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Monitoring the skin NADH changes during ischaemia and reperfusion in humans

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ABSTRACT

Nicotinamide adenine dinucleotide (NADH/NAD+) is involved in many important biochemical reactions in human metabolism, including participation in energy production by mitochondria. Flow Mediated Skin Fluorescence (FMSF) is a non-invasive method to study dynamic changes in the content of the reduced form of NADH by measuring the optical properties of NADH related to the emission of the autofluorescent light (460 nm) after an earlier excitation by ultraviolet light. This review summarises the available studies using this method to describe its potential and limitations.

Introduction

Nicotinamide adenine dinucleotide (NADH/NAD+) is the most important cofactor involved in energy metabolism in live human cells. NADH/NAD+ participates in glycolysis, the citric acid cycle and the mitochondrial respiratory chain, beta-oxidation, reduction of pyruvate to lactate, as well as the modification of RNA together with regulation of transcription. It is also a part of the second messenger system [1,2]. Some examples of NADH involvement in cellular metabolism are pictured in Figure 1.

Nicotinamide adenine dinucleotide exists in two forms: reduced NADH and oxidised NAD+. Both are found in the cytosol, cellular organelles, nucleus and the mitochondria [3]. However, the main site of NADH oxidation to NAD+ are the mitochondria. The architecture of a mitochondrion is outlined in Figure 1: an intermembrane space separates the outer and inner membranes and the mitochondrial matrix with the electron transport chain and genome are contained within said inner membrane [4].

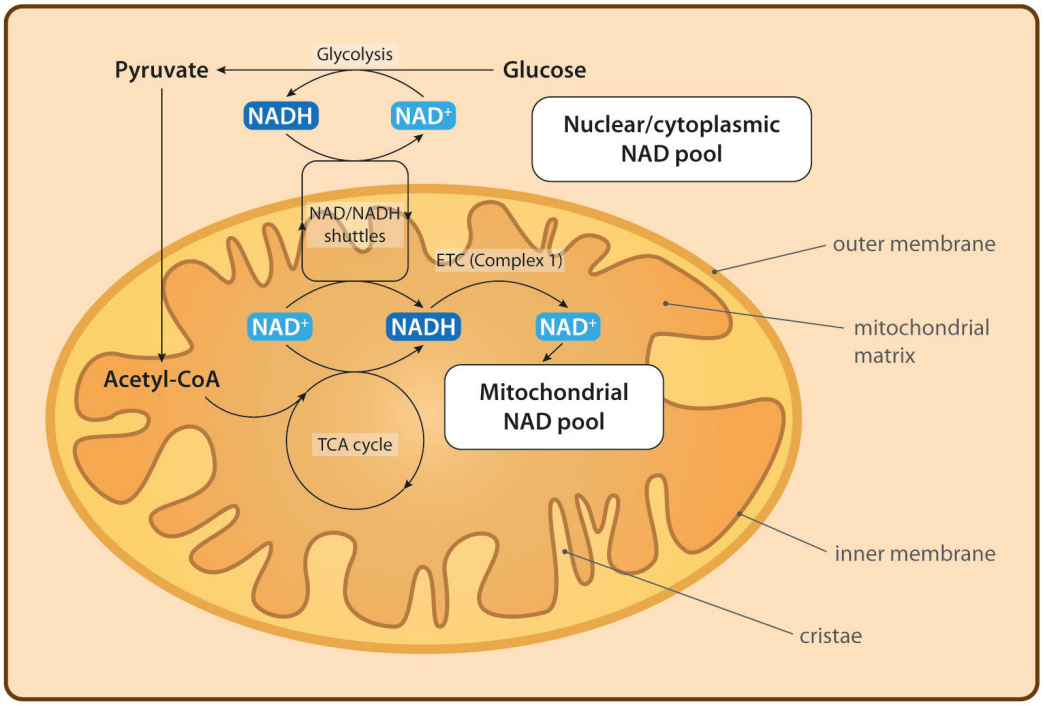

Figure 1. Metabolic pathways of NADH in cytosol and mitochondria. *Abbreviations: ETC – electron transport chain; TCA cycle – tricarboxylic acid cycle*

The intermembrane space is critical for storing protons involved in ATP production and both creatine kinase and adenine kinase are localised there. Both the outer and inner membranes are impermeable to most metabolites, including NADH. As NADH cannot cross the mitochondrial membranes, the transportation of high energy electrons from this molecule to the electron transport chain involves special reducing equivalents. The malate-aspartate and glycerophosphate cross membrane shuttles are two known pathways which transport reducing equivalents of NADH from cytoplasm to mitochondrial matrix.

