FSMW) was developed by Saucedo-zeni et al. They used a needle covered with a hydrogel and EpCAM. The tumor cells, which carry EpCAM antigens on their surface, attach themselves to the needle. After 30 minutes the needle is retracted and the collected CTCs can be further investigated.[70] Wang et al. used the same technique with another material, a biocompatible nylon. This needle was coated with carboxybetaine methacrylate and EpCAM, but is based on the same technique as the FSMW.[71]

In 2015, Zhang et al. claimed that these technologies would cause damage and invented the vein indwelling needle, a needle covered with polydimethylsiloxaan, polyvinylalcohol, poly(ethylene glycol) diacrylateaverage,

Mn 575 and anti-EpCAM antibodies. The needle stays in the cephalic or basilic vein for three days and binds the CTCs to the needle with the same mechanism as the other intravenous methods.[69]

All these intravenous methods have advantages over in vitro methods. In comparison to these methods, the intravenous approach can treat a much greater blood volume, the relatively low requirement of sensitivity reduces the threshold of the technology and there is a small false negative ratio. However, the counter part of this technique is the time it takes to have all the blood pass by the needle. Moreover, it is a relatively new approach in which data on collected CTCs is not available yet. It might be still too expensive for clinical use, because of the antibodies needed for this technology.[72] Another important disadvantage is the fact that epithelial markers like EpCAM are downregulated due to tumor heterogeneity.[2] An alternative method to eliminate CTCs for further investigation is dialysis. Dialysis is a filtration method based on cross-flow filtration. Size, shape and deformability play an important role in this filtration method, since CTCs are much bigger than other natural blood cells. The blood cells are filtered through small pores, while the CTCs can be isolated and collected for further research. A thin filter is used to filter a larger amount of blood volume in a short time frame. This device is developed by Viatar CTC Solutions Inc. and can be used if a patient has a concentration of CTCs that is more than 5 CTCs/7,5 mL. However, a great disadvantage of this approach is the repetition of the treatment and the impact it has on a patient. Since this technique is very invasive, a new approach needs to be developed.[71][73]

The first existing method with the actual intention to eliminate the CTCs from the bloodstream without killing healthy cells, is the Spaser, a nanoparticle of about 20 nm in diameter and coated with folic acid. Multiple Spasers will be inserted into the bloodstream, oral or intravenously. Since tumor cells are in possession of a large amount of folate receptors, in contrast to human blood cells, the Spasers bind to the CTCs and are able to kill them. This effect is caused by an external laser, which heats up the Spasers and causes a shockwave that breaks the membrane of the CTCs. This approach is a promising technique for future elimination of CTCs.[61][74]

3.8 Rubefacients

To reach the maximal achievable skin perfusion exploration has been done in vasodilator rubefacients. First there will be an explanation about the drugs chosen for the experimental part. Secondly, the active substances of each rubefacient will be elaborated.

3.8.1 Drug choice

The main goal of the experiment is to find a rubefacient which give a higher perfusion and a longer effect prolongation than Midalgan. Till now, the BMPI group has only used Midalgan for their research on skin perfusion. In the exploration for competitive alternatives, options for different active ingredients proved to be countless.[75][76] Therefore, a structural categorization is necessary.

A rubefacient is an agent that reddens the skin by producing active or passive hyperemia and it is mainly found as a topical analgesic. Topical analgesics (TA) are categorized as either a rubefacient, a non-steroidal anti-inflammatory drug (NSAID) or a miscellaneous group.[76] The rubefacient TA is further divisible into three main subcategories: counter irritants, salicylates and capsaicin.[77]

Midalgan has been examined to make a proper comparison with the other selected rubefacients. Because the original idea of BMPI was based on the use of a capsaicin containing rubefacient and this molecule forms a distinct category, this drug deserves extensive testing. The concentrations 0,025%, 0,075% and 0,1% were chosen. Secondly, Tigerbalm is an over the counter, traditional drug that increases skin perfusion and is therefore an interesting drug to take into account. A pharmacist at Center Pharmacy Enschede was contacted to verify that Tigerbalm would be effective. These four drugs have got different action mechanisms, making it interesting for comparison. 'Tantum' was recommended by B.A. Fischer, because the action mechanisms could be placed between Capsaicin and Midalgan. The action mechanisms of Midalgan, Capsaicin cream with 0,025%, 0,075% and 0,1% capsaicin, Tigerbalm and Tantum are discussed below.

As elaborated below, these four drugs each have their own particular mechanism of action, making it interesting to compare them. Lastly, the BMPI research group suggested specifically to research 'Tantum', which could be placed between Capsaicin and Midalgan in terms of mechanism of action. These mechanisms are discussed in the next chapter.

3.8.2 Action Mechanisms

For artificially enhancing skin perfusion, different mechanisms and drugs are possible, all of which focus on increasing vasodilation of capillaries in the skin. The net vascular tone is a result of the balance between vasoconstrictor and vasodilator factors acting on vascular smooth muscle cells. Mostly, vasodilation depends on endothelial factors, which are local paracrine mediators deriving directly from the vessel wall endothelium. Stimulation of endothelium dependent factors such as NO reduces intracellular calcium content of the VSMC, leading to relaxation of vascular smooth muscle and, therefore, vasodilation.[78] Endothelium independent factors directly influence the VSMC, without interfering with the endothelium in their metabolic pathway. Also, the autonomic nervous system provides factors that may play a role in vasodilation through the effect of catecholamines. However, this vasodilation is based on the alleviation of vasoconstriction and only plays a small role in human physiology.[79] Finally, the efficacy and efficiency of the rubefacient also depend on the carrier to which the rubefacient is added.[80] The used drugs contain different active substances of which an overview can be found in table 3. The different pharmacodynamic properties are discussed below.

Capsaicin

Capsaicin is a alcohol soluble compound produced by the chili pepper and is known to cause temporary erythema, pain and irritation on the skin.[81][82] This erythema hints at increased perfusion. Capsaicin is a selective agonist of the 'transient receptor potential vanilloid 1-receptor' (TRPV₁). The TRPV₁-receptor is a transmembrane receptor-ion channel complex that is normally only activated by temperatures higher than 43 degrees Celsius, pH lower than 6, and endogenous lipids. These receptors are expressed on A-δ and C-fibers that, on stimulation, send depolarization signals to the brain. The impulses are perceived as warming, tingling, itching, stinging, or burning.[83] The TRPV₁-receptor therefore is a nociceptor. Capsaicin binds to this nociceptor more strongly than environmental agonists, causing a loss of response to normal sensory input like pain and itch. This process is the basis for the paradoxical pain-relieving effect of capsaicin. It is called defunctionalization and is thought to be caused by depletion of the neurotransmitters in these capsaicin-sensitive sensory nerves.[83]

More importantly erythema is induced by releasing vasoactive neuropeptides.[84] The cardiovascular system is rich in TRPV₁-receptors that regulate the release of neurotransmitters such as calcitonin gene-related peptide (CGRP) and substance P.[85] CGRP is considered one of the most potent vasodilator agents, playing an important role in both physiological and pathological blood pressure control.[86] Although the antihypertensive effects of capsaicin on the general circulation are intensely investigated in literature, the vasodilator effects on the microcirculation are less established. The mechanisms by which CGRP dilates blood vessels vary in different vascular beds.[87]

Midalgan

Midalgan has two vasoactive components: Methyl-Nicotinate and Glycol Salicylate.

Methyl Nicotinate (MN) is a nicotinic acid methyl ester, used as rubefacient in cosmetics to produce temporary, inflammation-like erythema.[83] Like capsaicin, its pain-relieving effects are based on occupying the sensory nerves, functioning as a so called counter-irritant. During absorption in the dermis, MN is hydrolyzed to nicotinic acid by enzymes. This molecule appears to have acute vasodilator effects by assessing a drop in blood pressure.[88] Nicotinic acid is the metabolite of all nicotine esters, including benzyl nicotinate found in Tantum.[80] It activates the G protein-coupled GPR109A receptors on various cells, such as epidermal Langerhans cells. Langerhans cells also occur in the papillary dermis, particularly around blood vessels.[89] These cells generate prostaglandins such as Prostaglandin D₂ and E₂, which stimulate their receptors on VSMC in dermal arterioles, causing vasodilation. Other research hints at the involvement of prostaglandins as well.[90] However, GPR109A receptors on other cell types might have a hemodynamic effect as well and effects on other receptors cannot be ruled out. Therefore, the exact mechanisms of action of MN remain unclear.[91]

Research suggests that the vasodilation reached using MN topically is comparable to sodium nitroprusside (SNP) and ACh iontophoretically induced vasodilation in diabetic patients.[92] Iontophoresis is a method to electrostatically force a drug into skin tissue, in order to reach a high concentration. The SNP and ACh are, respectively, endothelium independent and dependent vasodilators. Also, iontophoresis causes transcutaneous electrical nerve stimulation, inducing direct axon reflex vasodilation in the skin.[93] These three mechanisms

combined make this method a highly effective one. Therefore, it is remarkable that the topical application MN is able to reach comparable vasodilation, albeit in diabetic patients.

Glycol salicylate (2-hydroxyethyl salicylate) is a benzoate ester formed from the condensation of salicylic acid with ethylene glycol. It is used in TA to relieve mild to moderate muscle and joint pain.[94] Its action mechanism is complex and still debated.[95] Salicylates are NSAID, like aspirin.[94] Therefore, one would expect an anti-inflammatory effect and resulting reduction in perfusion. However, salicylates are difficult to categorize and there is still no common agreement about the mechanisms of action of salicylates.[96] In topical analgesics its role could be binary, functioning as a counter-irritant rubefacient on contact with the skin first, and as a pain relieving NSAID later upon absorption in the skin.[76][94] Another systematic review concludes that current research on salicylates in rubefacients is limited and concrete evidence of effect is lacking.[96]

Tigerbalm Red

The main vasoactive ingredient in Tigerbalm is menthol. Menthol activates thermoreceptors that express the transient receptor potential melastatin 8 (TRPM8) channel. These channels normally open in response to cool temperatures (8-28°C). Information from these cold thermoreceptors is carried via the same A-δ and C fibers as pain information.[97] Therefore, its pain-relieving effects rely on the counter irritant effect of competition with the pain signal, much like capsaicin. Where vasodilation following the capsaicin stimulation of heat receptors is a logical consequence, a vasodilation in reaction to a cold receptor stimulation seems less obvious. It is likely that activation of TRPM8 channels on sensory nerves elicits cool sensations, while vascular TRPM8 receptors mediate vasodilation.[98]

Dependent on the initial level of vascular tone, menthol can act as a vasodilator or vasoconstrictor.[98] The exact actions mechanisms are unknown, but cross talk between various pathways is shown. These include alterations in sensory nerve activation and endothelial factor levels of NO and EDHF for vasodilation and rho-associated protein kinase for vasoconstriction.[99]

Tigerbalm also contains camphor, which works in much the same way. Probably, the sensation of heat that camphor produces on the skin is caused by activation of the ion channels TRPV₃ and TRPV₁, while the cool sensation is due to activation of TRPM8.[100][101][102]

Tantum

Tantum contains capsaicin and benzyl nicotinate as active ingredients. Benzyl nicotinate works in similar fashion to methyl nicotinate.

3.9 Risks of maintaining a high perfusion

The risks of the experiment are twofold. On the one hand the increased perfusion itself might bring risks and on the other hand the increased dose and unorthodox use of medication can play a role. Medication is thoroughly tested before it is allowed to be sold in the Netherlands, hence the documented side effects provide for a reliable overview of risks.

Burns and pain are described for all rubefacients. This effect is enhanced when the medication is applied under moist clothing or directly after bathing or showering. More importantly, any water impermeable layer covering the treated skin can elicit these effects. Hyper sensibility to one of the substances in the medicine can express itself as itch or rash.[103] Application on mucosa is discouraged in general and usage only on intact, healthy skin is advised.

The over-the-counter medications Midalgan, Tantum and Tigerbalm were considered low risk.

Midalgan was tested by Lareb, a laboratory for assessing side effects. They found possible systemic effects of abdominal pain and abdominal cramps, that could be caused by a prostaglandin response. The effects were more intense in people with sensitive skin.[104] For Tantum specifically, only warnings for hypersensitivity are addressed in the patient information leaflet.[105] The risks of the active substance capsaicin are assessed below. The manual for Tigerbalm discourages application on mucosa and irritated skin.[106] No other specific health risks were found.

Capsaicin is the only drug used that needs prescription and the highest concentration in this experiment (0,1%) is not legal medication in the Netherlands. Used as a creme, a burning sensation should last around 20 minutes, followed by erythema of one to two hours. Often seen side effects (1-10%) are burning sensation, pain in extremity. Temporary and local skin reactions on application site are itch, papules, vesiculae, edema and dryness.[84]

Literature does not indicate any risks of maintained enhanced perfusion independent from the causal agent in healthy subjects. Applied on large surfaces, the patient might lose a lot of body heat. However, in combination with active CTCs, a new hypothetical risk could develop. As mentioned in chapter 3.4 CTC attachment, wall friction correlates positively with receptor ligand bonds, potentially increasing the chance of attachment and extravasation. If this process indeed facilitates metastasis, this can be seen as a major drawback and risk of consequently increasing microcirculation perfusion, as in the skin.

Lastly, a dermatologist was asked, Dr. P.M. Ossenkoppele, for advice on assessing the risks of the drugs used and no insurmountable obstacles were addressed. Conclusively, it was considered safe to continue the experiment, using ourselves as test subjects.

4. Method

In this chapter the method of this research will be discussed. The design of this research, the participants, the time and place, instruments, procedures and data processing will be clarified.

4.1 Design

Functioning as a 'proof of concept' study for further research on a possible transdermal CTC treatment, this experiment aims at finding the most effective rubefacient as stated in the main question and in sub-questions 2.1, 2.3, 2.4 and 2.5. The conclusions and recommendations of all sub-questions of this explorative study can be used in future research on skin perfusion and skin perfusion measurements. The rubefacients are the independent variables of this research, because they will be responsible for the change in the dependent variable, 'perfusion of the skin'. The second independent variable is the reapplication of the rubefacient and influences the dependent variable 'perfusion of the skin' as well. A correctly timed reapplication will increase the absolute perfusion by prolongation of the enhanced perfusion. A conceptual model is shown below in figure 8.

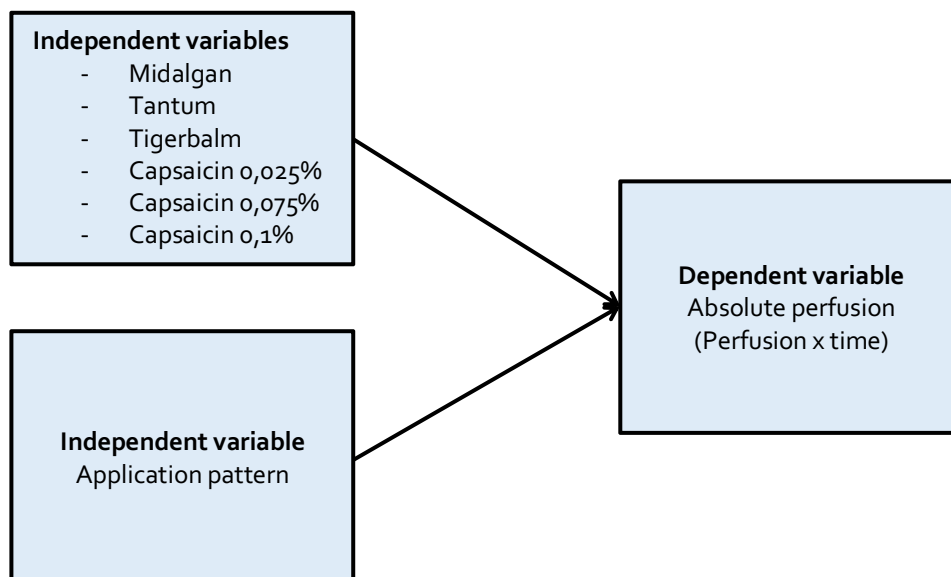


Figure 8: Conceptual model of cause and effect in the variables.

Because the BMPI research group is interested in infrared measurements to provide extra data points for their research, a few infrared measurements have been done. They are compared with the perfusion measurements in chapter 5.2. In this way the relation between temperature change and perfusion can be examined.

4.2 Subjects

By limitations of the Medical Research Human Subjects Act (WMO), it is not allowed to use more than two subjects without accordance of the METC. The test subjects used in these experiments were two healthy volunteers from the University of Twente, of which details can be found in table 2.

	Test subject 1	Test subject 2
Age	21	23
Gender	Female	Male
Origin	Caucasian	Caucasian
Medication	Methotrexate 1/w, Folic acid 6/w, Adalimumab 0.5/w, Pred forte eye drops 7/w, Ethinylestradiol levonorgestrel mylan hormonal contraceptive pill 7/w.	Antihistaminic Cetirizine on indication
Allergies	-	Hay fever

Table 2: Information about test subjects.

4.3 Materials and environment

The experiments took place in the laser lab of the BMPI research group, during the months May and June of 2018. The temperature and humidity fluctuated between respectively 22,5 - 24,7 °C and 39 - 66%.

To measure the perfusion of the skin, the PeriFlux system 5000, Perimed AB, was coupled with laser Doppler probes. These probes were attached to the skin of the volar forearm during regular measurements and on the upper back during the back measurement. The probes used were one '407 Small Straight' probe, three probes '408 Large Straight' and one probe '457 Thermostatic Small Angled'. Two PeriFlux systems were used during the experiments, one used Probe 407 and Probe 457, the other PeriFlux systems used three Probes 408. The probes were fixed with Elastomull® haft 6cm x 20 m stretched. The infrared measurements were performed with the infrared camera, FLIR C2.

The used rubefacients are Capsaicin cream with 0,025%, 0,075% and 0,1% capsaicin concentration, Midalgan, Tantum and Tigerbalm, which can be found in table 3. More in depth details of these drugs are addressed in the literature research. After each measurement the probes were cleaned with ethanol. During the measurements of the perfusion in the arm, the arm was fixed in a VacuForm pillow. During the measurements of the back, the subjects sat upright in a massage chair.

Rubefacient	Active ingredient	Active ingredient
Capsaicin cream 0,025%	Capsaicin	
Capsaicin cream 0,075%	Capsaicin	
Capsaicin cream 0,1%	Capsaicin	
Midalgan Xtra warm	Methyl-nicotinate	Glycol-salicylate
Tantum warmte extra warm	Capsaicin	Benzyl-nicotinate
Tigerbalm Red	Menthol	

Table 3: Used rubefacients and their active ingredients.

