Evaluating the microcirculation in early phase clinical trials: novel methodologies and interventions

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CHAPTER VIII

GENERAL DISCUSSION

Summary of findings

Nitric oxide, the smallest known signaling molecule produced by mammalian cells[1] is an intracellular and extracellular messenger involved in an extensive array of physiological processes.[2] First discovered as the mediator of acetylcholine or bradykinin-induced vasodilation and known as 'endothelial-derived relaxing factor',[3] the central role of NO in vascular functioning became clear. This initially led to great interest in using drugs affecting the NO system for treatment of cardiovascular disorders. This area of interest later expanded, as it was shown that NO also fulfils functions in the central nervous system and immunological pathways. In the past decades this resulted in investigations on treatments of a wide range of conditions, varying from neurodegenerative diseases to sepsis.[4–5] Measurements of NO bioavailability and the effects of NO are vital for the success of early phase clinical trials of compounds targeting it, since early pharmacodynamic biomarkers can guide and enhance further development of drugs.[6] This thesis explored the use of vascular imaging as a biomarker to measure NO-dependent processes and, by proxy, NO bioavailability. The addition of imaging to the pharmacodynamic measurements performed in clinical trials might be worthwhile due to the advantages of non-invasiveness and the measurement of end organ physiological function as opposed to metabolite or biomarker concentrations.

In the first half of this thesis, potential imaging methods were explored and validated. In **Chapter II**, the effects of a mixed meal tolerance test, a metabolic challenge proven to induce subclinical vascular changes in otherwise healthy volunteers,[7] were assessed in healthy elderly volunteers with 4 different imaging tools. Laser speckle contrast imaging (LSCI) combined with occlusion – reperfusion and local thermal hyperemia, sidestream darkfield imaging (SDFM) and passive leg movement (PLM) ultrasonography were performed before and after administration of the mixed meal tolerance test and before and after a 12-week treatment regimen of 13 g of dietary fiber, administered once daily. The employed imaging modalities showed high inter- and intraindividual variability, but a change in occlusion – reperfusion measured with LSCI, possibly induced by the mixed meal tolerance test, was detected. This manifested as a distinct pattern of reduced post-occlusive blood flow after consumption of the mixed meal, returning to baseline at the end of the 6-hour measurement period. Further standardization of imaging protocols and use of robust challenges known to affect vascular function may improve the performance of the imaging techniques that were used, enhancing their ability to detect pharmacodynamic effects of physiological challenges and drugs on the vasculature.

In **Chapter III**, patients diagnosed with mitochondrial disease and matched healthy volunteers underwent imaging assessments as well as assessment of various blood-based biomarkers, including *ex vivo* assessments of mitochondrial function in peripheral blood mononuclear cells. In this study, PLM and flow mediated skin fluorescence detected differences in femoral artery blood flow and reduced nicotinamide adenine dinucleotide (NADH) fluorescence, respectively, when comparing patients and matched volunteers. These results indicate that PLM and FMSF can differentiate between healthy and disordered function of the vasculature, although PLM showed high inter-subject variability, consistent with the variability of this method in Chapter II. In contrast, *ex vivo* assessments of mitochondrial function in peripheral blood mononuclear cells (PBMCS) did not distinguish healthy volunteers and patients. This may be a result of the purifying selection pressure for these cells, as PBMCS with diminished mitochondrial function may have a shorter lifespan in the bloodstream compared to PMBCS with adequate mitochondrial function.[8] Patients with mitochondrial disease display different levels of disease severity, and not all tissues are similarly affected by the mitochondrial DNA mutations,[9] probably resulting in the high inter subject variability found in the patients with mitochondrial disease and the failure of several *ex vivo* and *in vivo* assessments to discriminate between healthy and diseased. To combat this problem, usage of a combination of imaging modalities, assays of mitochondrial function and biomarkers in serum or plasma may be advisable in future clinical trials to fully capture the effects of drugs intended to treat this patient population.