The indirect, shuttle-based transportation of high energy electrons from NADH through the shuttling substrates across the mitochondrial membrane occurs only in aerobic conditions. Therefore, during anaerobic conditions (such as ischaemia), NADH produced by glycolysis accumulatesin thecytoplasm, and its reducing equivalents cannot be passed further down to the mitochondrial matrix through the inner membrane. NADH inside mitochondria cannot be oxidised by Complex I to NAD+ and thus the redox state changes in favour of NADH. Therefore, the amount of NADH may be utilised as a surrogate marker for absolute (hypoxia) or relative (increased metabolism) oxygen deprivation [3].

The NADH content can be measured in several ways. Some of the examples include spectrophotometric, fluorometric [3,5] and bioluminescent enzyme assays [6,7]. Interestingly, exciting NADH with ultraviolet (UV) light in the 320–380 nm range produces autofluorescent light emission in the 420–480 nm range with peak intensity at 450–460 nm - this optical feature has been widely used for measuring NADH concentration or content in solutions, cells and tissues [3].

Chance applied the UV/fluorescence-based method to monitor the NADH amount in cells and tissues or concentration in liquids [8]. The first device that allowed for such measurement was developed in 1954 by Theorell and Nygaard [9], and in 1962 Chance et al. studied NADH content in the brain and kidneys of anaesthetized rats [10]. In 1966 Chance showed that the primary source of 420–480 nm UV photoemission is the NADH and not the oxidised NAD+, thus validating this method for NADH quantification [8,10]. Since then, the fluorescence method has been commonly used in many in-vivo studies. For instance, Mayevsky et al. studied NADH with this method in rats [11] and humans [12].

Mayevsky et al. showed that the majority of 460-nm fluorescence comes from mitochondria with an irrelevant contribution of cytoplasm on human and animal skin models [3]. Dunaev et al. showed that the UV and fluorescent light penetrates only the most superficial skin layers — up to 0.5 mm in depth — with the majority of photoemission coming from the depth up to 0.3 mm [13]. Thus, most of the fluorescence comes from the epidermis, which lacks a direct blood supply. Nutrients and oxygen are delivered to the epidermis directly from dermis and via dermal microcirculation [3,13]. Figure 2 outlines the penetration of UV light and the origin of the fluorescence derived from NADH in human skin.