4.4 Procedure

This thesis exists of two parts, a literature study and an experimental part, both described in the following paragraphs.

4.4.1 Literature

To answer the sub-questions about CTCs a literature research was done. The articles used are found in Scopus, PubMed, Google Scholar and Google. Different search terms related to circulating tumor cells and perfusion of the skin have been used. Articles were sorted on relevance or date (newest). Articles were only used when written in English or in Dutch but may be published all over the world.

4.4.2 Experiment

The experimental part of this research exists of three measurement protocols. Primarily, two subjects have been measured at the same time, one subject with three large straight probes and one subject with the thermostatic small angled probe and small straight probe. To receive a valid result, the skin had to be clean and the subject

was not allowed to use any body lotion or other rubefacient on the day of the measurement. All the performed measurements are shown in table 4.

Measurement	Test subject	Arm	Amount
BM	1	Left	0 mL
BM	2	Right	0 mL
M1Mi	1	Right	1 mL
M1.1Mi	2	Left	1 mL
M1.2Mi	2	Right	0,5 mL
M2.1Mi	1	Left	2 x 0,5 mL
M2.2Mi	1	Left	2 x 0,5 mL
M2.1Mi	2	Left	2 x 0,5 mL
M2.2Mi	2	Left	2 x 0,5 mL
M1TR	1	Left	0,5 mL
M1.1TR	2	Left	0,5 mL
M1.2TR	2	Right	0,5 mL
M1TAN	1	Left	0,5 mL
M1.1TAN	2	Left	0,5 mL
M1.2TAN	2	Right	0,5 mL
M2TAN	1	Right	2 x 0,5 mL
M2TAN	2	Left	2 x 0,5 mL
M3TAN	1	Left	0,5 mL
M1Cao.025%	1	Left	0,5 mL
M2Cao.025%	2	Left	2 x 0,5 mL
M1Cao.075%	1	Right	0,5 mL
M1Cao.075%	2	Left	1 mL
M2Cao.075%	1	Left	2 x 1 mL
M2Cao.075%	2	Left	2 x 1 mL
M1Cao.1%	1	Right	1 mL
M1Cao.1%	2	Right	1 mL

Table 4: All performed measurements with each rubefacient. Code: M=measurement, first digit = first, second or third measurement (M1,M2,M3 respectively), second digit (optional) = repetition of an experiment. Rubefacient used: Mi = Midalgan, TR = Tigerbalm, TAN = Tantum, Ca[x,xx%] = Capsaicin [with its concentrations].

Measurement 1 (single application)

This measurement was performed on the volar forearm. An example of the setup can be seen in figure 11. The probes were cleaned with ethanol and were fixed to the arm using the elastic bandage Elastomull®. First, a baseline measurement of five minutes was performed, after which the rubefacient was applied. The measurement was stopped after the perfusion had almost returned to baseline level. During some experiments an infrared camera measured the temperature of the skin. The infrared recording was paused while applying the rubefacient to not disturb the measurement. A timeline of this measurements can be seen in figure 9.

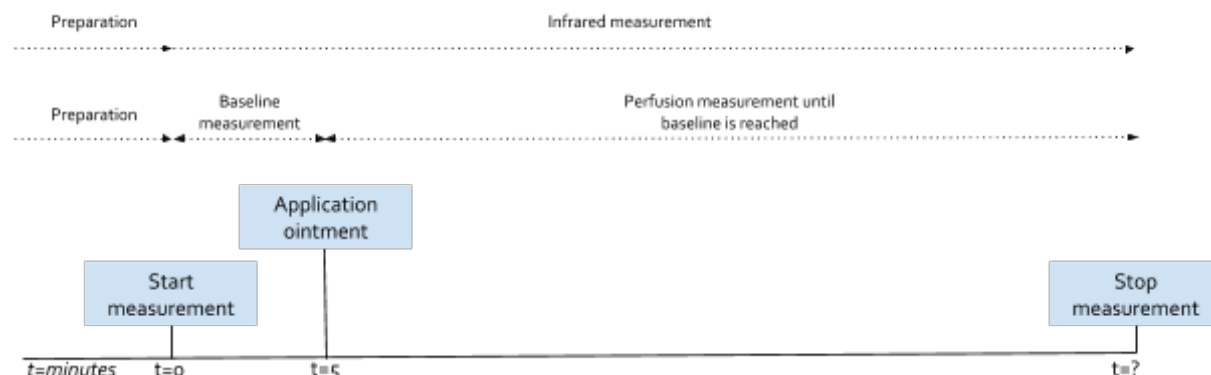
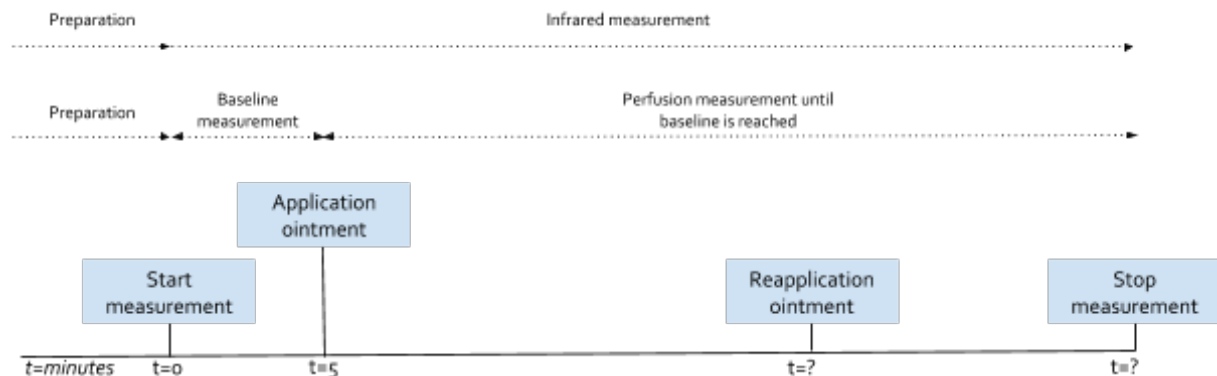


Figure 9: The protocol timeline of measurement 1 and 3.

Measurement 2 (reapplication)

Measurement 2 was also carried out on the volar forearm and starts with a baseline measurement of five minutes, after which the rubefacient was applied. The results of measurement 1 were used to determine the optimal moment for reapplication for each rubefacient. The measurement was stopped after the baseline was reached after reapplication. During the experiment an infrared camera measured the temperature of the skin. The infrared record was paused while applying and reapplying the rubefacient. A timeline of this measurement can be seen in figure 10.



Measurement 3

Measurement three was only performed with Tantum on the back of the test subject. Two probes were placed between the scapulae and one halfway on the back, as seen in figure 11. The probes were cleaned with ethanol and were fixed to the skin with Elastomull®. First, a baseline measurement of five minutes was performed, after which the rubefacient was applied. During the measurement an infrared camera measured the temperature of the skin. B.A. Fischer performed an additional procedure in this measurement, in which two metal bars were pressed on the skin for ten seconds. Thence, the warming of the skin can be assessed in future research. The record was on pause while applying the rubefacient. The timeline of this measurement is similar to that of measurement 1 and can be found in figure 9.

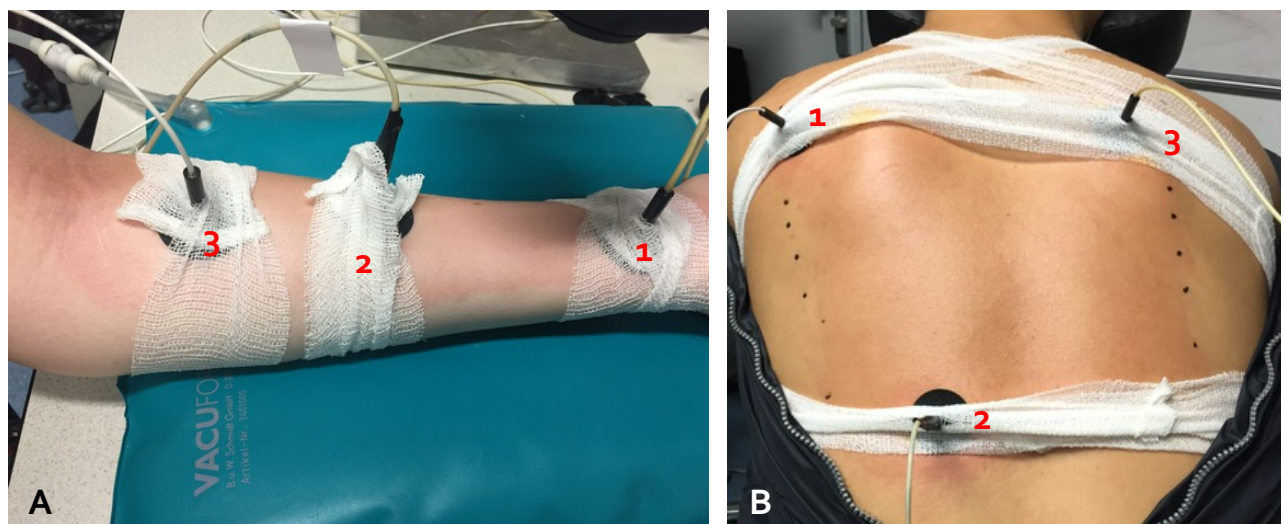


Figure 11: Set up of the measurement at the (A) volar forearm and the (B) back.

4.4.3 METC

The BMPI research group wants to perform a study to fit their COMSOL model with different test subjects. To carry out this study, approval from the METC Twente is needed for which an official request needs to be written. To gain insight in the requirements for an METC request, C.D. Lammertink, MSc. was contacted. The required

documents were identified with online research and a checklist was made using the website of the Central Committee on Research Involving Human Subjects (CCMO) and the METC Twente.[107][108]

4.5 Data processing

The *.csv data were first exported from Perisoft to Microsoft® Excel, after which they were analyzed and filtered in MATLAB. The infrared data were analyzed in FLIR tools+, exported to Excel and also filtered in MATLAB. The used MATLAB scripts can be found in Appendix I.

As there were high levels of noise present, a moving average filter was used. This filter is a low pass Finite Impulse Response filter. The filter takes N samples from the input signal and calculates the average, thereby forming a single data point. All the calculated average values create a filtered signal. A larger window size gives a smoother output signal and makes a sharp peak blunter.[109]

Several frequencies were present in the signal and a moving average filter with one window size is not able to smooth all of these frequencies. As seen in the Fourier spectral analyses in figure 56, 57, 58 and 59 in Appendix IV, there was no consistent pattern of noise frequencies. Figure 12 shows a representative power spectrum on which the filter method was based.

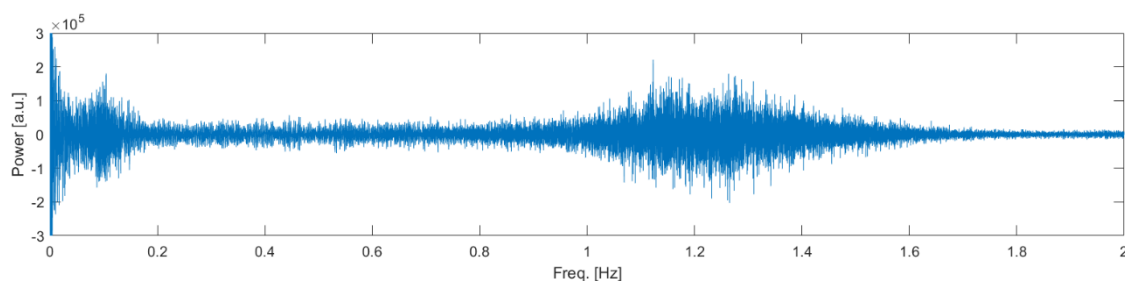


Figure 12: A representative spectrum example taken from Tantum measurement 1 test subject 1.

A cloud of frequencies was found around 1-1,4 Hz in several measurements. This would translate into 60-84 beats per minute, which likely is the heartbeat. Moreover, in some measurements noticeable peaks were seen just below 0,1 Hz, mostly around 0,08 Hz. With a sample frequency of 32,25 Hz, one wave of this frequency would translate into a period of approximately 400 samples. There is no physiological process to explain these frequencies, however Bernardi et al. described this 10-second rhythm. This rhythm could be dependent on sympathetic control, and of respiration-related, high-frequency fluctuations, due to the transmission of mechanical chest activity.[110]

As seen in Appendix IV, some signals showed a less expressive distribution of these frequencies. Finding a suitable window size is difficult in signals with an irregular frequency distribution. To eliminate the impact of the 0,1 Hz frequency, a moving average filter with a window size of $N = 400$ was used in the data processing, because this is the length of samples in one period. The average of one period in a wave equals the equilibrium position. Also, higher frequencies are averaged in this way, resulting in a low pass filter effect.[109] Lower, physiological frequencies, in which the actual perfusion information is concealed, are not affected with this filter. Hence, this method approaches the desired representation of the signal.

5. Results

In this chapter, the results of the experiments and of the METC research are presented.

5.1 Perfusion measurements

Baseline measurements have been performed to see the perfusion under normal conditions and if there is any influence from e.g. the probes. These results can be found in Appendix II, figure 31.

The raw data from all the measurements can be found in Appendix II. Next to this raw data the average signal of the two probes can be found.

These average signals have been used to compare the different rubefacients. For this purpose, a moving average filter has been used. In every measurement, first a baseline of five minutes can be seen. Thereafter, some artefacts will show up, this is when the rubefacient was applied. After the application, the graph shows the progress of the perfusion as measured.

5.1.1 Single application of various rubefacients

In this section the single application of the rubefacients is discussed for each test subject. In figure 13 and 14 the perfusion of Midalgan, Tigerbalm and Tantum is compared, in figure 15 and 16 the perfusion of Capsaicin 0,025%, 0,075% and 0,1% is compared for each subject.

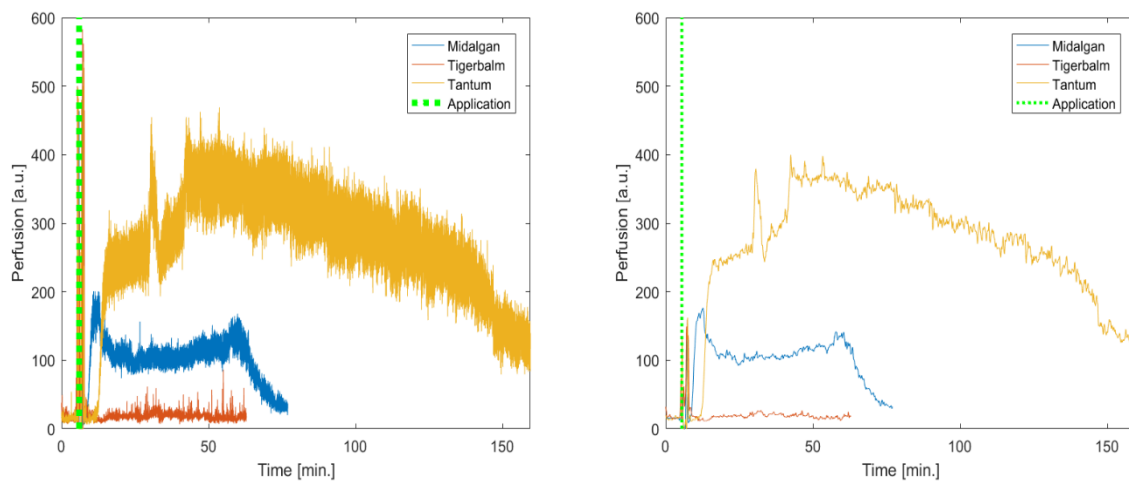


Figure 13: Average signal (l) and moving average filter (r) of Midalgan, Tigerbalm and Tantum from test subject 1.

As seen in figure 13, Tigerbalm does not cause any visible increase of perfusion. Midalgan induces an increased perfusion for approximately one hour. Tantum causes a way higher perfusion then Midalgan and increases the perfusion for a longer amount of time.

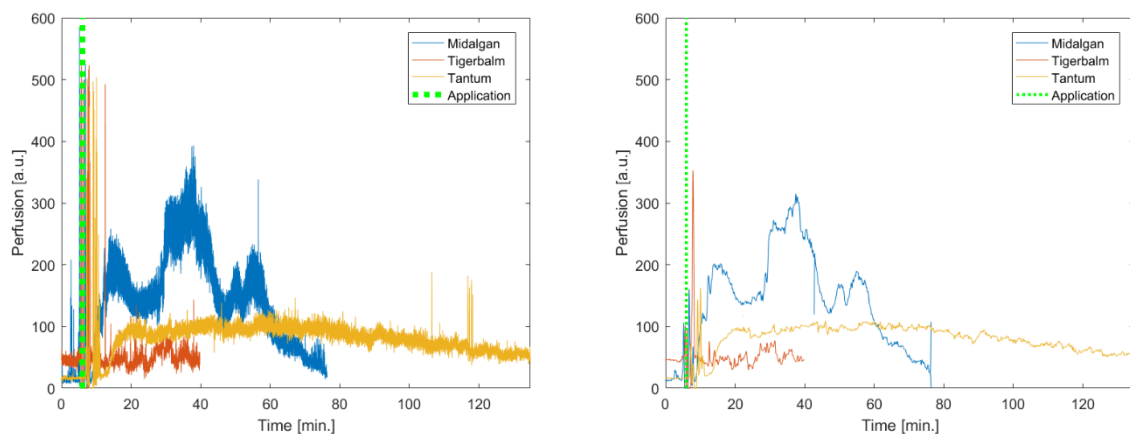


Figure 14: Average signal (l) and moving average filter (r) of Midalgan, Tigerbalm and Tantum from test subject 2.

As shown in figure 14, Tigerbalm does cause a minor increase of perfusion. However, the other drugs cause a larger perfusion. The perfusion caused by Midalgan is the highest, but the increased perfusion caused by Tantum lasts longer.