Chapter IV investigated the effects of a patch containing titanium dioxide on local skin microcirculation. The hypothesized mode of action of this patch is the absorption of emitted body heat and re-emission as infrared radiation (wavelength between 50 and 1000 µm).[10] This is purported to have health benefits and used in therapeutic modalities such as saunas, heat-emitting lamps and heating garments.[11] Using LSCI, SDFM, near infrared spectroscopy, multispectral imaging, and thermography it was shown that the far infrared patch induced a short-lived increase in local skin perfusion, and longer lasting increases in oxygen consumption and skin temperature. The increase in skin temperature may have been the result of mechanical occlusion by the fabric of the patch, but since the increase in skin perfusion did not correlate temporally with the raise in temperature, it more likely was a direct effect of the far infrared radiation

emitted by the patch. Increased oxygen consumption and increased mitochondrial function are possible effects of far-infrared radiation[12–13] and hence may also be a result of the titanium dioxide contained in the patch. The study provides a proof-of-concept for the theoretical mode of action of the patch, and thereby evidence that imaging can be used to detect treatment effects of experimental devices on the microcirculation

The second section of this thesis describes clinical trials with compounds affecting the NO-soluble guanylyl cyclase (sGC)-cyclic guanosine monophosphate (cGMP) system. First, **Chapter V** describes the results of a first-in-human trial of zagociguat, an sGC stimulator developed for the treatment of neurodegenerative conditions, showing it to be safe and tolerable in single doses up to 50 mg and multiple doses of up to 15 mg administered once daily. Zagociguat concentrations were also detected in cerebrospinal fluid, making this compound the first in its class to penetrate this compartment in humans, a crucial aspect for the intended treatment indication. Several of the adverse events that occurred with increased frequency in the zagociguat-treated group when compared to placebo in this study, as well as lower blood pressure readings in the zagociguat-treated participants, point to the mechanism of action of zagociguat working as intended. However, central nervous system (CNS) pharmacodynamic tests did not reveal any effects of the compound in the young, healthy population examined in the trial.

The results of a proof-of-concept study in healthy elderly with the same compound, using extensive pharmacodynamic testing, including blood and cerebrospinal fluid biomarkers, a battery of neurocognitive tests, magnetic resonance imaging and PLM, are described in **Chapter VI**. Here, as in the first-in-human trial, blood pressure lowering effects of zagociguat were seen, but pharmacodynamic testing did not reveal any CNS effects of the compound, and the exploratory PLM assessment showed no effects of the drug on systemic NO bioavailability. These results show that even though compounds may be pharmacologically active at the target site, as shown for zagociguat by reduced blood pressure, detection of meaningful pharmacodynamic effects on target organs, i.e. the brain, can be difficult in healthy volunteers who likely have optimal function in the assessed system.

Finally, in **Chapter VII**, the effects of a phosphodiesterase 2 (PDE2) inhibitor on cGMP levels in cerebrospinal fluid were examined. This study confirmed that this PDE inhibitor can reach the cerebral compartment and elevate cGMP by blocking its degradation, although not in a dose-dependent manner.

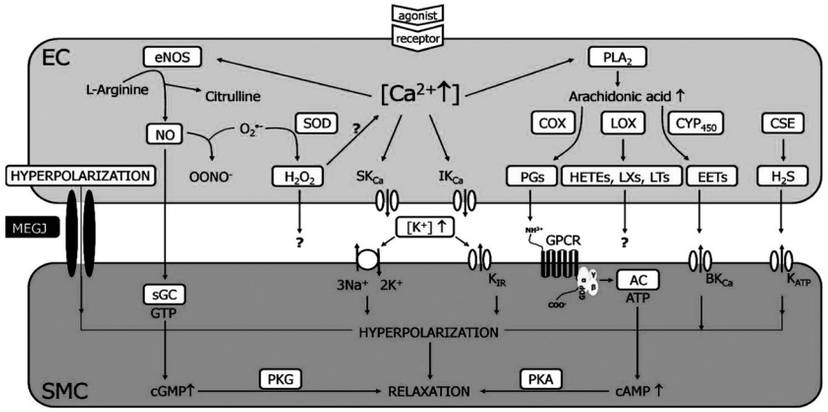
Furthermore, cGMP levels showed a rise-and-fall pattern in both placebo-treated participants as well as participants receiving study drug, confirming the circadian rhythm in cGMP production suggested in earlier studies.[14] PDE inhibitors have been studied for the treatment of central nervous system disease for decades, and this study adds a proof-of-concept for PDE2 inhibition to increase cGMP signaling in the brain.