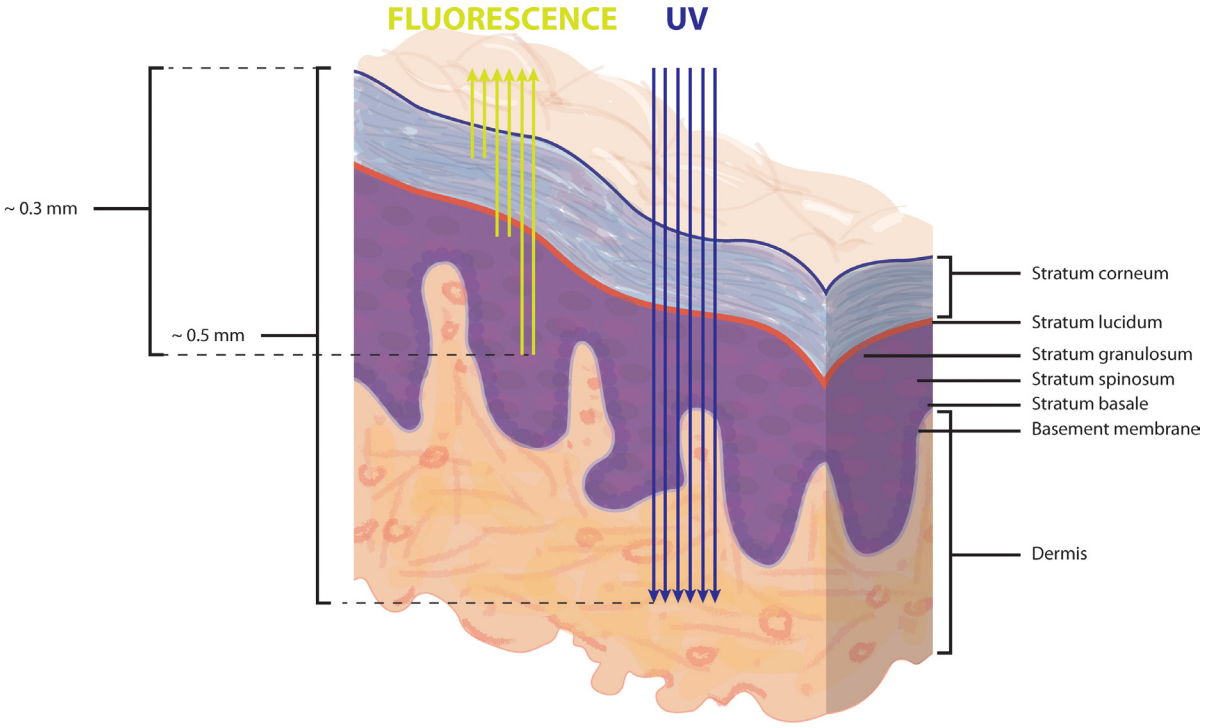

Figure 2. Penetration of the ultraviolet (UV) light through different skin layers and the source of the fluorescent light specific for NADH. Maximum penetration is about 0.5 mm, but the majority of the autofluorescence typical for NADH comes from the depth up to 0.3 mm

Dynamic measurement of skin NADH content during ischaemia and reperfusion challenge

Cellular energy production in aerobic conditions depends on oxygen availability. During transient ischaemia, blood flow is ceased, leading to a decrease in oxygen supply. This leads to cellular metabolism switching into the anaerobic mode. The electron transfer through the electron transport chain stops [14], the activity of mitochondrial Complex I (NADH dehydrogenase), responsible for the NADH oxidation to NAD+, decreases and then ceases [15,16]. As a result NADH accumulates in mitochondria. Restoration of oxygen delivery to mitochondria and its matrix rapidly reverses this process. Complex I activity recovers and NADH is immediately oxidised to NAD+. Therefore, following ischaemia-induced hypoxia, reperfusion induces a rapid decline in NADH concentration [15,17].

The phenomenon of NADH accumulation during ischaemia and its reduction during reperfusion has been used by Piotrowski et al. to measure the dynamic changes in the NADH accumulation using the UV excitement and fluorescent emission method [18]. The method — termed Flow Mediated Skin Fluorescence (FMSF) — involves continuous measurement of 460-nm fluorescence during ischaemia and reperfusion challenge. More specifically, the proposed FMSF method studies the dynamic changes in the 460-nm fluorescence after an earlier 340-nm ultraviolet excitation of the human forearm skin during a TIAR. Figure 3 shows a typical curve produced during FMSF plotting the intensity of photo emissions during an ischaemia-reperfusion episode.

During ischaemia, there is a gradual increase until the plateau of the 460-nm fluorescence intensity. The restoration of blood flow leads to a rapid decline in the strength of this signal (Figure 3) followed by its gradual recovery to the reference values. There are some grossly visible oscillations of the 460-nm fluorescence. Those are present at rest and reperfusion but not during ischaemia [19] (Figure 4). Their nature is unclear and under investigation. The FMSF method is simple, fast and allows for real-time, dynamic and non-invasive measurement. The AngioExpert device (manufactured by Angionica in Lodz, Poland) is currently the only available device employing the FMSF method.

Measurement of the flow-mediated skin 460-nm fluorescence

To record the FMSF with the AngioExpert device, the forearm of a study participant is immobilised

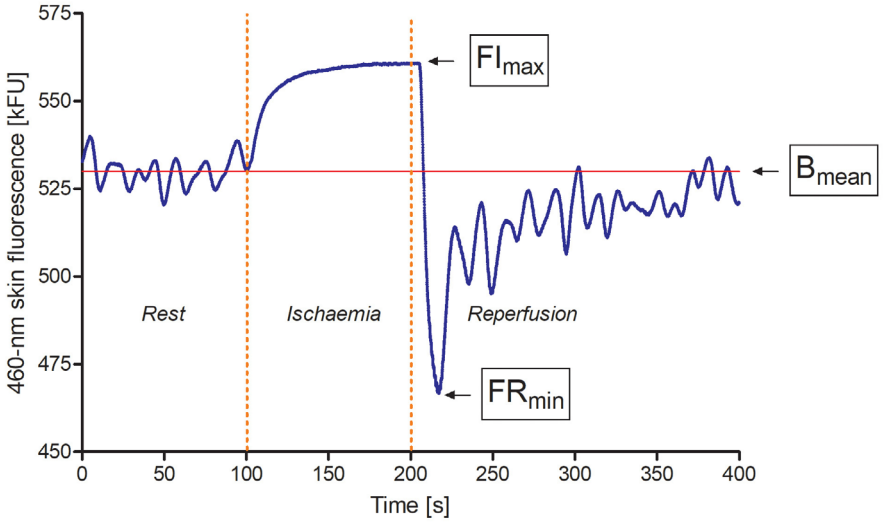

Figure 3. Typical changes of 460-nm forearm skin fluorescence during rest, ischaemia and reperfusion. At rest, NADH content is relatively stable, although some fluctuations are visible. During ischaemia, there is a rapid increase in NADH content. Reperfusion leads to fall of NADH, followed by slow recovery to the baseline level. For the abbreviations, please refer to the main text