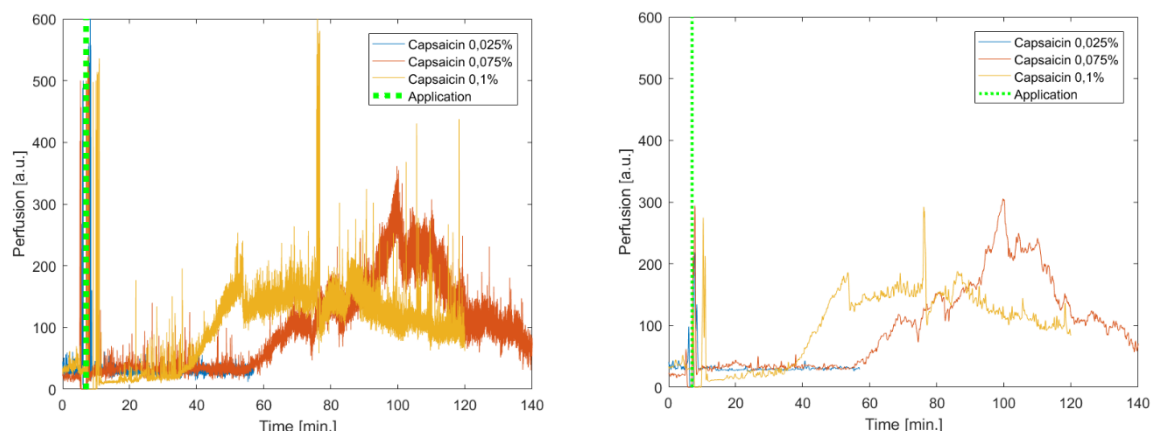


Figure 15: Average signal (l) and moving average filter (r) of Capsaicin 0,025%, 0,075% and 0,1% from test subject 1.

Figure 15 shows that Capsaicin 0,025% has a very little effect. Both Capsaicin 0,075% and Capsaicin 0,1% have an effect, since a high perfusion was found. However, it takes a long time for Capsaicin 0,075% and 0,1% to start increasing the perfusion.

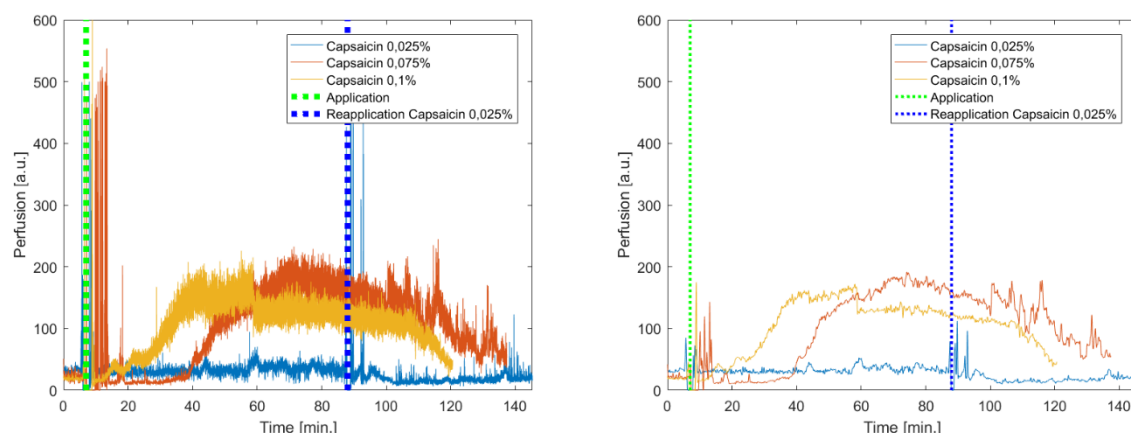


Figure 16: Average signal (l) and moving average filter (r) of Capsaicin 0,025%, 0,075% and 0,1% from test subject 2.

Capsaicin 0,025%, as seen in figure 16, has a little to no effect on the perfusion and was therefore directly reapplied during the first measurement. Capsaicin 0,075% and 0,1% have a similar effect and both cause an increased perfusion for a longer amount of time. However, Capsaicin 0,1% shows an earlier effect than Capsaicin 0,075%.

5.1.2 Reapplication of potential rubefacients

Midalgan, Tantum and Capsaicin 0,075% have been applied for a second time to see if the increased perfusion could be endured.



Figure 17: Average signal (above) and moving average filter (below) of reapplication of Midalgan, Tantum and Capsaicin 0,075% from test subject 1.

Figure 17 shows that both Midalgan and Tantum have been reapplied after 50 minutes. The perfusion rises again after reapplying Tantum, and therefore stays active for an increased amount of time. The increased perfusion caused by Midalgan rises a bit after reapplying the rubefacient but decreases fast after that. Capsaicin 0,075% has been reapplied after 100 minutes, an effect of this can be seen. However, this means that the increased perfusion persists longer, but it is still not high compared to the other rubefacients.

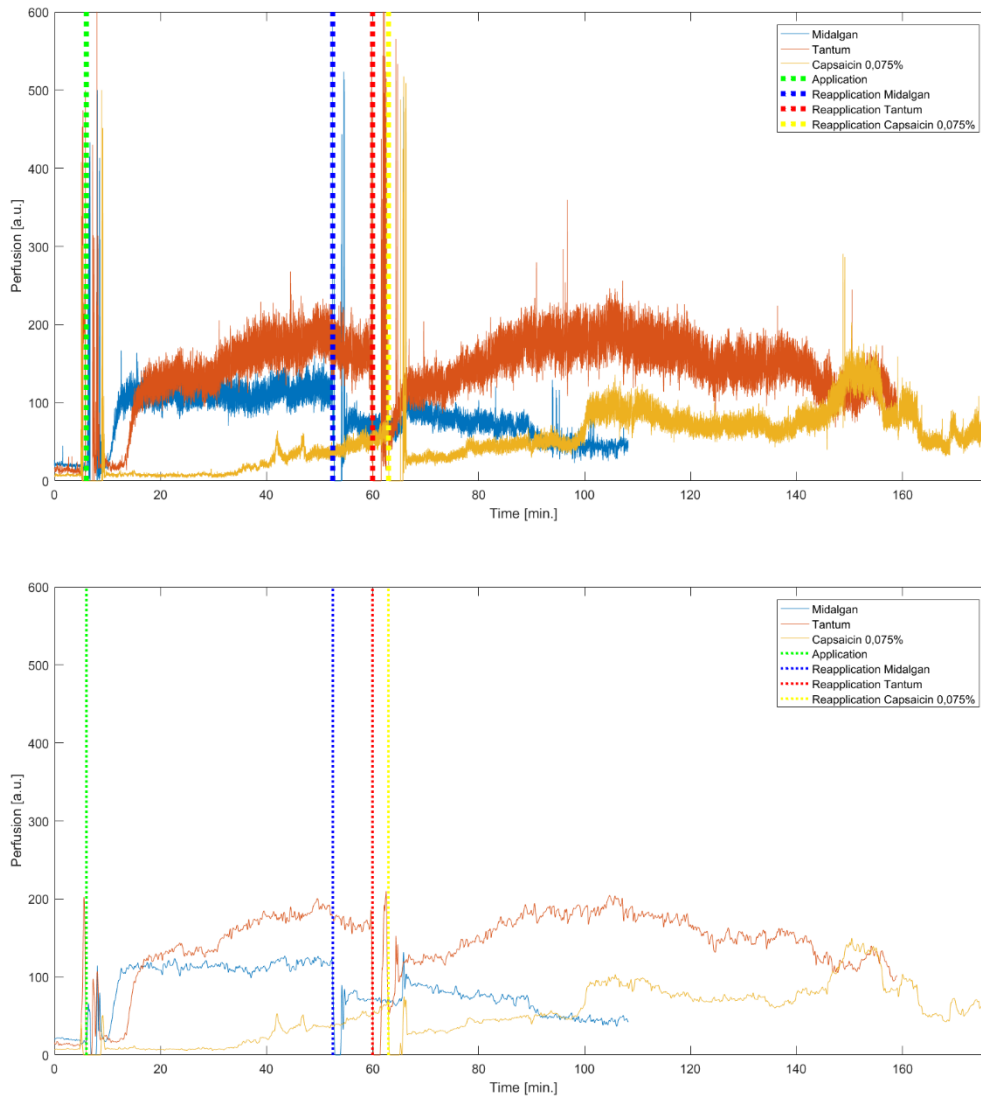


Figure 18: Average signal (above) and moving average filter (below) of reapplication of Midalgan, Tantum and Capsaicin 0,075% from test subject 2.

All rubefacients have been reapplied after 50 to 60 minutes, as seen in figure 18. After reapplying Midalgan, the perfusion decreases fast. After reapplying Tantum, the perfusion increases for a second time and this upsurge is maintained for a long time. The reapplication of Capsaicin 0,075% also has an increasing effect which lasts for a long amount of time. However, the gained overall perfusion is lower than the perfusion achieved by Tantum.

5.1.3 Back measurement

For this measurement the most promising rubefacient was used, which was Tantum. This measurement was performed on the back of the subject. Due to its large surface and superficial blood circulation, the back is an appropriate place to measure the perfusion. An overview of the probes on the back is given in figure 11B.

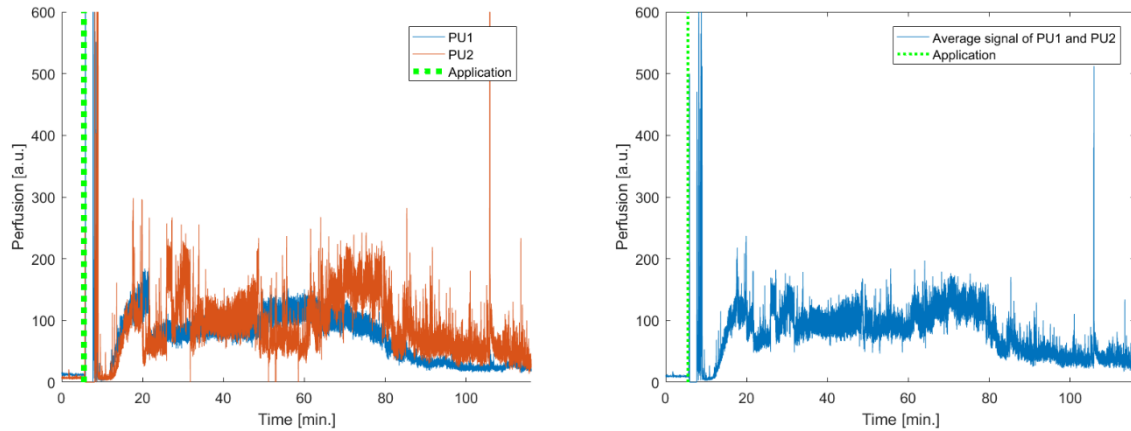


Figure 19: Back measurement test subject 1.

In figure 19, the two different probes both show approximately the same amount of increased perfusion. The raise in perfusion starts quite fast and lasts long. The amount of perfusion increase is lower than seen in the previous experiments performed on the lower arm.

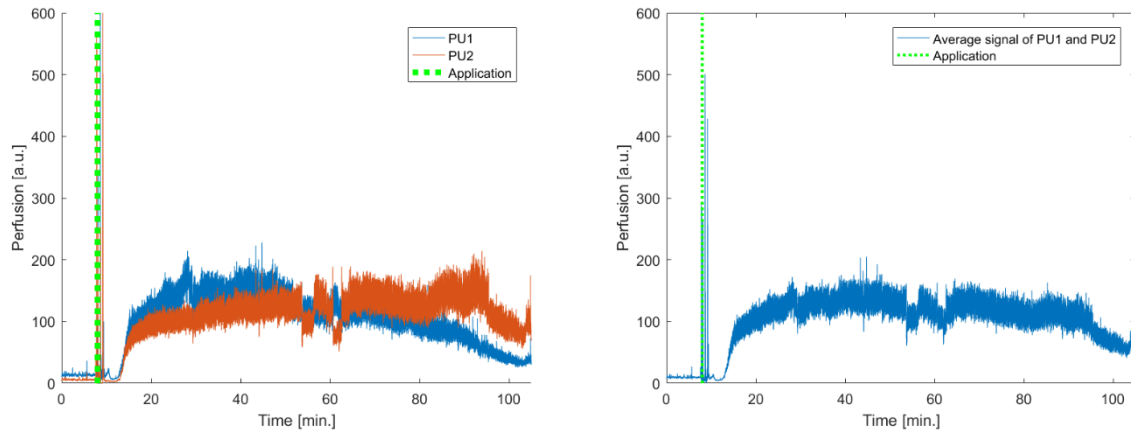


Figure 20: Back measurement test subject 2.

The application of Tatum on the back of test subject 2 as presented in figure 20, shows about the same results as in the other test subject. The perfusion is increased for around 80 till 90 minutes and is quite stable.

Baseline corrected measurements

To give a proper comparison of the different rubefacients, the perfusion in arbitrary units as measured by the Perimed have been corrected using the corresponding baseline. This means that for each measurement the data is divided by its baseline. This means that the baseline itself is 1, and the increase in perfusion can be seen on the y-axis as a factor of this baseline, as can be seen in figure 21 and 22.

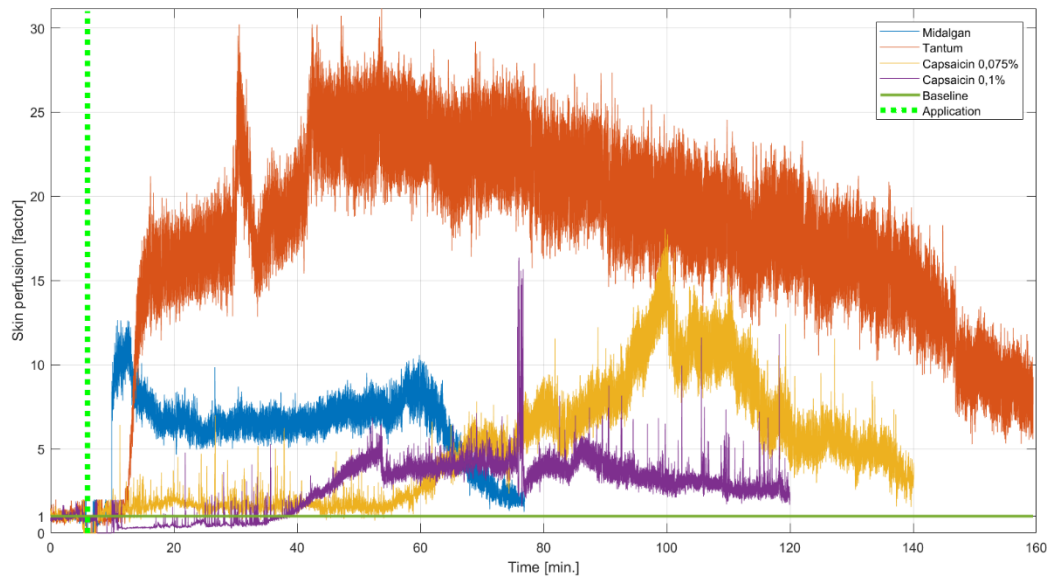


Figure 21: Baseline corrected comparison of Midalgan, Tantum, Capsaicin 0,075% and 0,1% from test subject 1.

Tantum causes a high increase of perfusion in comparison to the other rubefaciants as seen in figure 21. This increase also lasts for the longest amount of time. Midalgan and Capsaicin 0,075% cause a similar increase. However, Midalgan has a fast effect and ends early while Capsaicin 0,075% only starts increasing the perfusion after an hour. Capsaicin 0,1% stays active for a long time but does not increase the perfusion with the same factor as the other rubefaciants.

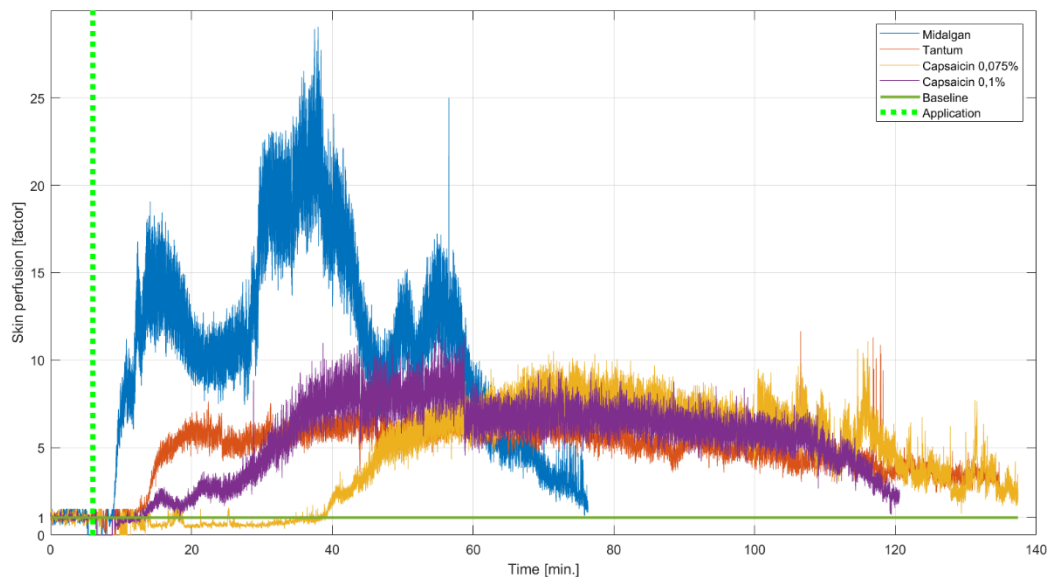


Figure 22: Baseline corrected comparison of Midalgan, Tantum, Capsaicin 0,075% and 0,1% from test subject 2.

As seen in figure 22, Midalgan increases the perfusion with a much higher factor than the other rubefaciants. However, its activated period is quite short, especially in comparison to the other rubefaciants. Tantum, Capsaicin 0,075% and Capsaicin 0,1% all have a similar effect. They increase the perfusion with a factor of approximately 7,5 and this increase lasts for about 100 minutes.

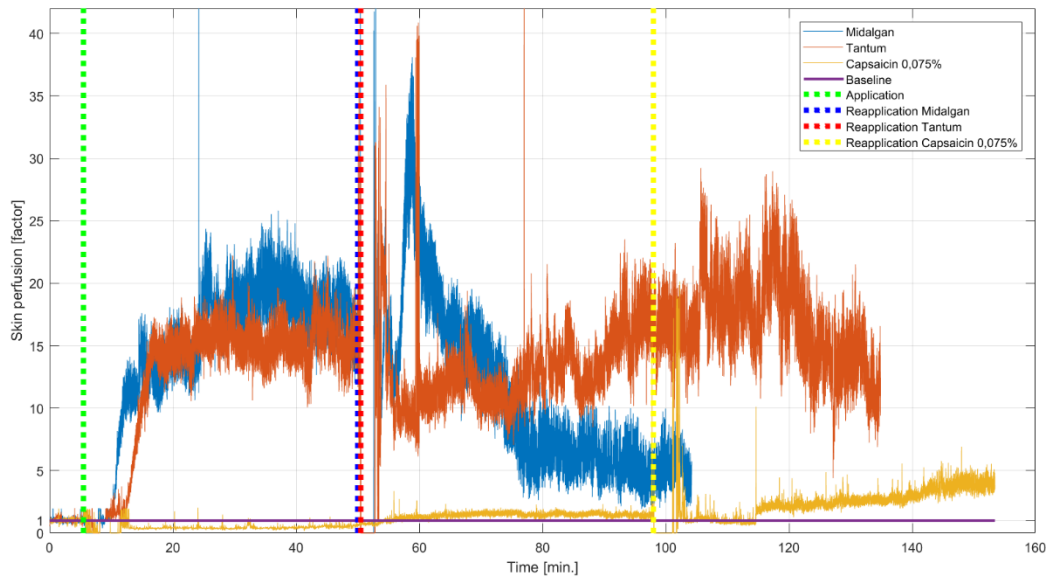


Figure 23: Baseline corrected comparison of reapplication of Midalgan, Tantum and Capsaicin 0,075% from test subject 1.