PATHWAYS INVOLVED IN ENDOTHELIAL FUNCTION

The NO-sGC-cGMP pathway has been extensively discussed in the introduction of this thesis and is the main target of both the therapeutic interventions and imaging methods employed in the described studies. However, endothelial function is a multifactorial physiological process that is influenced by NO-independent pathways and a large variety of intermediary enzymes and messenger molecules, a selection of which is shown in Figure 1.[15] The pathways involved in endothelial function include but are not limited to sensory innervation, cyclo-oxygenase activity, endothelial-derived constrictive factors such as endothelin, thromboaxane A_2 and angiotensin II,[16] and endothelium dependent hyperpolarizing factor.[17]

Given the multitude of factors involved in endothelial physiology, a multimodal approach to evaluate endothelial function may be advisable. All the components of the endothelial system interact with each other, with both positive and negative feedback loops, regulation by endothelial-derived and systemic factors and cell self-regulation. Therefore, measurement of blood-based biomarkers can be prone to misinterpretation, since, for example, an increase in relaxing factors might be an indicator of healthy endothelial function, or the result of compensatory mechanisms during endothelial dysfunction. Similarly, as discussed in the introduction of this thesis, the actions of NO can be beneficial and detrimental to the endothelium depending on quantity and location. Hence, methodologies that measure the result of the complex interactions within the endothelium, i.e., endothelial function, can provide an intermediary between proximal cell- or blood-based biomarkers and clinical endpoints. The imaging methods employed in the studies in this thesis might achieve this by assessing NO-dependent vasodilation (PLM, LSCI combined with local hyperthermia), general vascular function (LSCI combined with occlusion-reperfusion), density and perfusion of the microcirculation (SDFM), tissue oxygenation and blood flow (NIRS) and metabolic activity (FMSF). In the following section, benefits and drawbacks of each individual imaging method will be discussed.

FIGURE 1 Pathways involved in endothelium-dependent vessel relaxation.



AC=adenylyl cyclase; CAMP=cyclic adenosine monophosphate; CGMP=cyclic guanosine monophosphate; COX=cyclooxygenase; CSE=cystathionine-lyase; CYP450=cytochrome P450 epoxygenase; EC=endothelial cell; eNOS=endothelial nitric oxide synthase; EETS=epoxyeicosatrienoic acids; GPCR=G-protein coupled receptor; HETES=hydroxyeicosatrienoic acids; H2O2=hydrogen peroxide; H2S=hydrogen sulfide; KCA=Ca^{2+}-activated potassium channel with small (SKCA), intermediate (IKCA) or big conductance (BKCA); KATP=ATP-sensitive potassium channels; KIR=inwardly rectifying potassium channel; LOX=lipoxygenase; LTS=leukotrienes; LXS=lipoxines; MEGJ=myoendothelial gap junctions; NO=nitric oxide; OONO−=peroxynitrite anion; O2−=superoxide anion; PGS=prostaglandins; PKA=protein kinase A; PKG=protein kinase G; PLA2=phospholipase A2; sGC=soluble guanylyl cyclase; SMC=smooth muscle cell; SOD=superoxide dismutase. (Adapted from Schmidt et al.[15])

SYNTHESIS OF FINDINGS BY IMAGING METHOD

The studies contained in this thesis show that imaging can be used to detect effects of both metabolic challenges and pharmacological treatments, and that the possibility to detect no-mediated effects in early phase clinical trials can be increased by including imaging modalities in their design.