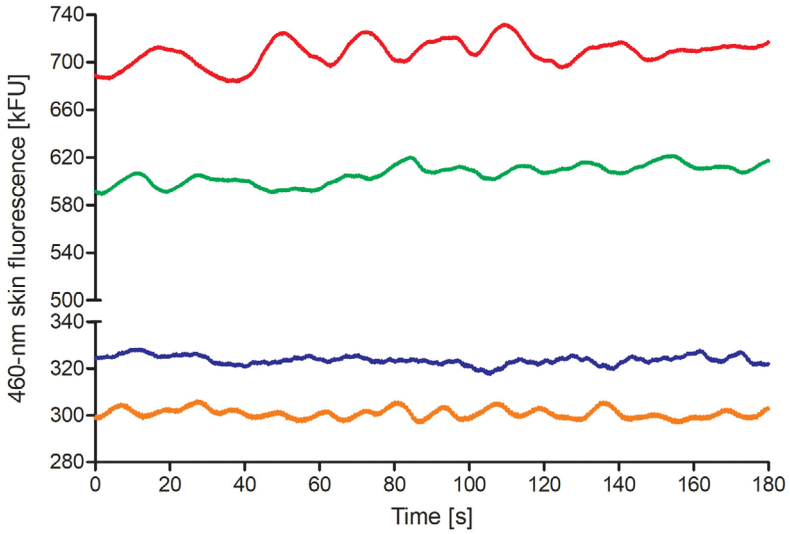

Figure 4. Four samples of the 460-nm fluorescence intensity and oscillations recorded in different young and healthy volunteers resting in a sitting position. There are natural differences in the baseline 460-nm fluorescence intensity between different people, depending for example, on the melatonin amount in the skin. All oscillations of different amplitude and frequency occur during normal blood flow. These oscillations disappear when blood supply is ceased during ischemia (please compare Figure 4 and Figure 6)

in special support to minimise recording noise. To induce TIAR, a deflated brachial cuff is placed on the studied arm for the baseline recording, and then it is inflated to 60 mmHg above Systolic Blood Pressure (SBP) for at least 100 seconds (in some studies 300 seconds [20]) to induce transient and controlled ischaemia. To allow for reperfusion, the brachial cuff is rapidly and wholly deflated.

The fluorescence data is obtained from a UV emitter-sensor device. The forearm completely covers the UV light emitter and fluorescence sensor. As the UV sensor is sensitive to movement, the study participant is required to remain still, and the forearm support is employed to improve the quality of readings. An example recording of 100-second ischaemia is shown in Figure 4. This procedure is safe and commonly applied in human studies [21,22], including both studies on healthy volunteers and studies on patients with an active disease process.

To quantify the FMSF response, we have proposed and use the following parameters [19,21]:

- Bmean [kFU] – mean fluorescence at 460 nm recorded before each ischaemia as the baseline value;
- FImax [kFU] – the maximal 460-nm fluorescence that increased above the baseline during each controlled forearm ischaemia;
- FRmin [kFU] – the minimal 460-nm fluorescence after decreasing below the baseline during each reperfusion;
- Imax [kFU] – the difference between FImax and Bmean;
- Rmin [kFU] – the difference between Bmean and FRmin;
- IRampl [kFU] – the maximal range of the 460-nm fluorescence change during ischaemia and reperfusion;
- Clmax – Imax/IRampl ratio showing the relative contribution of the NADH increase during ischaemia to the maximal change in NADH during TIAR.

So far, the FMSF method has been applied in several studies, including both healthy people and patients with various diseases. Table 1 summarises all available publications describing this method.

Literature analysis and our current experience helped us identify some limitations of the FMSF method, which we describe below.

Limitations related to the 460-nm fluorescence method of measuring NADH

Skin properties

Skin parameters vary between individuals and ethnic groups, and it appears that the fluorescence intensity is affected by the melanin content of the skin. The percentage melanin content of the basal layer of the epidermis varies between 1% and 43% [23]. The study by Dremin and Dunaev suggests that the fluorescence signal decreases as the skin pigment increases [24]. Thus, comparing the results of skin fluorescence between participants from different ethnic groups at nominal value is inappropriate without appropriate statistical modelling that would account for this confounder. Additionally, the fluorescence signal is affected by epidermis thickness which varies between body sites. Epidermis thickness measured by Robertson and Rees using confocal microscopy differs between 55.6 µm (upper back) to 62.5 µm (back of the hand) [25]. Therefore, data comparison and pooling can only be attempted with measurements taken at the same body site.