In figure 23, the reapplication measurements are shown. The increasing factor caused by Capsaicin 0,075% is much lower than other rubefacients. Tantum increases the perfusion for the longest amount of time and has overall the highest amount of increase.

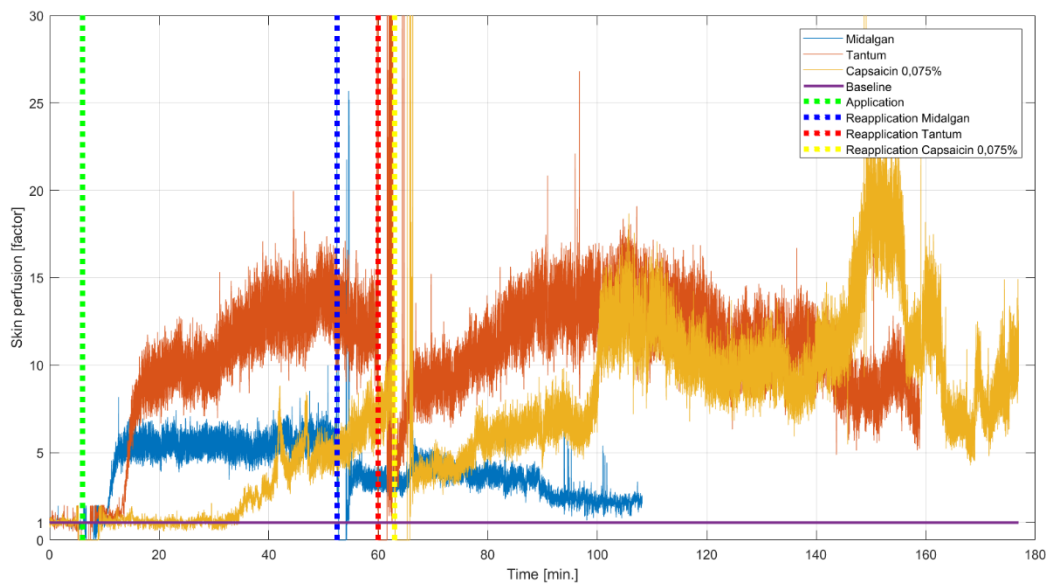


Figure 24: Baseline corrected comparison of reapplication of Midalgan, Tantum and Capsaicin 0,075% from test subject 2.

The least effect is caused by Midalgan, as can be seen in figure 24. Tantum and Capsaicin 0,075% last approximately for the same amount of time and reach the same factor of increase. However, the effect caused by Tantum starts earlier so this causes in the end the most increase of perfusion.

5.2 Infrared measurements

During the reapplication measurements of the most promising drugs, being Capsaicin 0,075%, Midalgan and Tantum, infrared measurements have been performed. The results of this measurement will be discussed and compared with the average signal of the corresponding perfusion measurements below.

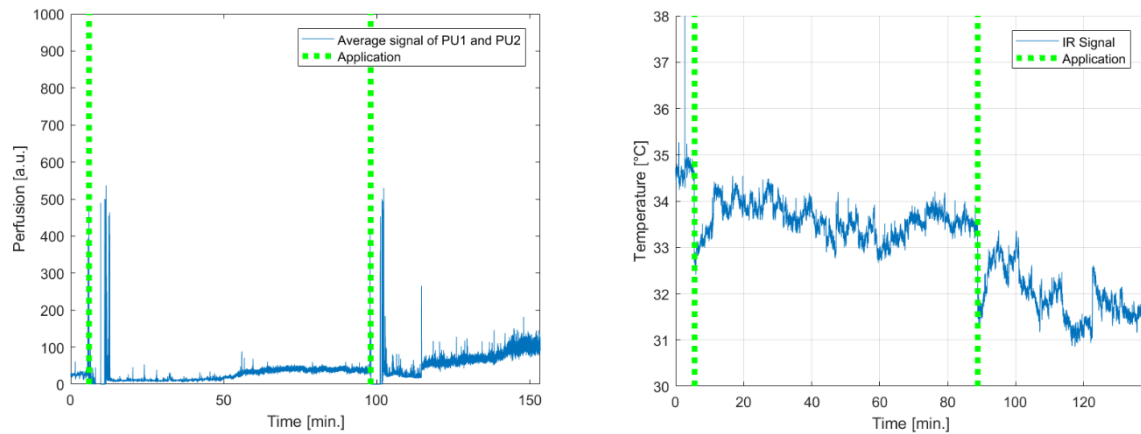


Figure 25: Infrared measurement and perfusion measurement of Capsaicin 0,075% from test subject 1.

As seen in figure 25, the temperature of the skin is at baseline between 34°C and 35°C. After application of the Capsaicin cream 0,075%, the temperature falls to 33°C and thereafter increases immediately to 34°C. Until the reapplication of the Capsaicin, after 90 minutes, the temperature stays stable. Meanwhile, the perfusion of the skin increases 45 minutes after application of the rubefacient and has not been decreased at the reapplication. After reapplication first the perfusion falls, but gradually increases again. The temperature falls, increases and then gradually decreases to 31°C.

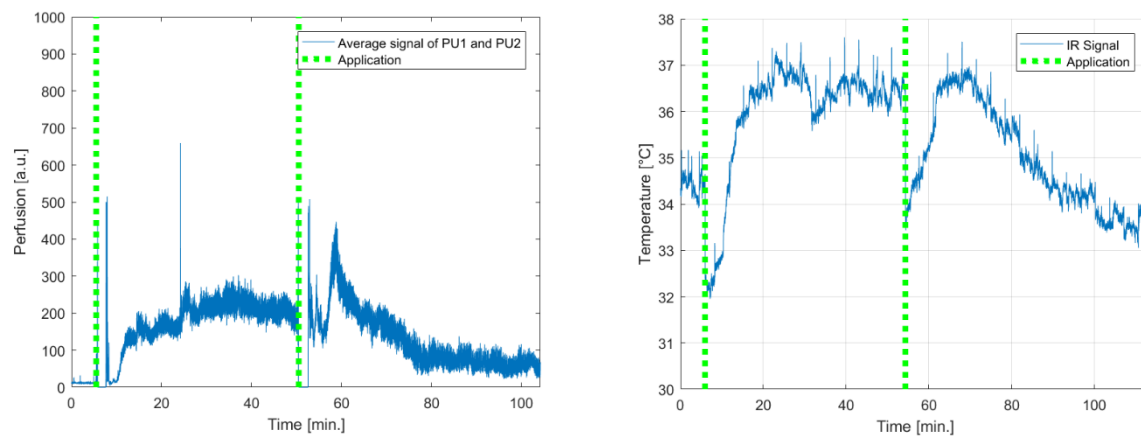


Figure 26: Infrared measurement and perfusion measurement of Midalgan from test subject 1.

As presented in figure 26, the baseline of the measurement is between 34°C and 35°C. The temperature falls to 32°C after application of Midalgan. In 20 minutes the temperature increases to 37°C and fluctuates between 36°C and 37°C until reapplication. At the same time, the perfusion increases immediately after application of the Midalgan and remains stable. After reapplication there is seen an overshoot in the perfusion, but gradually decreases. 95 minutes after the first application, the perfusion is still increased. The temperature falls to 34°C after reapplication and immediately increases to 37°C again. After this maximum of 37°C, the temperature gradually decreases.

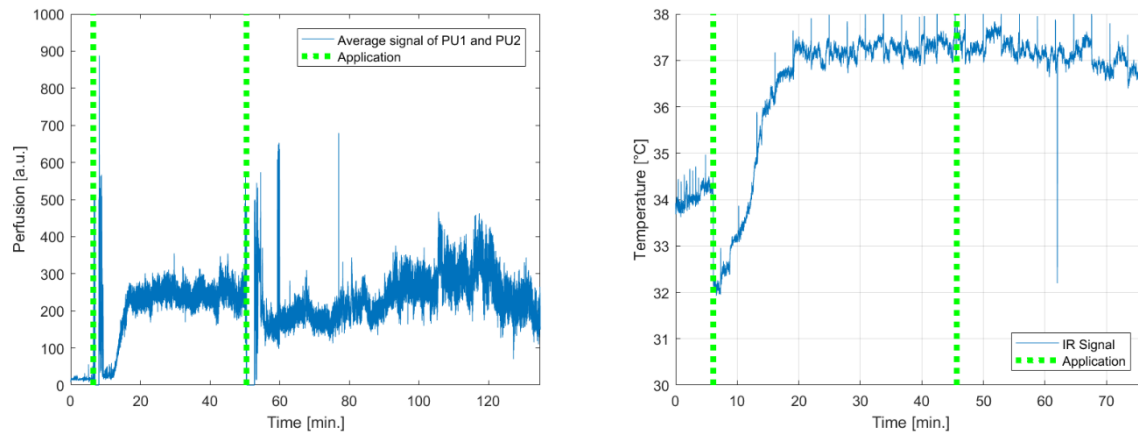


Figure 27: Infrared measurement and perfusion measurement of Tatum from test subject 1.

As shown in figure 27, after application the temperature decreases with approximately two degrees and thereafter increases to about 37°C. Until the end of the measurement, the temperature remains 37°C. The perfusion increases 5 minutes after application and has not been decreased at reapplication. After reapplication the perfusion remains increased.

5.3 METC

After a research, all the documents needed for the approval from the METC Twente were found and collected in a Google Drive document. An overview of this documents can be found in Appendix V. A lot of test subjects are needed to fit the COMSOL model made by the BMPI research group. The intended experiment takes approximately two hours and requires participants to use a medical rubefacient. Besides that, the participants will undergo a cooling procedure to examine the heating process. The impact on the required test subjects of these factors combined result in the need for an approved METC request. To receive the approval, the documents assessed in Appendix V need to be filled in and handed to the METC Twente. This process will not be performed by this research group but will be outsourced.

6. Discussion

The discussion contains three chapters. First the interpretation of the results will be discussed, second the weaknesses of the experiment and finally the METC request.

6.1 Interpretation of results

The interpretation of results is subdivided into a description of the deviations from the original plan, an assessment of the actual results and an evaluation of the infrared data.

Description of deviations from the plan

One of the used PeriFlux devices has three flowmetry gates. However, during the experiments data gained from perfusion unit (PU) 3 gave improbable and later impossible results. An example of this can be found in Appendix III, figure 52. Other researchers in the BMPI group experienced similar deviation. Therefore, PU 3 was considered rogue and it was decided not to use any data gained from PU 3 in the results. Raw data of this gate can be requested at the researchers.

In the results PU 1 and PU 2 were averaged and taken as one result. This is a correct representation of the perfusion, since both probes were attached to the same arm under the same circumstances in each measurement. Therefore, they represent the same measurement and can be processed accordingly. However, as seen in the raw data in Appendix II, the outcomes of the different gates can differ substantially. This effect has not been fully explained. The possibility of superficial veins influencing the measurement to this degree seems improbable but should be further investigated.

The reapplication measurement of Midalgan has been performed twice on both test subjects. During the first experiment the reapplication had a different effect on the perfusion of the two test subjects. In test subject 2, no revival of perfusion was seen after reapplication, whereas test subject 1 did show renewed response. See Appendix II, figure 43 and 44. This results in ambiguous outcome and this measurement has therefore been repeated to get a better idea of the real general effect of reapplying Midalgan. Since it was not clear how this lack of perfusion revival can be explained, and these effects did not repeat consequently, the consistent data from the second attempt were used in the final comparing results.

Another measurement (Midalgan measurement 2 test subject 2) had to be aborted and repeated, because the baseline measurement showed a steady increase in perfusion, instead of the required flat line. This was later explained as the effect of leftover rubefacient from the previous measurement on the probe, due to bad cleaning of the probe (see Appendix III, figure 55). This indicates the necessity of thorough cleaning of the probe with ethanol.

In chapter 3.9 risks of maintaining a high perfusion as found in the literature were described. Self-reported complaints indeed include pain and burning sensation, but no severe side effects were experienced. Capsaicin 0,1% caused the most pain. Therefore, these measurements were not continued after the first experiment, due to too much discomfort in test subjects.

Tigerbalm measurement 1 in subject 2 was repeated after improbably high perfusion results were acquired in only one test subject and no perfusion increase in the other. A leftover effect from Midalgan or the interactive effect with Midalgan from earlier that day was presumed and the measurement was repeated on test subject 2. See Appendix III, figure 53.

The infrared results have a somewhat misleading time axis compared to the perfusion measurements, as the measurements were paused during application moments. The perfusion measurements were not. This should be taken into account when comparing both graphs.

Results addressing the research question

For the interpretation of results, the best overview is given in figure 21, 22, 23 and 24. The first interesting finding is that Tantum shows a much higher increase in perfusion than Midalgan in test subject 1, see figure 21. Tantum also lasts longer in test subject 1. However, in test subject 2 a less distinct difference is seen in figure 22.

Although Tantum lasts longer, Midalgan clearly reaches higher maxima for a substantial amount of time. Tantum single application is effective for 140 minutes and most measurements stop there. This includes the reapplication measurements. One example, however, shows Tantum being at full effectiveness after 160 minutes in a reapplication test. This is an indication that reapplication can be an effective method of enhancing the duration of effect of this rubefacient. In Midalgan it is easily seen that the second application reboosts the effect, thereby increasing the duration of effect approximately from 60 to 100 minutes, see figure 21 and 22 in comparison to 23 and 24.

In a normal application measurement in figure 22 and in a reapplication measurement in figure 23, a strange result is seen for Capsaicin 0,075%. Directly after the first application, the perfusion is constantly lower than baseline and seems stable. Only after approximately 40 minutes, some increase in perfusion is seen. This could be caused by some unknown physiological reflex, but a measurement barrier induced by the rubefacient is not excludable. This idea is supported by the fact that reapplication in general (figure 23 and 24) often induces a slight drop in perfusion as well. This reapplication moment can be recognized by the spikes around 50 minutes. The possibility of a wrongly assessed baseline should be taken into account, in which the apparent baseline after application is the actual baseline. This offset in baseline makes Capsaicin 0,075% to appear as a weaker vasodilator than in reality, although probably not to a relevant degree. More relevant, yet unexplained, is the aspect of ambiguous outcome between test subjects in the measurements of this drug. In the reapplication experiment of test subject 1 only after 110 minutes, which is after the second application, some effect was seen. This does not match with test subject 2, in which effect starts after 30 minutes. More test subjects are needed to provide for more reliable outcomes.

The range of perfusion signal is expressed as a rapidly fluctuating wave in the plot. Figure 28 zooms in at this wave, where 7-8 periods are visible in a timeframe of 6 seconds, or a frequency of about 1,2 Hz or 70 beats per minute. Most likely this is the heartbeat. Interestingly, this range increases with an increase in perfusion over time. This could indicate that the heartbeat has a greater effect on perfusion in bigger or more filled vessels.

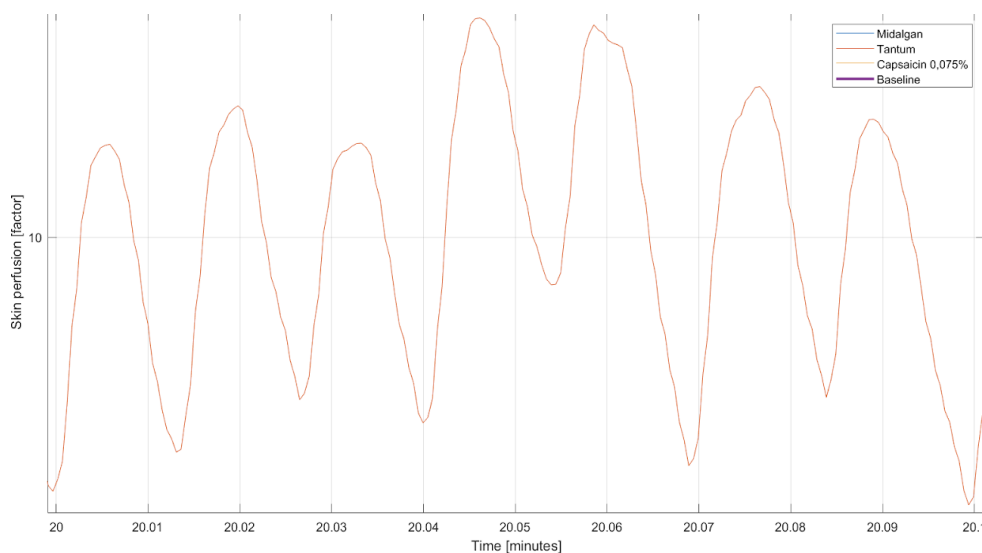


Figure 28: Supposed heartbeat frequency in Tantum measurement 2 from test subject 2.

IR measurement data and perfusion

The infrared assessment was not an anticipated part of this research, which is why not every perfusion measurement is accompanied by an IR measurement. However, it is interesting to analyze these measurements for any remarkable results. For example, a rise in perfusion concomitant with a drop in temperature would indicate a gap in the current knowledge on this physiology. As written in chapter 5.2, only the IR-measurements of the three contending rubefacients are discussed, since the temperature effects of these drugs might be relevant in future research by the BMPI research institute.

Unexpected results were obtained for capsaicin 0,075%, where a decrease of temperature after reapplication was seen for 50 minutes simultaneous with an increase in skin perfusion. This is important knowledge for the BMPI research group, since its objective is to correlate perfusion and temperature in a predictive model.

However, the corresponding perfusion measurement is somewhat unreliable, since PU1 and PU2 show divergent values. Another unexpected result for capsaicin 0,075% was to find a consistent lower temperature after reapplication in the second measurement, whereas temperature remained steady for at least 140 minutes in the first experiment.

For all rubefacients a decrease in temperature is recorded immediately after application moments. This is probably due to the energy absorbing effects of the cold, water containing rubefacient. However, the reflective layer of rubefacient could affect the measurement by blocking infrared radiation from the skin, hindering it to reach the infrared camera.