LSCI was the imaging method with the largest technical reproducibility, showing low inter- and intrasubject variability in **Chapter II**. LSCI was also able to identify responses elicited by mixed meal challenge tests and 12-week fibre administration (**Chapter II)**, measure differences between patients with mitochondrial disease and healthy volunteers (**Chapter III**) and assess the effects of a far-infrared radiation patch (**Chapter IV**). This indicates that LSCI is a valid method to detect expected changes in physiological processes. LSCI might therefore be candidate for inclusion in studies investigating a wide range of compounds aiming to modulate vascular function. A caveat to widespread application of LSCI is that in its current form, it can only be performed in dedicated centers equipped with the necessary expensive devices, experienced operators, and controlled environments. If those requirements are met, LSCI is a technique with good technical reproducibility, and selected LSCI parameters show biological validity, i.e., response to intervention or discrimination between functional and dysfunctional vascular function.[18–20] Moreover, LSCI can measure a large range of skin perfusion values, i.e., skin perfusion during complete blood flow occlusion as well as during inflammatory conditions,[21–22] and can be combined with many local reactivity challenges such as occlusion-reperfusion, local thermal hyperemia and iontophoresis of vasoactive compounds.[23–24] The combination with these challenges also makes LSCI attractive, since it allows evaluation of different physiological pathways, including cyclooxygenase activity, sensory innervation,[25] and the effects of locally administered vasoconstrictors or dilators.[26] Although laser doppler flowmetry can achieve similar goals, the advantages of LSCI in this regard are temporal as well as spatial resolution,[27] and arguably higher sensitivity.[18] However, the cost of a laser speckle imaging device can be prohibitive, although low-cost LSCI devices show some promise in limited context.[28] The LSCI method is very sensitive to influences of temperature and movement of the imaged area, necessitating not only careful instruction of participants in a clinical study, but also dedicated rooms which minimize the possibility of outside interference with measurements. Moreover, LSCI measures blood flow through spatial analysis of changes in speckle pattern, resulting in arbitrary perfusion units instead of units easier to understand in the context of blood flow, e.g., mL/min. The physics of speckle pattern analysis make the translation to clinically useful units challenging,[29] and the relation between changes in speckle pattern and physiological blood flow is poorly understood.[30] The use of LSCI has remained mainly in carefully controlled clinical trials, although innovations in post-image processing[31] and machine learning[32] show promise for use of LSCI in a clinical setting, for example to identify poorly perfused tissue regions intraoperatively.[33]

The PLM technique was employed to assess the effects of a mixed meal challenge (**Chapter II**) determine differences in vascular function between patients with mitochondrial disease and healthy volunteers (**Chapter III**) as well as to measure potential vasodilatory effects of sGC stimulation (**Chapter VI**). PLM could discriminate between healthy volunteers and patients with mitochondrial disease, confirming that PLM can distinguish populations with different

cardiovascular risk factors or characteristics.[34–37] However, no effects of sGC stimulation or mixed meal administration were detected by PLM, and the technique showed high variability across all studies. The lack of detected treatment effects may be a function of sample size or absence of meaningful macrovascular effects of either sGC stimulation or a mixed meal, but since no positive control was employed in the described studies, this is impossible to ascertain. Alternatively, PLM may not have been able to detect treatment effects due to its operator dependency, a well-known confounder for ultrasonography, particularly when a stable image of a blood vessel with an appropriate insonation angle of 60° during the entire recording is vital.[38] Nevertheless, given PLM's clinical relevance and high correlation with NO bioavailability, the technique might still be a worthwhile addition in clinical studies. Based on the studies in this thesis, this is only possible if the method is significantly improved in consistency by performing the procedure with experienced operators adhering to strict guidelines.[39]

SDFM was used in the assessment of microcirculatory disturbances caused by a mixed meal challenge (**Chapter II**), as well as measurement of differences in sublingual microcirculation between patients and healthy volunteers (**Chapter III**) and effects of a far-infrared emitting patch (**Chapter IV**). SDFM, as a technique to visualize red blood cells and their movement through blood vessels in real time, enables the identification of vessel density and perfusion changes in the microcirculation. SDFM can also be used to calculate glycocalyx thickness, but this requires complicated analyses which were not employed in the studies in this thesis. SDFM has mainly been employed in the study of critically ill subjects such as patients with sepsis,[40] or undifferentiated patients admitted to the ICU,[41–42] or patients undergoing various types of surgery,[43–44] since microcirculatory changes are most likely to occur and most evident in these populations. Evidence of microcirculatory changes detected by SDFM has also been found in populations with elevated risk of cardiovascular disease.[45] However, in the studies in this thesis, SDFM did not detect microcirculatory effects of a mixed meal or a far-infrared patch, nor were differences in sublingual microcirculation found between healthy volunteers and patients with mitochondrial disease. Microcirculatory disturbances are possibly unlikely to occur in the healthy volunteers participating in these trials, a limitation which could be remedied by using a challenge to disturb the microcirculatory perfusion and reveal small potential effects of an intervention. Alternatively, if the proposed mechanism of the studied intervention is to affect angiogenesis rather than vessel perfusion, e.g., by inhibition of vascular endothelial growth factor,[46] measurements over a long period of time may be advisable. The studies in this thesis employed neither remedy, which may be an explanation for the negative findings in **Chapter II, III and IV**. In addition, recent developments in the SDFM technique, with novel equipment and analysis software currently available, allow for more in-depth assessments such as automatic measurement of glycocalyx thickness and red blood cell velocity. These innovations were not available or implemented in the described studies.[47] However, the advantages of SDFM such as minimal participant burden, good reproducibility, in particular when using multiple consecutive measurements,[48–49] portability, and ease of use may still make it a worthwhile inclusion in some clinical trials, especially when implemented according to the above recommendations.