NADH/NAD+ balance

The 460-nm fluorescence measures only NADH and not NAD+ content [3]. NAD+ does not possess optic properties similar to NADH and so does not allow for direct quantity measurement. However, in a short period, it is usually assumed that the total amount of NAD (sum of NADH and NAD+) remains close to constant [3]. Klein et al. confirmed the assumption in their study on dogs where the entire NAD content remained constant during the first 30 minutes of ischaemia [26]. However, during more extended ischaemia of over 30 minutes, the total NAD content depletes, and the interpretation of NADH/NAD+ becomes less reliable.

Uncertainty about cellular NADH origin

As already mentioned, NADH is present not only in the mitochondria but also in the cytoplasm and the nucleus. The 460-nm fluorescence measures total tissue or cellular NADH content. However, as shown by Anderson-Engels and Wilson, the 460-nm fluorescence, at least at resting aerobic conditions, comes primarily from mitochon-

drial NADH [3,27] with negligible input from the cellular NADH. This is supported by many other studies on various tissues [3,28,29]. It is therefore assumed that the 460-nm fluorescence informs about the redox state of mitochondrial NADH. It is also uncertain what is the main source of the 460-nm fluorescence increase — NADH from cytoplasm generated by glycolysis or NADH from the mitochondrial matrix as it cannot be oxidised to NAD+ as hypoxia/anoxia stop the function of the electron transport chain. Regardless the processes which have the largest contribution to the NADH increase during ischaemia, it is more certain that the sudden drop in its content during reperfusion is caused by rapid oxidation of this molecule to NAD+ in the mitochondrial matrix. In other words, the NADH drop during reperfusion appears to be mostly, if not exclusively, related to the function of the electron transport chain. For this reason, the rapid reduction in NADH content during reperfusion is a valuable index of the restoration of mitochondrial function after earlier ischaemia.

Uncertainty about the mitochondrial pool of NADH

NADH in mitochondria is divided into two pools: free NADH and protein-bound NADH. Protein-bound NADH makes up around 35% of the total NADH while contributing to almost 80% of the fluorescence signal [30]. Blinova et al. showed that the binding site of NADH molecules influences the intensity of NADH autofluorescence in mitochondria [31]. When NADH binds to the Complex 1, the NADH fluorescence is enhanced tenfold, but when it binds to other matrix enzymes, such as lactate dehydrogenase, then this fluorescence increases only 1.5–2 fold. The FMSF method does not inform about NADH relocation inside mitochondria.

Spontaneous oscillations of the 460-nm fluorescence

As already mentioned and shown in Figure 5, the resting 460-nm fluorescence is dynamic, with clearly visible oscillations. Their nature is unknown, they disappear during ischaemia and return, usually with a higher amplitude, during reperfusion. These oscillations are currently investigated by Angionica, i.e. the manufacturer of the AngioExpert device.

Measurement of the skin fluorescence as an index of microvascular function

Most of the studies on the post-UV skin 420–480 nm fluorescence repeatedly show that this method measures NADH content and mitochondrial function [3,8,10-13,18,19,21,23,24,28-32]. It is evident that mitochondrial function and oxidation of NADH to NAD+ strictly depend on the blood flow through circulation and supply of oxygen. In other words, less available oxygen impairs mitochondrial function and attenuates NADH oxidation. Mayevsky has suggested that the monitoring of NADH by the autofluorescence also reflects the role of microvascular circulation [3,11,12]. For this reason, it is never definite what the most probable cause of the observed change in NADH skin content — mitochondria, microcirculation or both is? In some studies, the 460-nm fluorescence is only used for measuring the microvascular circulation [22,33-36].

Limitations related to the FMSF method

Forearm skin as the target body part for studying

This method, due to the construction of the measuring table and forearm support, allows measuring the FMSF in the forearm only. Although the 460-nm fluorescence can be measured anywhere in the skin or other tissues [3], the Angio-expert device allows measuring the FMSF signal only using one of two forearms.

Atypical response

Typical changes of the FMSF signal occurring during ischaemia and reperfusion in healthy individuals are presented in Figure 4 and Panel A of Figure 5. However, sometimes there are atypical FMSF responses noted in healthy people. Some examples of such atypical FMSF curves are shown in panels B, C and D in Figure 5.