6.2 Weaknesses of the experiment

During the experiment there were expected weaknesses and unexpected weaknesses. They will be discussed in this chapter.

Expected weaknesses:

First off, the experiment was explorative, so different approaches and techniques were discovered during the process. Also, only two test subjects were included in this experiment and no placebo applicant was used. Therefore, statistical power and degree of hard evidence are low. Secondly, upon initiating the experiment, many confounders were known. These confounders can cause effect in one test subject and absence of effect in the other, making it difficult to draw conclusions. As touched upon in the literature research, confounding differences include gender, physical condition and skin type. Given the WMO legislation, only two test subjects were allowed which had to be chosen from the group of researchers. Therefore, some variation in physical conditions of these subjects was unavoidable. Both test subjects use medication (see table 2). Since test subject 1 uses an oral contraceptive, this might influence the perfusion of the skin, since hormones in the menstrual cycle could affect outcome. Subject 2 uses an antihistamine which inhibits an allergic inflammatory reaction and might also affect the controlled inflammatory reaction in the skin. It has not been proven in literature that those medication affect the perfusion of the skin. However, healthy subjects without medication would be preferred in future research.

As an important general point of interest, the arbitrary units in which the PeriFlux measures, only represent the actual physical perfusion to some degree. In chapter 3.3 is explained that perfusion itself influences measurement depth, and 100% linearity can therefore not be expected from these arbitrary units. However, according to various professionals, linearity is assumed, since a closer, substantiated approximation to physical reality is considered not possible for now.

Moreover, an expected weakness can be seen in the graphs of the experiment. Movement artefacts are visible in the results of the experiment, since the probes were sensitive for any movement. The artefacts can be seen as high peaks at the moment of application, reapplication and unexpected moments like sneezes.

Unexpected weaknesses:

It was found that the probe probably influences the effectivity of the rubefacient. The water permeability of a covering layer is important in this process, indicating a factor of sweating or evaporation to be in play. As shown in figure 29 the probes left a red circle, meaning an increased perfusion at the location of the probe holders. This process was isolated by replacing the probes after half an hour to check if the effects of Tantum would subside faster compared to matched measurement without drug. The results of this effect are shown in Appendix III, figure 54. This inaccuracy was difficult to avert, but it is important to take into account that actual skin perfusion of the entire forearm may be lower than the recorded perfusion underneath the probes.



Figure 29: Redness under the plastic probe holders.

For reasons explained in chapter 4.5 Data Processing, a window size of 400 was chosen. However, a window size of 1200 would filter the signal more smoothly, seemingly without loss of relevant information. An example of this is shown in figure 30. The power spectrum did not show any remarkable lower frequencies justifying the use of a larger window size. Therefore, the filter characteristics remain a point of discussion.

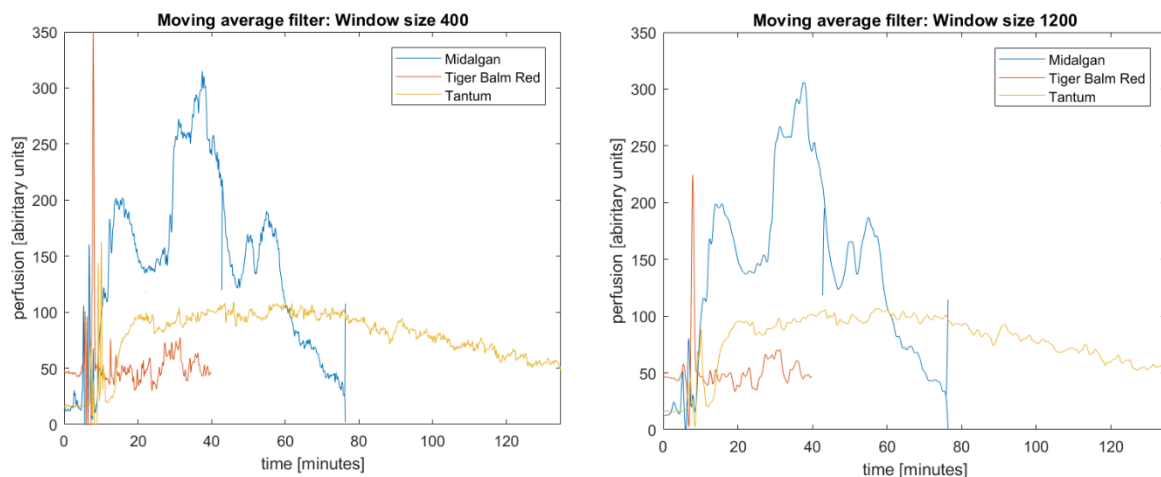


Figure 30: Example of increased window size of 1200. Measurement 1 test subject 1.

In this research rubefacients are analyzed for their efficacy in increasing the perfusion of the skin. For that reason, the influence of other perfusion increasing methods was left out of consideration. These methods include heating the skin with an infrared heat lamp, massaging or scrubbing the skin or pretreatment of the skin with alcohol. Especially for the alcohol solvable molecule capsaicin alcohol pretreatment could enhance the effects. These possible factors were left for another research to investigate.

Literature did not show any indication of physiological mechanisms losing sensitivity after consequent application of rubefacients. However, this effect cannot be excluded, and the possibility should be considered that intense testing for several days in a row can wear out the bodily response.

6.3 METC

A modest element of this thesis was to investigate the role of an METC application in future research of the BMPI group. The unorthodox use of (prescription) medication is a method that has some limitations in research. This experiment was limited by the legal maximum of two test subjects and future BMPI research will likely be restrained in a similar fashion. The procedure of an METC request contains different aspects. The most important contribution to the METC request from BMPI is this proof of concept study in its entirety. It can be used to write the protocol in the application of the final research. The use of prescription medication drastically increases the degree of required METC approval needed. Therefore, the use of an over the counter medication is recommended. However, an METC approval is still needed for further research according to the reasons mentioned in chapter 5.3. Formulating an METC request requires detailed knowledge about the intended research of the BMPI group. A protocol needs to be written in which all aspects of the experiment are assessed. Great amounts of literature discussed in this thesis can be used as support for this protocol. Lastly, the experiment part of this thesis can provide groundwork for the chosen rubefacients.

7. Conclusion

In response to the main question 'How can the therapeutic window in a transdermal treatment against CTCs be optimized, in terms of CTC behavior perfusion limits and timeframe?', Tantum shows the highest perfusion increase of a factor 10 to 25. Skin perfusion in a 20x20cm patch will therefore increase from resting perfusion to a perfusion that allows all 5,5 liters of blood to pass by in 40 to 100 minutes. This creates a workable time frame for a possible treatment.

The way CTC behavior affects the therapeutic window is a complex process. From the literature research can be concluded that the presence of CTCs in the lymphatic system cannot be ignored in a novel treatment method, since this system plays a main part in the pathway of metastasis. Secondly, the penetration depth of the therapy is paramount to the therapeutic window given the vessel plexuses. Furthermore, CTCs can enter the smallest capillaries and therefore reach the entire microcirculation. Finally, clusters of CTCs might need a different therapeutic approach than single cells, because of their size and higher risk of metastasis compared to single cells.

8. Recommendations

In this chapter, some possibilities for further research will be discussed. Based on the results and the discussion there are five interesting topics. Firstly, other substances that may give a higher perfusion can be tested. Secondly, alternative perfusion enhancement methods than rubefacients may give a higher perfusion and could therefore be tested. Thirdly, the place on the body which gives the highest absolute perfusion can be tested. As a fourth, more research on the safety of enhancing microcirculation regarding CTC attachment is required. Finally, further research on new detection methods may be necessary for the future treatment. These recommended topics can be further researched by other students or researchers from the BMPI group.

Other rubefacients

There might be other substances than Tatum which give a higher perfusion or are able to retain the perfusion for a longer period. Some substances had to be left out of consideration in this research because of the limited amount of time, for example benzydamine. Also, a combination of two rubefacients could be promising. For example, first applying Midalgan and then Tigerbalm gives an increased perfusion in comparison with only applying Tigerbalm. This was found by accident during this research and therefore its process has not been fully explained, see Appendix III, figure 53. Finally, the reason why Tatum proved to be so effective can be further investigated by researching the isolated effect of one of its active components, benzyl-nicotinate.

Different perfusion enhancement methods

Besides rubefacients, there are also other methods which might give a perfusion enhancement. For example, the use of an infrared light or a hot water bath will obtain a higher perfusion for a longer period. In this research the use of an infrared light was not possible, because of the use of an infrared camera during the measurements. Possibly, those devices would affect each other.

The place of the measurement

The regular measurements in this research were performed at the volar forearm and the last measurements were performed on the back of the test subjects. Due to its large and easy to reach surface and superficial blood flow, the forearm is an effective location to measure the skin perfusion. However, in future research the skin of the buttocks or scapula may be used, since this skin has a better circulation and therefore might give a higher perfusion.

The effect of an enhanced microcirculation regarding CTC attachment

An important issue is the effect of an enhanced microcirculation itself on the attachment of CTCs to the vascular wall. When an increased microcirculation would facilitate attachment, this would have a crucial impact on the effectiveness of the treatment. The attachment of CTCs is greater in smaller blood vessels and should therefore be a hypothetical danger for an increased amount of extravasation during an enhanced blood flow in the microcirculation. However, during an increased perfusion the blood flow rises which lowers the chance of CTC attachment. Currently, the risks are still unknown, making further research required.

A new detection method

As discussed earlier in this thesis, CTCs play an important role in current investigation. A lot of new detection and elimination methods have been developed in the past years and many will be developed in the future. As described in chapter 3.7 the Spaser is one of the newest approaches in eliminating CTCs and has great potential. It is therefore important for the final treatment of CTCs that this technique will be further evaluated.

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 - [109] Moving Average Filter (MA filter) – GaussianWaves 2010. <https://www.gaussianwaves.com/2010/11/moving-average-filter-ma-filter-2/> (accessed June 18, 2018).
 - [110] Bernardi L, Rossi M, Leuzzi S, Mevio E, Fornasari G, Calciati A, et al. Reduction of 0.1 Hz microcirculatory fluctuations as evidence of sympathetic dysfunction in insulin-dependent diabetes. *Cardiovasc Res* 1997;34:185–91. doi:10.1016/S0008-6363(97)00017-5.
 - [111] WMO-plichtig onderzoek | METC Twente n.d.

Appendix

There are five appendices. Appendix I contains the MATLAB scripts, Appendix II shows the raw data and the average signals gained from the MATLAB script. Appendix III contains the failed measurements. Appendix IV shows the Fourier spectral analysis of measurement 1 and Appendix V gives a short overview of the required METC documents.

Appendix I: MATLAB Scripts

Measurement 1 and 2

```
%% Script for measurement 1 and 2
```

```
Date: _____
```

```
Measurement: _____
```

```
Ointment: _____
```

```
Test Subject: _____
```

```
clear all
```

```
close all
```

```
% Enter data
```

```
data = xlsread('file-name.xlsx',2);
```

```
% Time - Seconds to minutes
```

```
seconds = data(:,3);
```

```
minutes = seconds/60;
```

```
% Plot data vs time
```

```
figure (1)
```

```
hold on
```

```
plot (minutes,data(:,1),minutes,data(:,2));
```

```
legend('PU1','PU2')
```

```
title('Title of the graph');xlabel('Time [min.]'); ylabel('Perfusion  
[a.u.]')
```

```
axis([0 inf 0 inf])
```

```
% plot average signal vs time
```

```
x = minutes;
```

```
y1 = data(:,1);
```

```
y2 = data(:,2);
```

```
yn = ((y1+y2)/2);
```

```
plot(x,yn)
```

```
legend('Average signal of PU1 and PU2')
```

```
title('title of the graph');xlabel('Time [min.]'); ylabel('Perfusion  
[a.u.]')
```

```
axis([0 inf 0 inf])
```

Average signal and moving average filter

```
%% script for average signal and moving average filter for all six
rubefaciens
% test person: _____
% measurement: _____
clear all
close all

% Enter data Midalgan, Tigerbalm and Tantum
data1 = xlsread('file-name-midalgan.xlsx',2);
data2 = xlsread('File-name-Tigerbalm.xlsx',2);
data3 = xlsread('File-name-Tantum.2.xlsx',2);

% Enter data Midalgan
seconds1 = data1(:,4);
minutes1 = seconds1/60;
x1 = minutes1;
y11 = data1(:,1);
y21 = data1(:,2);
yn1 = ((y11+y21)/2);

% Enter data Tigerbalm
seconds2 = data2(:,3);
minutes2 = seconds2/60;
x2 = minutes2;
y12 = data2(:,1);
y22 = data2(:,2);
yn2 = ((y12+y22)/2);

% Enter data Tantum
seconds3 = data3(:,3);
minutes3 = seconds3/60;
x3 = minutes3;
y13 = data3(:,1);
y23 = data3(:,2);
yn3 = ((y13+y23)/2);

% plot average signal for Midalgan, Tigerbalm and Tantum vs time in one
graph
plot(x1,yn1)
hold on
plot(x2,yn2)
hold on
plot(x3,yn3)
legend('Midalgan','Tigerbalm','Tantum')
title('AS - Midalgan, Tigerbalm, Tantum - test subject _ '); xlabel('Time
[min.]'); ylabel('Perfusion [a.u.]')
axis([0 inf 0 inf])

% Mark application moments
l = line([_ _], [0 600]); % Define application moments
l.LineStyle = ':';
t = l.Color;
l.Color = 'g';
u = l.LineWidth;
l.LineWidth = 4;
legend('Midalgan','Tigerbalm','Tantum','Application')

% Moving average filter
```

```

windowSize = 400;
b = (1/windowSize)*ones(1,windowSize);
a = 1;

yn1(isnan(yn1))=0; % the perflux produces it owns data, expressed as NaN,
this is set to zero for lack of alternatives.

N1 = filtfilt(b,a,yn1);
N2 = filtfilt(b,a,yn2);
N3 = filtfilt(b,a,yn3);

% plot moving average signal for Midalgan, Tigerbalm and Tantum vs time
plot(x1,N1)
hold on
plot(x2,N2)
hold on
plot(x3,N3)
legend('Midalgan','Tigerbalm','Tantum')
title('MAF - Midalgan, Tigerbalm, Tantum - test subject _ '); xlabel('Time
[min.]'); ylabel('Perfusion [a.u.]')
axis([0 inf 0 inf])

% Mark application moments
l = line([_ _], [0 600]); % Define application moments
l.LineStyle = ':';
t = l.Color;
l.Color = 'g';
u = l.LineWidth;
l.LineWidth = 2;
legend('Midalgan','Tigerbalm','Tantum','Application')

% Enter data capsaicin 0,025%, 0,075% and 0,1%
data4 = xlsread('file-name-capsaicin 0,025%',2);
data5 = xlsread('file-name-capsaicin 0,075%',2);
data6 = xlsread('file-name-capsaicin 0,1%',2);

% Enter data capsaicin 0,025%
seconds4 = data4(:,4);
minutes4 = seconds4/60;
x4 = minutes4;
y14 = data4(:,1);
y24 = data4(:,2);
yn4 = ((y14+y24)/2);

% Enter data capsaicin 0,075%
seconds5 = data5(:,4);
minutes5 = seconds5/60;
x5 = minutes5;
y15 = data5(:,1);
y25 = data5(:,2);
yn5 = ((y15+y25)/2);

% Enter data capsaicin 0,1%
seconds6 = data6(:,4);
minutes6 = seconds6/60;
x6 = minutes6;
y16 = data6(:,1);
y26 = data6(:,2);
yn6 = ((y16+y26)/2);

```

```

% plot average signal for Capsicum 0,025%, 0,075% and 0,1% vs time in one
graph
plot (x4,yn4)
hold on
plot(x5,yn5)
hold on
plot(x6,yn6)
legend('Capsaicin 0,025%', 'Capsaicin 0,075%', 'Capsaicin 0,1%')
title('AS - Capsaicin 0,025%, 0,075% and 0,1% - test subject _ ');
xlabel('Time [min.]'); ylabel('Perfusion [a.u.]')
axis([0 inf 0 400])

% Mark application moments
l = line([_ _], [0 600]);
l.LineStyle = ':';
t = l.Color;
l.Color = 'g';
u = l.LineWidth;
l.LineWidth = 4;
legend('Capsaicin 0,025%', 'Capsaicin 0,075%', 'Capsaicin
0,1%', 'Application')

% values for the moving average filter
N4 = filtfilt(b,a,yn4);
N5 = filtfilt(b,a,yn5);
N6 = filtfilt(b,a,yn6);

% plot moving average signal for Capsaicin 0,025%, 0,075% and 0,1% vs time
yn4(isnan(yn4))=0;
yn5(isnan(yn5))=0;
yn6(isnan(yn6))=0;

plot(x4,N4)
hold on
plot(x5,N5)
hold on
plot(x6,N6)
title('MAF - test subject _ - Capsaicin 0,025%, 0,075% and
0,1%');xlabel('Time [min.]'); ylabel('Perfusion [a.u.]')
legend('Capsaicin 0,025%', 'Capsaicin 0,075%', 'Capsaicin 0,1%')
axis([0 inf 0 inf])

% Mark application moments
l = line([_ _], [0 600]);
l.LineStyle = ':';
t = l.Color;
l.Color = 'g';
u = l.LineWidth;
l.LineWidth = 2;
legend('Capsaicin 0,025%', 'Capsaicin 0,075%', 'Capsaicin
0,1%', 'Application')

```

Normalized baseline

%% script for normalized baseline (after running script 2, run script 3)

% test person: _____

% measurement: _____

samplefreq = 32,25;

duurbaseline = 5*samplefreq*60;

% baseline corrected for Midalgan, Tantum, Capsaicin 0,075% and 0,1%

yn1(isnan(yn1))=0;

yn3(isnan(yn3))=0;

yn5(isnan(yn5))=0;

yn6(isnan(yn6))=0;