NIRS was used in this thesis to assess differences between patients with mitochondrial disease and healthy volunteers in **Chapter III** and to determine the effects of a far-infrared radiation emitting skin patch in **Chapter IV**. In its clinical application, NIRS is a relatively simple technique using the relative concentrations of oxygenated and deoxygenated hemoglobin to derive tissue perfusion and oxygenation.[50] NIRS has found application in clinical research on a wide range of disorders such as stroke,[51] burns,[52] central nervous system disorders[53–55] and vascular disease.[56–58] The implementation of NIRS varies widely, including measurement of cerebral perfusion and oxygenation,[59] peripheral oxygen saturation, muscle perfusion and oxygenation, and measurement of mitochondrial oxidative capacity.[60] The disadvantages of NIRS are similar to other imaging techniques: without a significant challenge to perfusion or oxygenation, NIRS will show optimal tissue saturation indexes for individuals participating in clinical trials. Moreover, to derive a measure of tissue oxygen consumption and hence mitochondrial oxidative capacity as well as to acquire information on vascular function, combination of NIRS with arterial and venous occlusive challenges is necessary.[61–62] Furthermore, accurate assessment of mitochondrial oxidative capacity may need blood volume corrections,[63] which were not employed in the studies in **Chapter III** and **IV**. However, given these limitations, NIRS provides unique information on vascular function visá-vis the other imaging modalities used in this thesis: since near-infrared radiation can pass relatively easily through different tissues,[64] NIRS penetrates skin, underlying muscle and, in the case of cerebral assessments, the skull, thereby providing information on perfusion and oxygenation of deeper tissues. SDFM and LSCI imaging can only assess perfusion and microcirculatory status

in superficial tissues such as the skin or sublingual blood vessels, which creates a niche for NIRS as an imaging endpoint for use in clinical trials evaluating perfusion in deep tissues, without the burdensome use of magnetic resonance imaging or other advanced imaging techniques.

FMSF was solely employed in the study described in **Chapter III**, where significant differences in NADH skin fluorescence between patients with mitochondrial disease and healthy volunteers were found using this unique technique. FMSF uses a different physiological approach to assess vascular function and microcirculation: rather than directly visualizing blood vessels or blood cells, FMSF measures NADH fluorescence, thereby deriving an estimate of the concentration of this compound in the superficial skin cells.[65] Since NADH is central to many cellular processes, in particular redox metabolism and oxidative phosphorylation, its concentration is directly influenced by the supply of oxygen to the cells, i.e., blood circulation. When combining FMSF with an occlusion-reperfusion challenge, the metabolic shift of skin cells to glycolysis, response to reperfusion, and oscillations in blood flow can be evaluated.[66–67] The latter, termed 'flow motion' is of particular interest, since the oscillations in NADH fluorescence reflect movement of blood vessels, i.e., vasomotion, which is critical to vascular homeostasis.[68] Vasomotion can be differentiated into categories by the frequency of vessel movements through Fourier transformation,[69] resulting in cardiac, respiratory, neurogenic, myogenic, and endothelial components.[70–71] The endothelial component can be measured with various imaging methods with sufficient temporal resolution, such as laser doppler flowmetry and FMSF, and may be a useful clinical tool in the assessment of microvascular endothelial function.[72] FMSF can provide unique physiological information when compared to the other imaging methods investigated in this thesis. Moreover, the FMSF technique is highly standardized and displays good reproducibility and sensitivity to blood flow changes.[73] However, since FMSF is a relatively novel technique which necessitates expensive equipment, its use is limited to specialized clinical research centers. In addition, FMSF necessarily must be combined with an occlusion-reperfusion challenge to yield useful information, which may be a significant subject burden especially when repeated measurements are needed. FMSF-derived assessments of vascular function are indirect since no oxygenation or true flow values are measured. This issue is compounded by the fact that, although FMSF does provide some metabolic information, by itself it cannot show a complete picture of the status of cellular respiration, since NAD+/NADH ratio is a vital parameter to assess cellular redox status which cannot be derived from NADH concentrations alone. Combination of FMSF with imaging or biomarkers providing additional information on either blood flow or tissue metabolism is therefore advisable.