Most commonly, the atypical FMSF response is limited to the ischaemic part of the recording. So far, there is no clear explanation for this phenomenon.

Ambiguous and variable duration of the ischaemic challenge

For the FMSF method, the optimal time for ischaemia duration is not established, and differ-

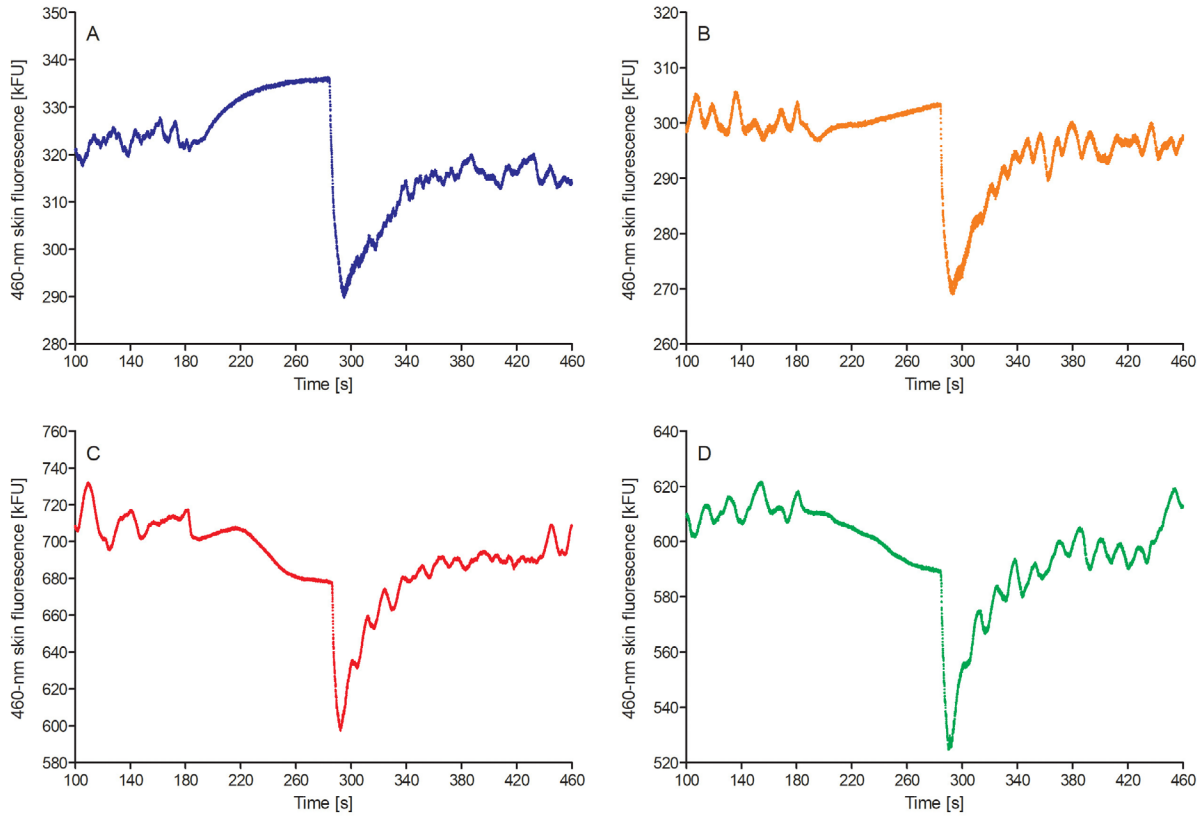

Figure 5. Examples of 460-nm skin fluorescence responses during ischaemia-reperfusion. Graph A represents the typical response, with an increase of FMSF signal during ischemia, its rapid fall during reperfusion, followed by restoration of the fluorescence to the baseline level. Graph B, C and D represent atypical responses found in young, healthy individuals

ent times are applied in various studies ranging from 100 seconds to 300 seconds [20]. A significant factor limiting ischaemia duration is numbness onset in the studied arm and forearm during ischaemia. Some patients may report pain of different severity, usually mild. Most commonly, however, the sensation experienced during the reperfusion phase is described by the participants as a transient feeling of warmth and itching lasting for 15–30 seconds. For these reasons, it is important to inform the participant about the potential uncomfortable symptoms which are, fortunately, short-lasting, tolerable and carry no risk.