% yn1 Midalgan

yaveragebegin1 = median(yn1(1:duurbaseline));

figure(1)

normy1 = yn1/yaveragebegin1;

filterpuntD = 60*samplefreq*9; %start measurement after application

drempelwaardeD = 1.5

for n = 1:filterpuntD;

 if (normy1(n,1) > drempelwaardeD);

 normy1(n,1) = 1;

 end

end

plot(x1,normy1)

hold on

% yn3 Tantum

yaveragebegin3 = median(yn3(1:duurbaseline));

normy3 = yn3/yaveragebegin3;

filterpuntD = 60*samplefreq*12;

drempelwaardeD = 1.5

for n = 1:filterpuntD;

 if (normy3(n,1) > drempelwaardeD);

 normy3(n,1) = 1;

 end

end

plot(x3,normy3)

hold on

% yn5 Capsaicin 0,075%

yaveragebegin5 = mean(yn5(1:duurbaseline));

normy5 = yn5/yaveragebegin5;

filterpuntD = 60*samplefreq*19;

drempelwaardeD = 1.5

for n = 1:filterpuntD;

 if (normy5(n,1) > drempelwaardeD);

 normy5(n,1) = 1;

 end

end

plot(x5,normy5)

hold on

% yn6 Capsaicin 0.1%

yaveragebegin6 = mean(yn6(1:duurbaseline));

figure(1)

```

normy6 = yn6/yaveragebegin6;
filterpuntD = 60*samplefreq*11;
drempelwaardeD = 1.5
for n = 1:filterpuntD;
    if (normy6(n,1) > drempelwaardeD);
        normy6(n,1) = 1;
    end
end

plot(x6,normy6)
hold on

plot(x5,1*ones(size(x5)), 'LineWidth', 2);

grid
title('Baseline corrected comparison measurement _ - test subject _')
xlabel('Time [min.]')
ylabel('Skin perfusion [factor]')
ylim([0,inf])
set(gca, 'YTick', sort([1, get(gca, 'YTick')]));
legend('Midalgan', 'Tantum', 'Capsaicin 0,075%', 'Capsaicin 0,1%', 'Baseline')

```

Infra-Red Data

%% Script for the infrared data

Date:_____

Measurement:_____

Ointment:_____

Test Subject:_____

clear all

close all

% Enter data

data = xlsread('filename-compleet.xlsx',2);

seconds = data(:,1);

minutes = seconds/60;

x1 = minutes;

figure (1)

grid

hold on

plot (minutes,data(:,2));

title('title of the graph');xlabel('Time [min.]'); ylabel('Temperature [°C]')

axis([0 inf 0 inf])

% mark application moments

l = line([__ __], [0 50]) % define application moments with data cursor

s = l.LineStyle;

l.LineStyle = ':';

t = l.Color;

l.Color = 'g';

u = l.LineWidth;

l.LineWidth = 4;

Appendix II: Raw data

Baseline measurements

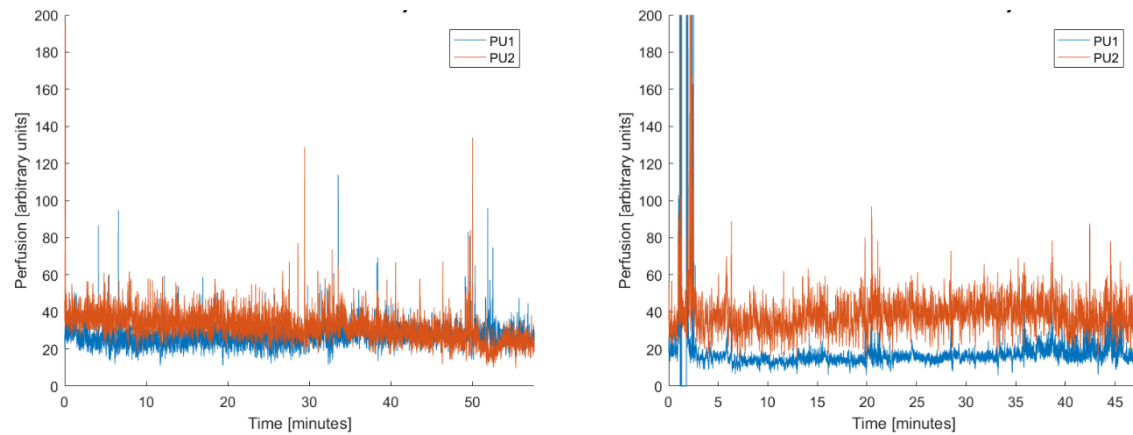


Figure 31: Baseline measurements from test subject 1 (l) and 2 (r)

Raw data

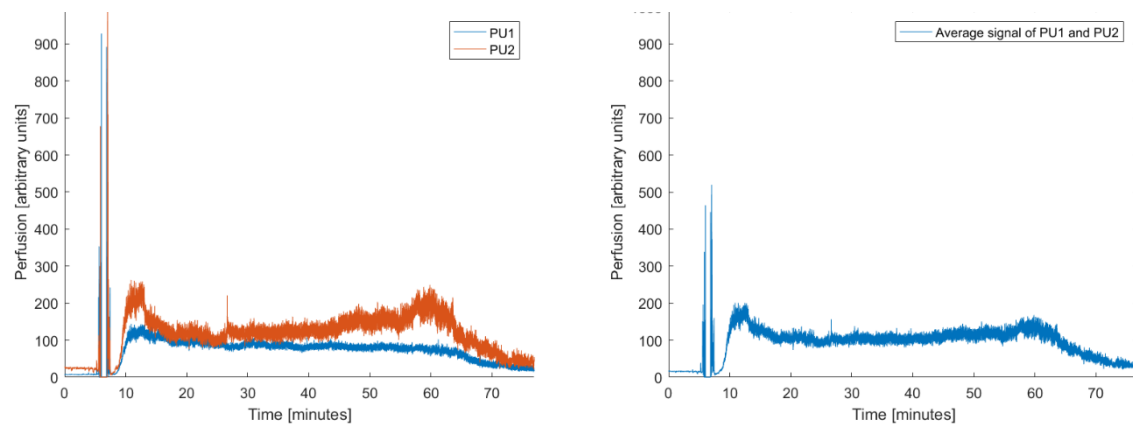


Figure 32: Application of Midalgan from test subject 1

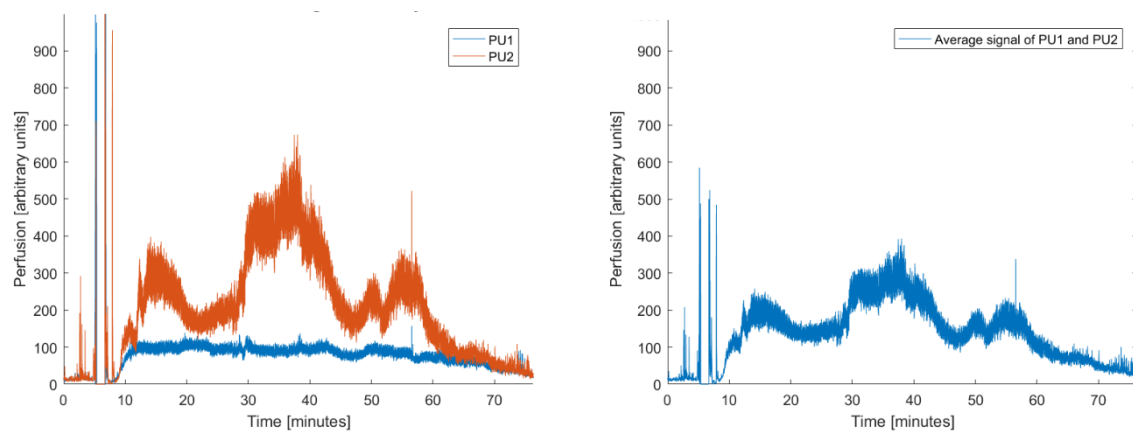


Figure 33: Application of Midalgan from test subject 2

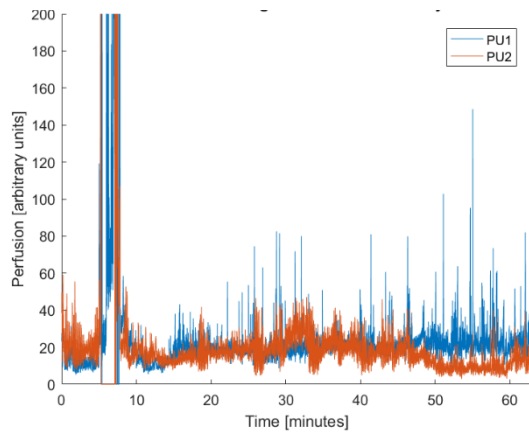


Figure 34: Application of Tigerbalm from test subject 1

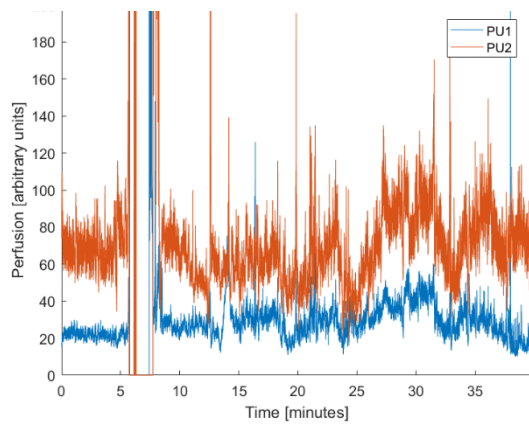
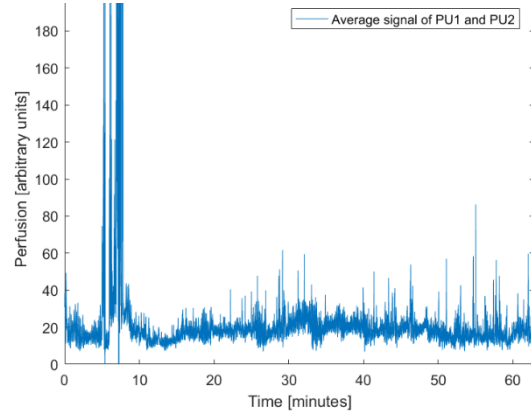


Figure 35: Application of Tigerbalm from test subject 2

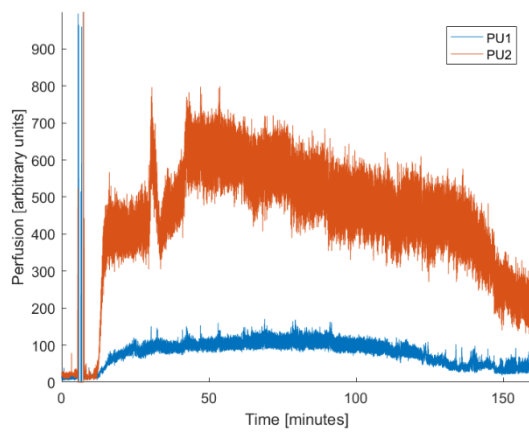
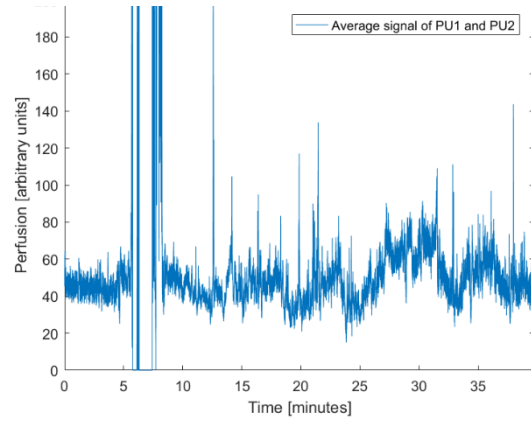
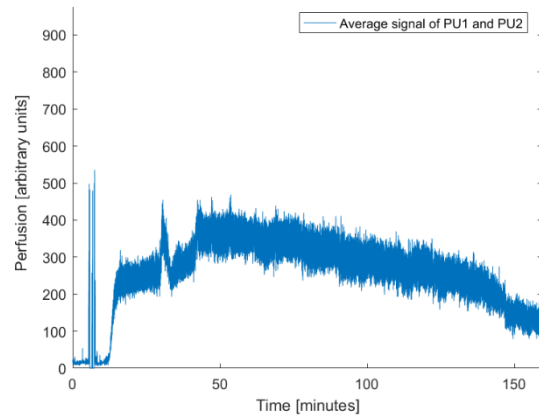


Figure 36: Application of Tantum from test subject 1



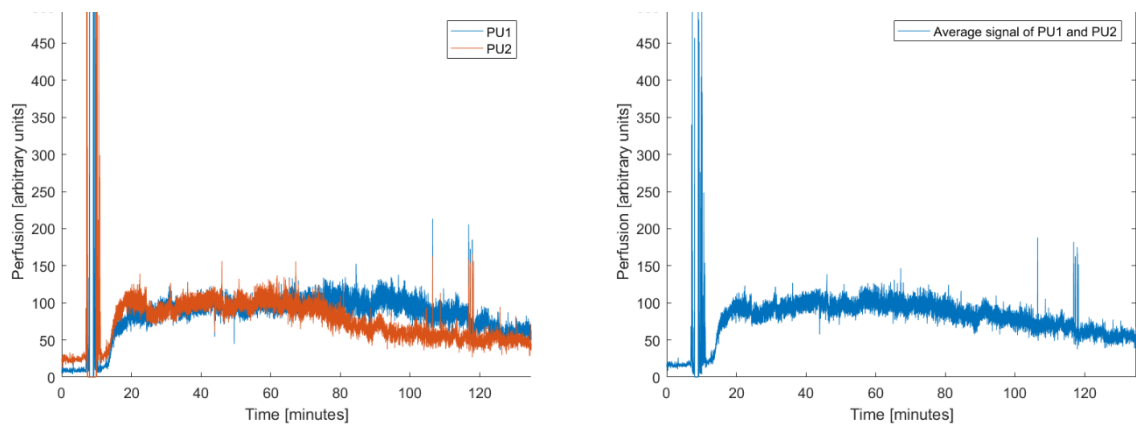


Figure 37: Application of Tantum from test subject 2

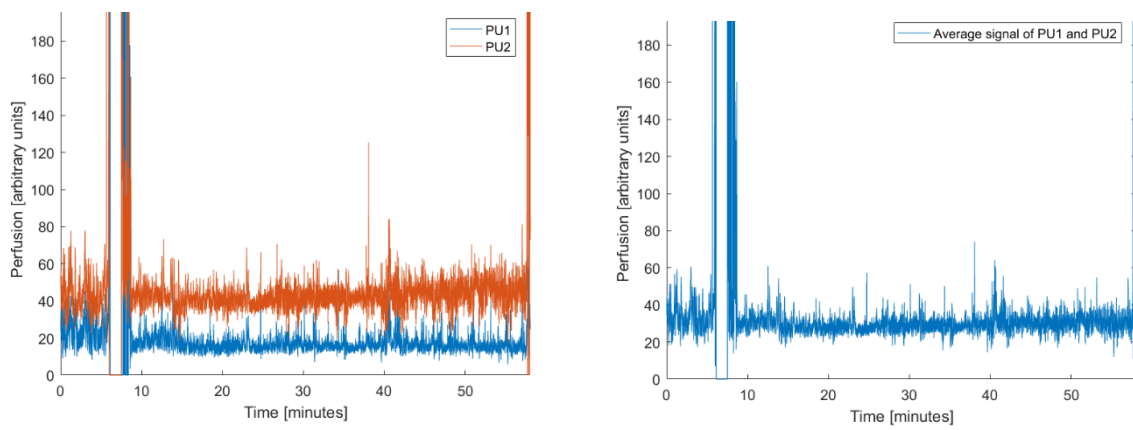


Figure 38: Application of Capsaicin 0,025% from test subject 1

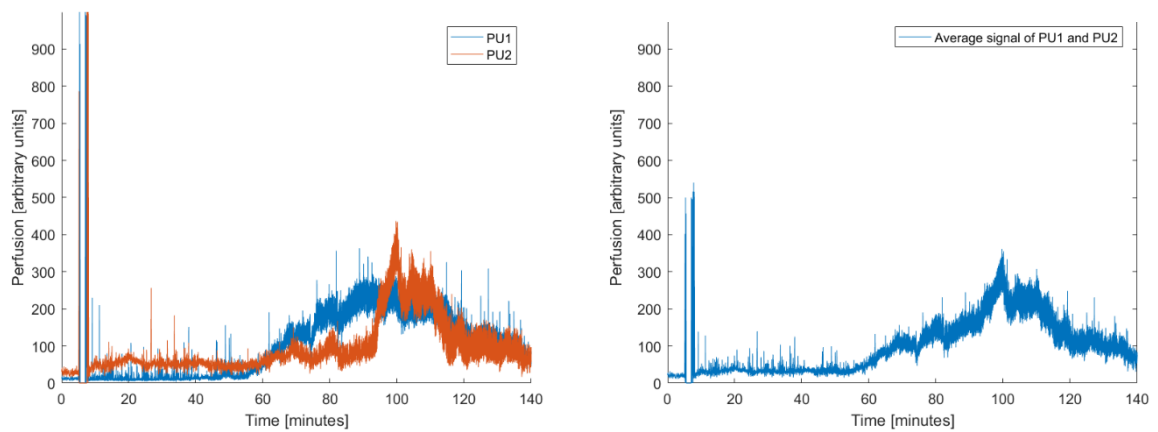


Figure 39: Application of Capsaicin 0,075% from test subject 1

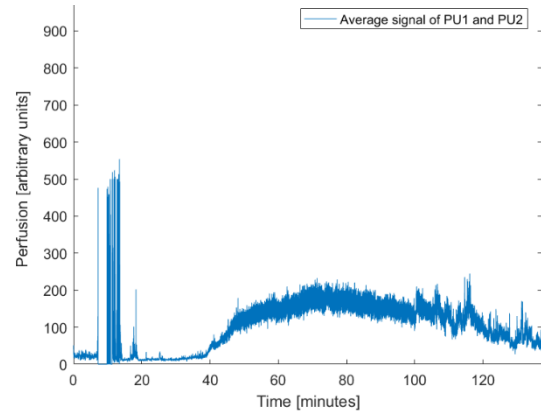
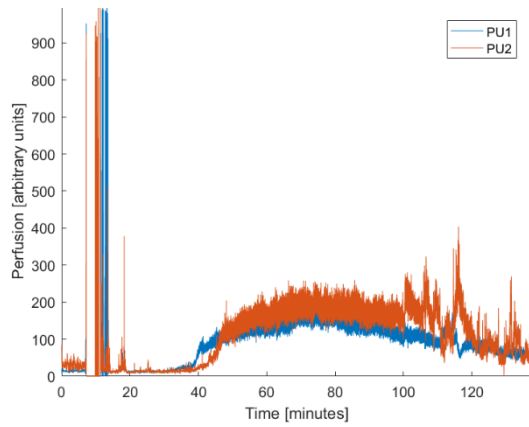