RECOMMENDATIONS FOR FUTURE STUDIES

All imaging modalities used in the studies described in this thesis have different benefits and drawbacks. The disadvantages can often be remedied by selecting the methodology to be fit for measuring the intended mechanism of action in tissues that are likely to be affected, by combining pharmacodynamic tests with physiological challenges that disturb homeostasis or create endothelial dysfunction and by incorporating both functional imaging as well as upstream biomarkers in blood or cells in the design of clinical studies. A limitation of the studies described in this thesis is that the employed imaging modalities were not compared to the existing gold standard for endothelial functional measurement, flow mediated dilation (FMD), due to the logistical challenges associated with implementing this technique in the described studies, and only limited data on blood-based biomarkers was collected, specifically in the study described in **Chapter III**. The first half of this thesis thus serves as a pilot for exploring the usefulness of imaging for assessment of endothelial function. The next step in developing these techniques would be to benchmark them against FMD and established biomarkers for endothelial function, such as adhesion molecules and coagulation factors.[74]

When several imaging techniques are combined in the same trial, these can complement each other, providing supportive information and revealing different mechanisms of action. For example, in **Chapter IV**, the combination of both LSCI, NIRS and thermography in the same study revealed that a far-infrared radiating patch does not only increase skin blood flow, but that this is likely not purely due to skin warming, and coincides with an increase in tissue metabolism, thereby providing a credible hypothesis for the mechanism of action of the investigated patch. The combination of an array of imaging methods also revealed differences in both baseline metabolic characteristics as well as NO bioavailability in **Chapter III** of this thesis.

On the other hand, some of the studies in this thesis may have benefited from the inclusion of additional imaging methodology to assess pharmacodynamic effects of the investigated drugs. For example, in **Chapter V**, no functional endothelial testing in the form of imaging was conducted, which may have been missed opportunity, since these could have further supported

potential systemic target engagement in the vascular system. The influence of the sGC stimulator on local and systemic NO bioavailability, as well as general vasodilation, could have been assessed through inclusion of respectively LSCI with local thermal hyperemia, PLM, and LSCI with occlusion-reperfusion. In **Chapter VI**, PLM was included as an exploratory endpoint, but similarly LSCI might have provided more information on upstream or downstream effects in the NO-sGC-cGMP pathway. In this trial the inclusion of a challenge designed to reduce endothelial or vascular function, for example infusion of lipopolysaccharide,[75] would also have been useful to improve the likelihood of finding pharmacodynamic effects, since detection of treatment effects on the vasculature in individuals without endothelial dysfunction has proven difficult, especially with relatively short treatment regimens. Similar recommendations could be made for the study in **Chapter VII**, although here the most important final common pathway of NO signaling, cGMP, was directly measured in a relevant pharmacokinetic compartment and shown to increase with treatment. The addition of imaging modalities such as LSCI and PLM could have confirmed not only the proof-of-mechanism of PDE-mediated cGMP elevation, but also provide information on whether the induced cGMP elevation caused increased cGMP signaling resulting in improved vascular function.

In conclusion, a variety of imaging methods show some promise to investigate the endothelium, specifically investigations of drugs aiming to influence NO signaling. Obviously, each modality has different requirements and caveats, demanding careful consideration of the study design, technical aspects, sensitivity, variability, and feasibility. The research described in this thesis could aid researchers investigating drugs targeting the NO system to tailor the imaging package exactly to their study objectives. Preferably, this should be combined with circulating markers of endothelial function to establish a coherent picture of both biochemical and functional effects of the investigated drug.

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