Ambiguous interpretation and terminology of the reperfusion phase

The term proposed by the Angionica for the reperfusion phase was hyperaemia. Undoubtedly, as shown in Figure 6, during the first 20 seconds of reperfusion, there is a transient increase in the blood flow through the microcirculation. However, normalisation of the blood flow to the reference level is much faster than the normalisation of FMSF signal, which takes about 2–5 minutes to recover to the baseline level. It suggests that terming the entire reperfusion phase as "hyperaemia" is not right to the physiological phenomena occurring.

So far, most of the published studies with FMSF employ the prototype AngioExpert device, which is under development. Although there were no significant changes made to the construction, UV light emitter and fluorescent sensor, the proposed parameters used to quantify the FMSF were changing over time. This is a natural consequence of a very young method, and for this reason, there are significant differences in the definitions and names of parameters describing the same FMSF curve. On the other hand, Angionica appears flexible and puts no limits for the use of different descriptors (see Figure 4, compare with studies listed in Table 1).

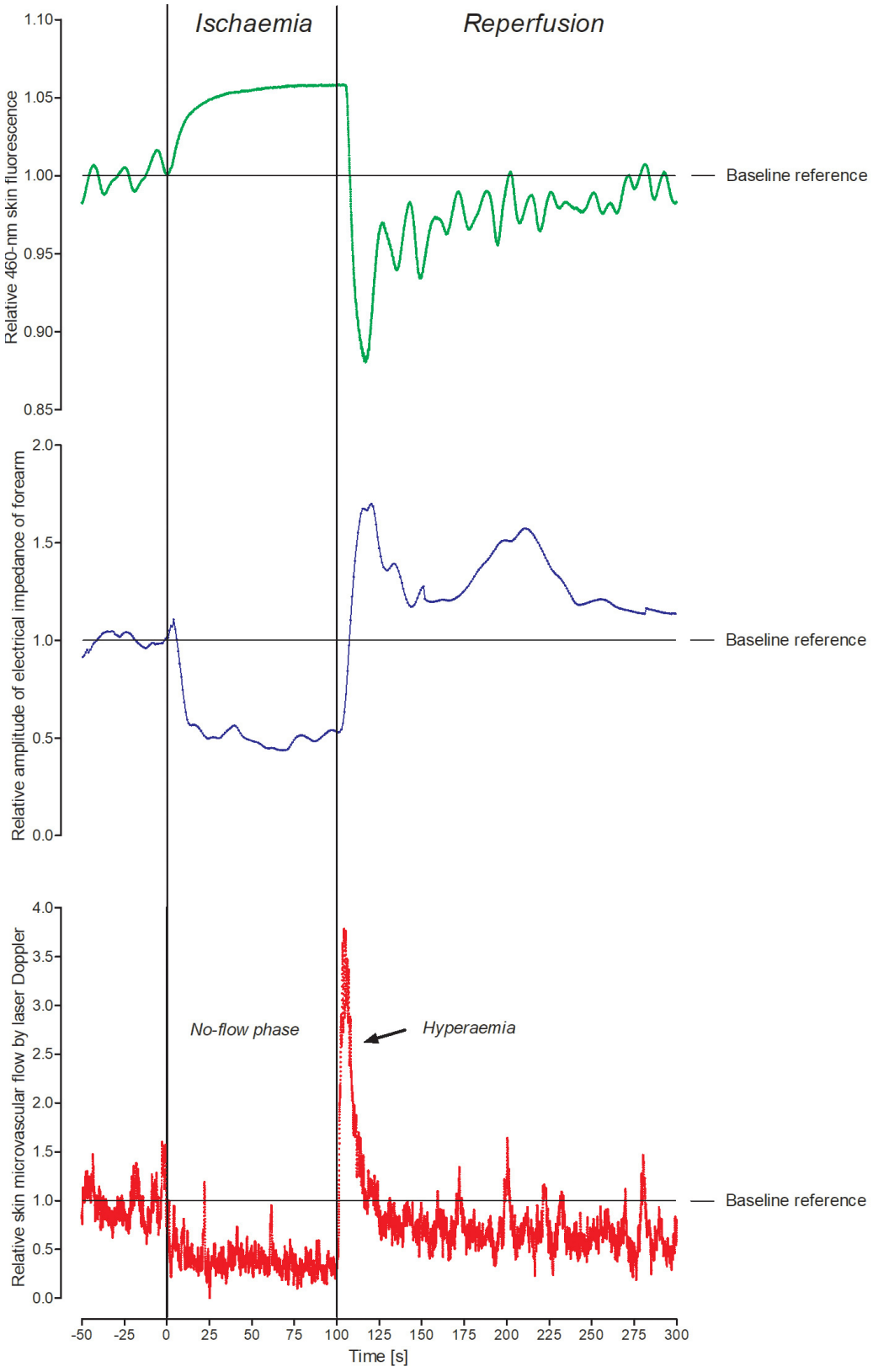

Figure 6. Example of simultaneous recording of FMSF, electrical impedance (showing the amount of water in the forearm) and laser Doppler (presenting the blood flow through skin microcirculation) during ischemia-reperfusion. The signals are synchronised according to the beginning and end of ischaemia. As shown, the dynamics of the changes in all three signals are not identical. It means that the FMSF signal is not a simple derivative of blood flow as assumed by some other authors [19,21,23,26]. FMSF is a more complex signal influenced by both NADH metabolism in mitochondria, the function of skin microcirculation and probably some other, yet unknown factors (e.g. water accumulation in the skin)

Table 1. Summary of studies using the FMSF method

Authors	Studied subjects	Summary
Piotrowski et al. [22]	11 healthy volunteers and 11 patients with cardiovascular diseases	FMSF curves have different characteristics between healthy volunteers and patients with cardiovascular disease
Hellman et al. [20]	18 healthy volunteers and 18 patients with coronary artery disease	Reduction of the value of NADH descriptors during reperfusion in patients with coronary artery disease compared with healthy subjects.
Tarnawska et al. [24]	28 patients with coronary artery disease	Ischaemic and reperfusion parts of the FMSF curves are blunted in patients with advanced coronary artery disease and diabetes. NADH fluorescence in patients with CAD is associated with plasma endothelial markers.
Bugaj et al. [21]	121 highly trained athletes	Exercise to exhaustion increases the skin NADH content at rest, during ischaemia and reperfusion but reduces the magnitude of NADH increase during ischemia both on men and women.
Katarzynska et al. [25]	31 healthy volunteers and 40 patients with type 1 diabetes (DM1)	The 460-nm fluorescence drop during reperfusion is weaker in patients with type 1 diabetes than in healthy people. This reperfusion-induced drop of fluorescence attenuates with age in diabetic patients.