Figure 40: Application of Capsaicin 0,075% from test subject 2

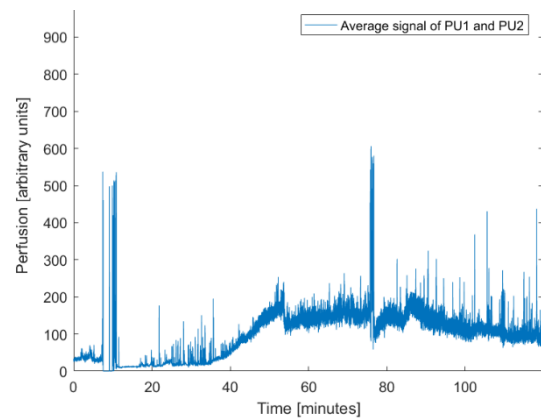
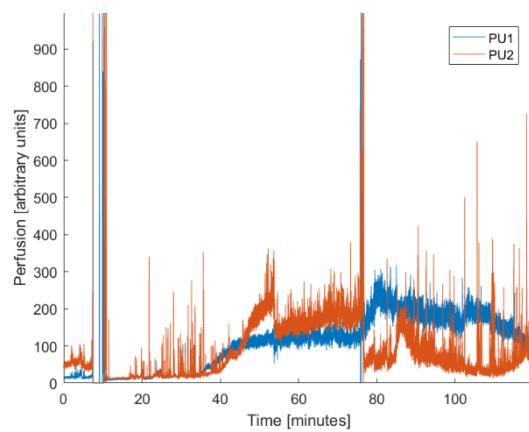


Figure 41: Application of Capsaicin 0,1% from test subject 1

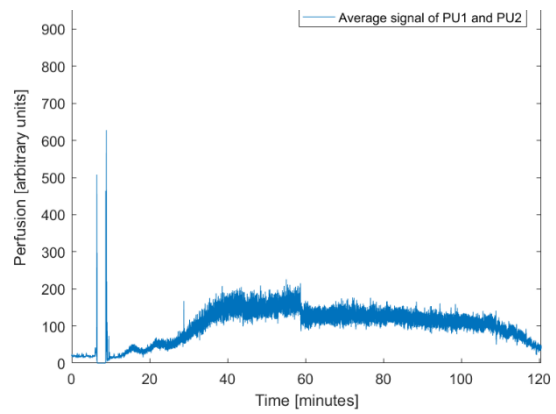
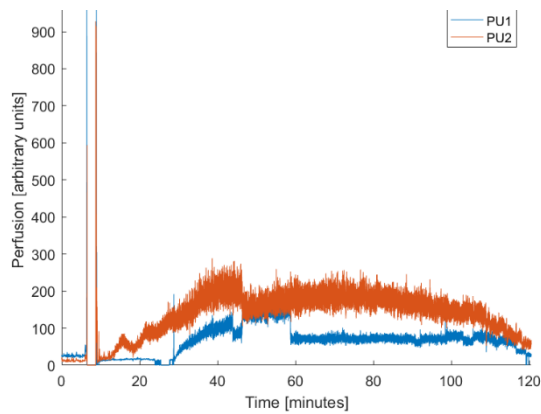


Figure 42: Application of Capsaicin 0,1% from test subject 2

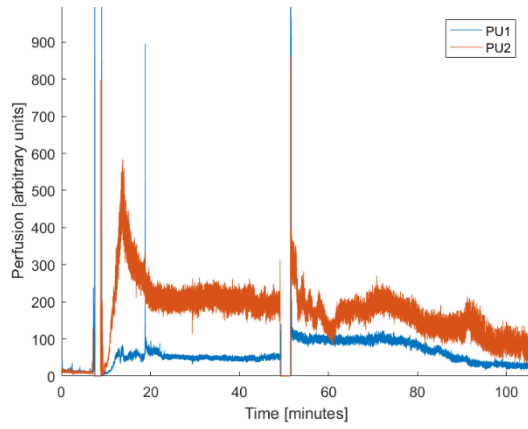


Figure 43: Reapplication of Midalgan from test subject 1

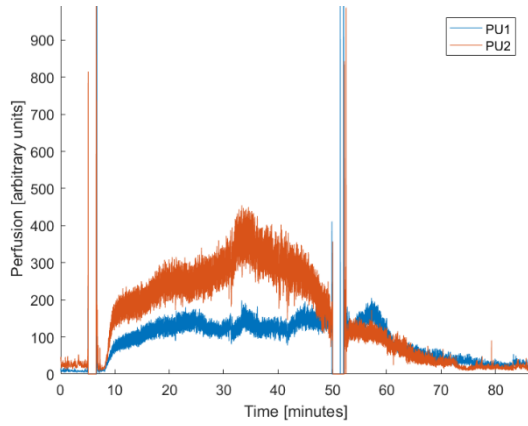
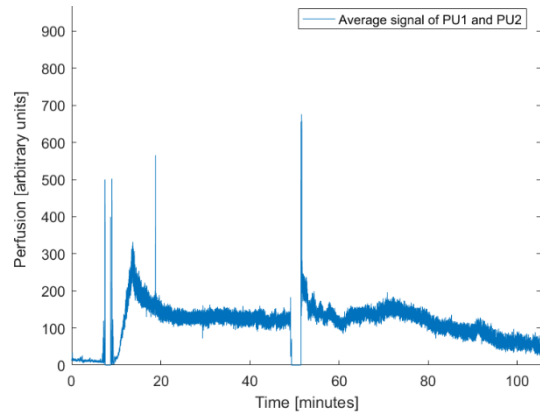


Figure 44: Reapplication of Midalgan from test subject 2

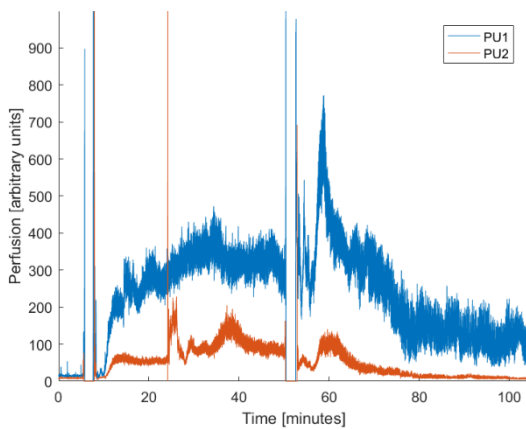
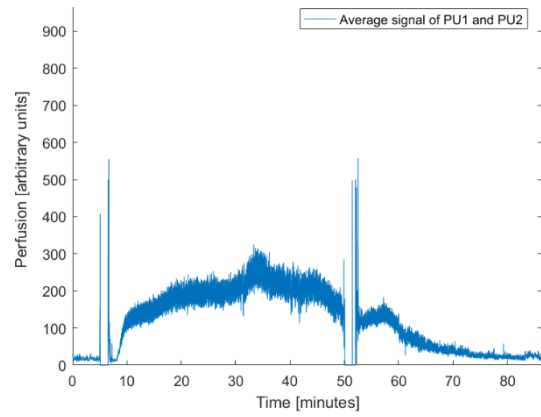
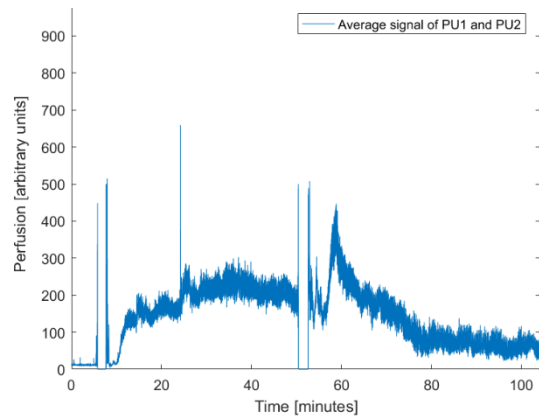


Figure 45: Reapplication of Midalgan from test subject 1 (repeat)



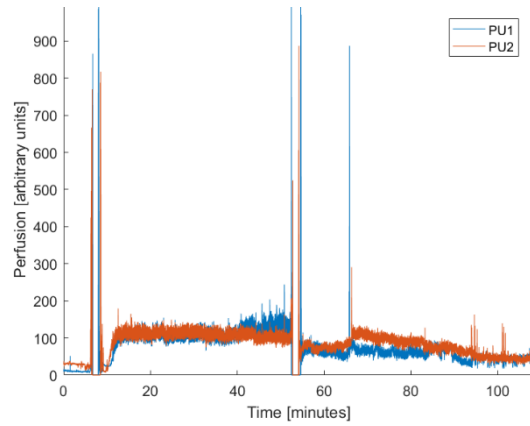


Figure 46: Reapplication of Midalgan from test subject 2 (repeat)

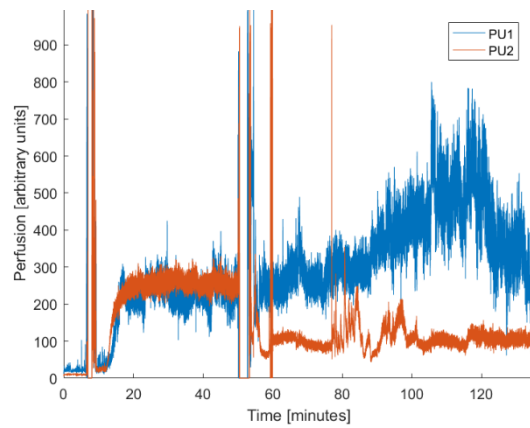
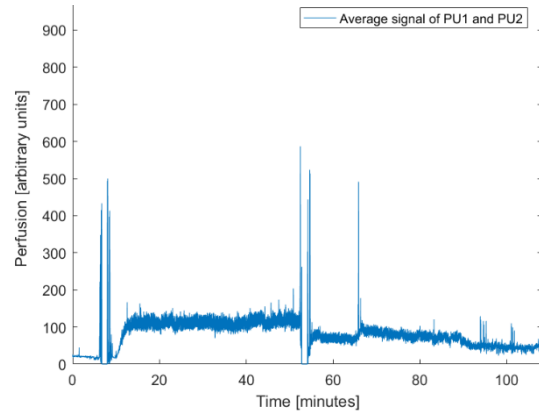


Figure 47: Reapplication of Tantum from test subject 1

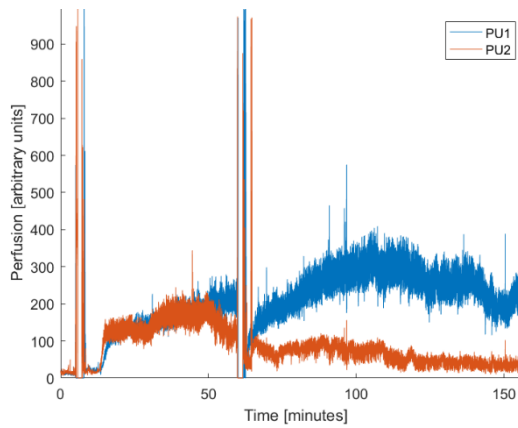
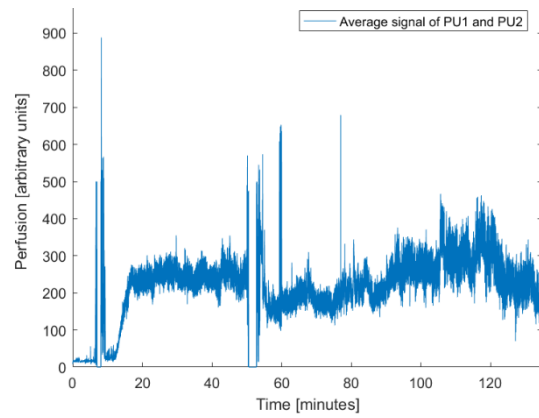


Figure 48: Reapplication of Tantum from test subject 2

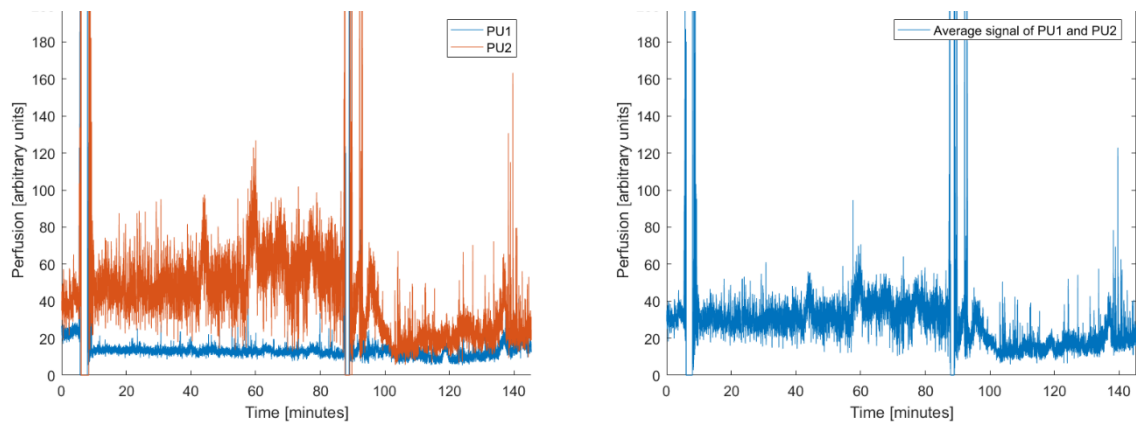


Figure 49: Reapplication of Capsaicin 0,025% from test subject 2

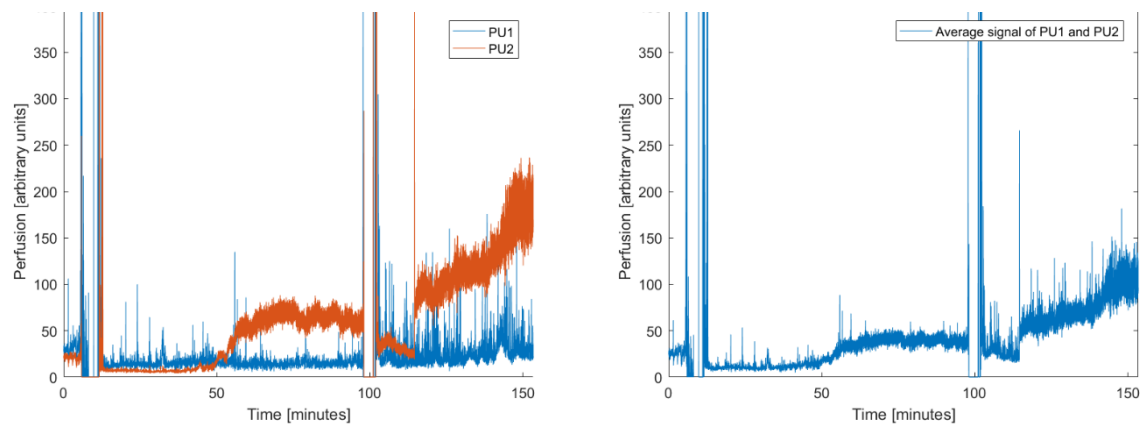


Figure 50: Reapplication of Capsaicin 0,075% from test subject 1

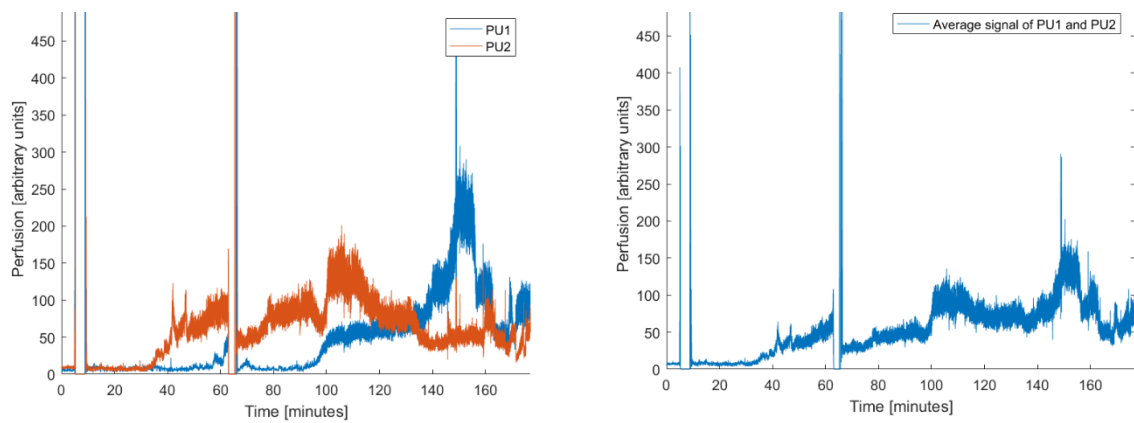


Figure 51: Reapplication of Capsaicin 0,075% from test subject 2

Appendix III: Failed measurements

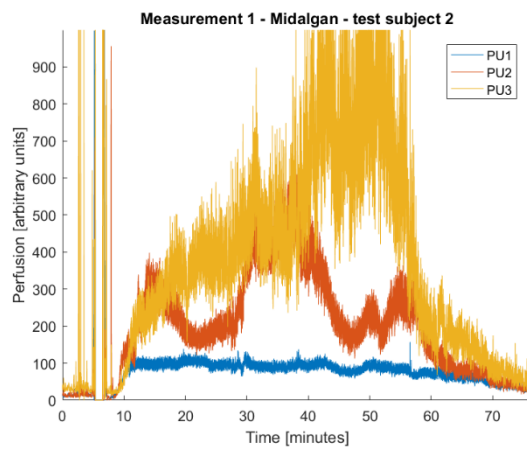


Figure 52: Example of values gained with PU₃

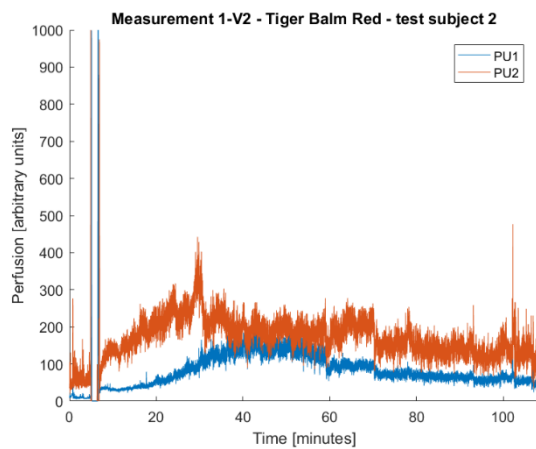


Figure 53: Application of Tigerbalm from test subject 2 with presumed effect of earlier used Midalgan

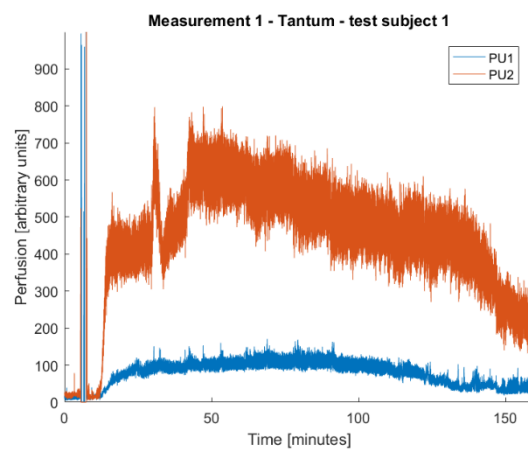
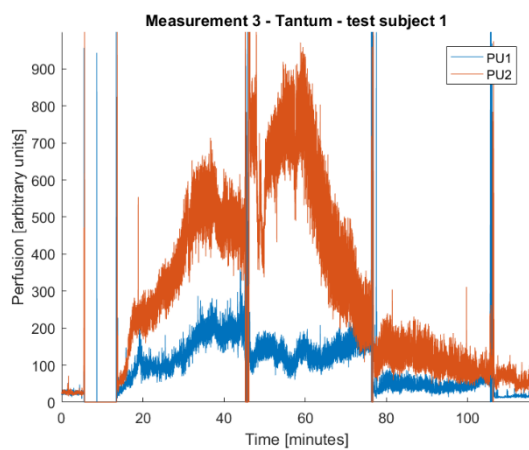


Figure 54: Application of Tantum from test subject 1 with shifting of the probes (l) compared to not moving the probes (r)

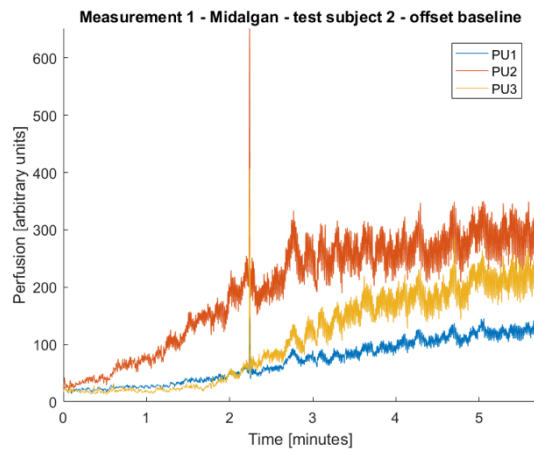


Figure 55: Application of Midalgan from test subject 2 with not sufficient cleaned probes

Appendix IV: Fourier spectral analysis of signals of measurement 1

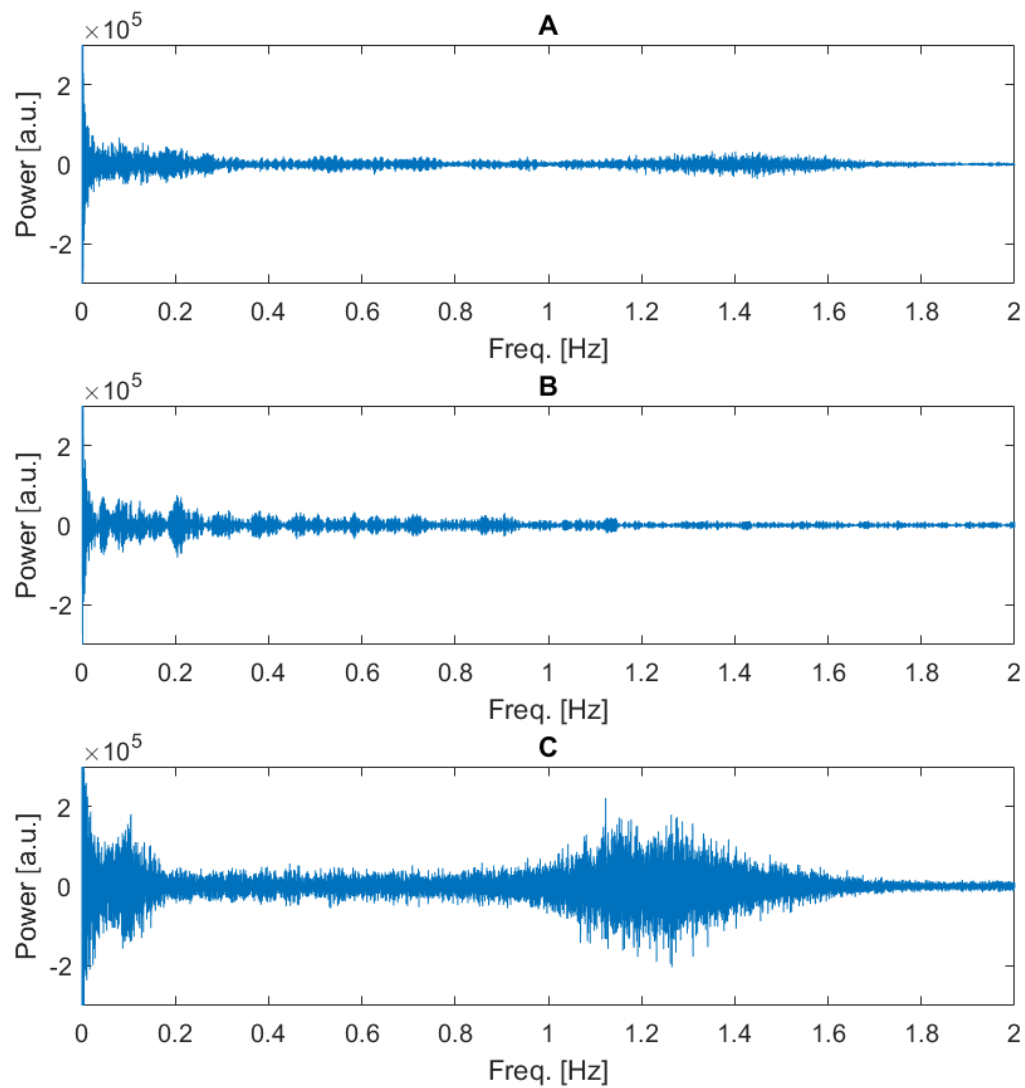


Figure 56: Fourier spectral analysis of all drugs from measurement 1, test subject 1. A=Midalgan, B=Tigerbalm, C=Tantum

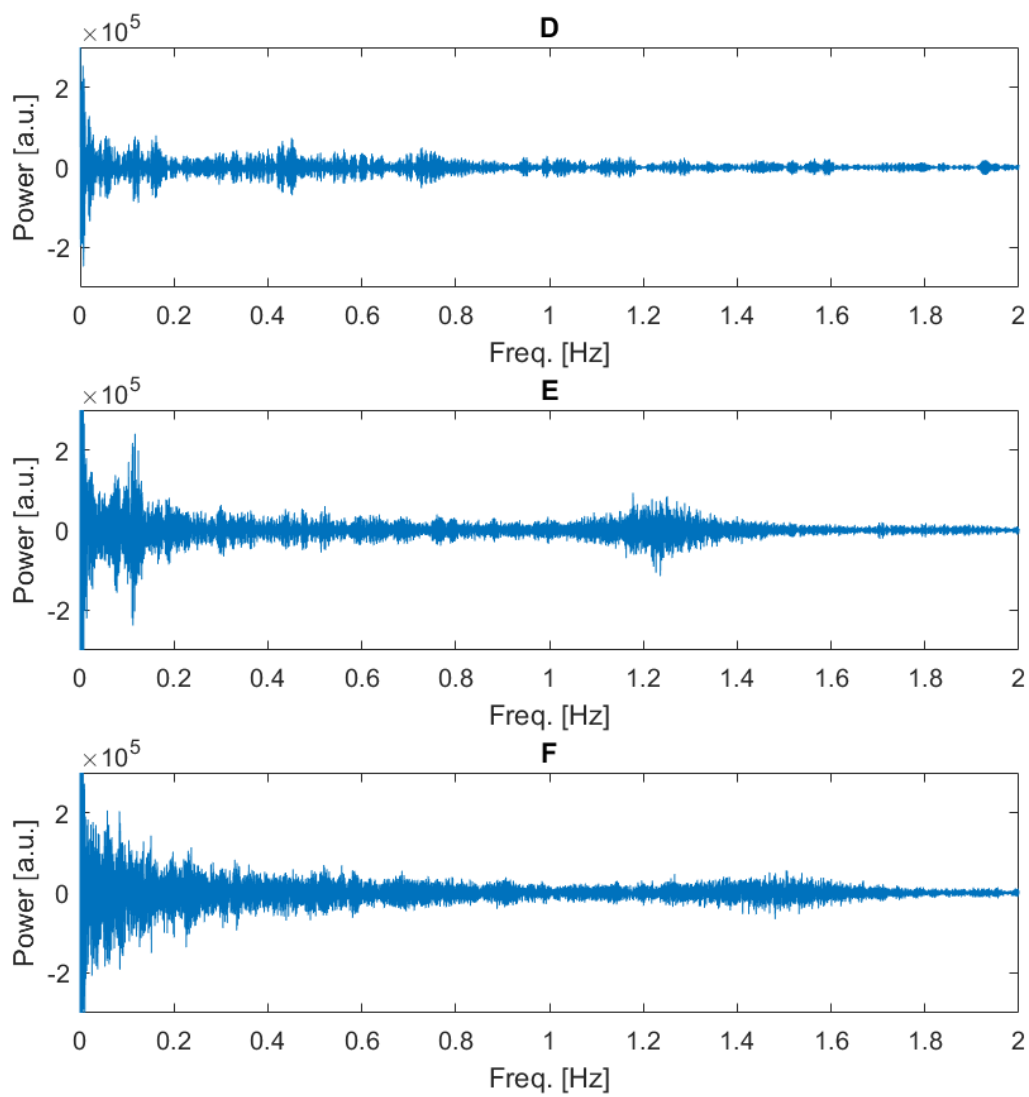


Figure 57: Fourier spectral analysis of all drugs from measurement 1, test subject 1. D=Capsaicin 0,025%, E=Capsaicin 0,075%, F=Capsaicin 0,1%

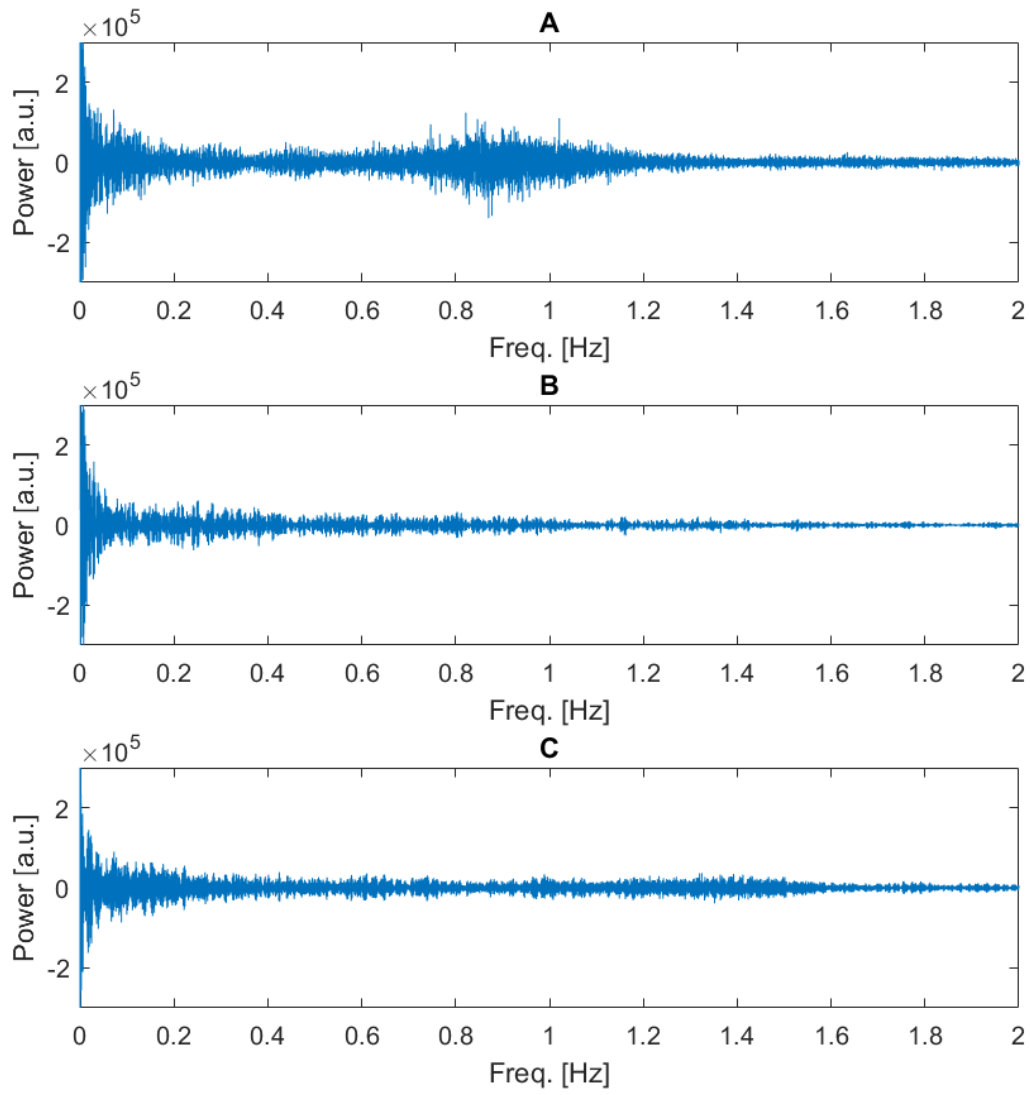


Figure 58: Fourier spectral analysis of all drugs measurement 1, test subject 2. A=Midalgan, B=Tigerbalm, C=Tantum

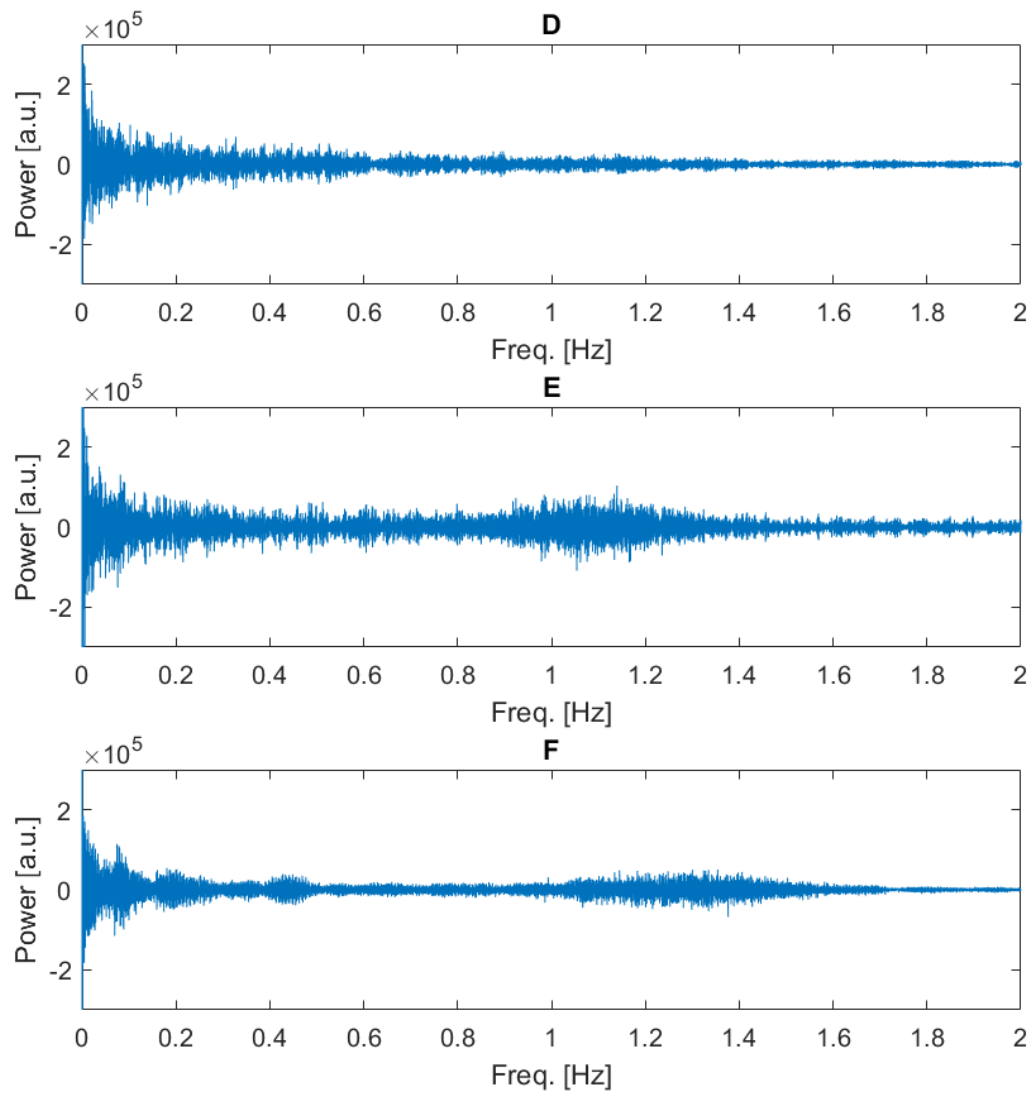


Figure 59: Fourier spectral analysis of all drugs measurement 1, test subject 2. D=Capsaicin 0,025%, E=Capsaicin 0,075%, F=Capsaicin 0,1%

Appendix V: METC

All documents needed to write an METC request have been collected in a folder which will be presented to the BMPI research group. This folder can also be requested at the researchers. The documents in this folder are written in Dutch, because the request also needs to be in Dutch. In this Appendix a short overview will be given of the documents in this folder and their content.

METC checklist

This checklist contains all the documents that need to be delivered for a request. The checklist is derived from the METC Twente and is enriched with information that is specific for this research.^[111] More information about the purpose of the other documents stated below is also described in this checklist.

Sectie A: Brieven

This folder contains two letters that need to be filled out. In here, one can indicate what is requested from the CCMO and where certain information can be found.

Sectie C: Onderzoeksprotocol

This folder contains template documents for a research protocol. In this protocol all aspects of the research need to be assessed and explained.

Sectie D: Productinformatie

This folder contains information about the required Investigator's Brochure. This is a summary of all clinical and pre-clinical information about the used products.

Sectie E: Proefpersonen

This folder contains a template for the information letter that needs to be send to the test subjects.

Sectie G: Verzekeringscertificaten

This folder contains documents that need to be filled in and describe the risks for the test subjects and potential insurances.

Sectie I: Informatie deelnemende centra

This folder contains a form that needs to be filled in with detailed information about the researcher.

Sectie K: Andere relevante documenten

This folder contains a Template Clinical Trial Agreement and a document about potential sponsoring.