Bogaczewicz et al. [26]	34 healthy volunteers and 36 patients with systemic lupus erythematosus	Patients with systemic lupus erythematosus have a reduction restoration of NADH during reperfusion compared to healthy people.
Majewski et al. [27]	20 healthy volunteers, 23 patients with asthma, 26 patients with chronic obstructive pulmonary disease (COPD)	The 460-nm fluorescence increase during ischaemia is reduced in patients with COPD patients compared with healthy people. The 460-nm fluorescence drop during reperfusion is weaker in COPD patients compared to asthma and healthy subjects.
Sibrecht et al. [19]	One healthy individual	Hyperaemic phase (measured by laser Doppler) of post-ischaemic reperfusion lasts app 20 seconds while the fluorescence signal during reperfusion recovers to baseline after over 200 seconds. Flow-related changes in the microvascular circulation contribute only to the early phase of reperfusion part of the FMSF curve.
Sibrecht et al. [28]	99 healthy volunteers	Post-ischaemic preconditioning reduces NADH skin content and its increase during the following ischaemia episodes
Nizinski et al. [29]	58 healthy volunteers	An increased BMI is accompanied by a faster and higher increase of NADH during ischaemia and a delayed NADH recovery during reperfusion.
Filberek [30]	99 healthy volunteers	Men have more intense skin fluorescence during ischaemia and higher contribution of ischaemia to whole fluorescence change (CI_{max}).

Conclusion

The 460-nm fluorescence intensity reflects the skin NADH content, which is mainly oxidised in mitochondria. However, mitochondrial function strictly depends on the oxygen supply by the skin microcirculation. Therefore it remains uncertain whether the dynamic alterations in this fluorescence recorded by the FMSF during TIAR reflect the function of either mitochondria or microvascular circulation or most probably both.

The FMSF is a unique scientific method to study the skin mitochondrial NADH content and its dynamic changes during transient and controlled ischaemia and reperfusion. This method is non-invasive, allows measuring NADH changes in a real-time and can be applied in many clinical and physiological scenarios. Although the FMSF has some limitations, its non-invasive character and scientific potential seem to merit further investigation. One of the most attractive features of this method is an opportunity to study mitochondrial function and/or microvascular circulation during the clinically relevant challenge such as ischaemia and reperfusion. Another interesting feature is that the reduction in the 460-nm fluorescence during the reperfusion reflects the restoration of mitochondrial function after earlier ischaemia. For many reasons, the FMSF appears to have a lot of potential for implementation in physiological, clinical and pharmacological studies.

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Conflict of interest statement
The authors declare no conflict of interest.

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