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RESEARCH ARTICLE

Fluorescence Spectroscopy With Temperature Functional Tests in the Assessment of Markers of Intracellular Energy Metabolism: Spatial Heterogeneity and Reproducibility of Measurements

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ABSTRACT

The fluorescence intensities of the cellular respiratory cofactors NADH (reduced nicotinamide adenine dinucleotide) and FAD^{++} (oxidized flavin adenine dinucleotide) reflect energy metabolism in skin and other tissues and can be quantified in vivo by fluorescence spectroscopy (FS). However, the variability of physiological parameters largely determines the reproducibility of measurement results and the reliability of the diagnostic test. In this prospective study, we evaluated the interday reproducibility of NADH and FAD^{++} fluorescence intensity measurements in the skin of 51 healthy volunteers assessed by the FS at baseline, after local cooling (10°C) and heating of the skin (35°C). Results showed that the fluorescence amplitude of NADH (AF_{NADH}) in forearm skin was the most reproducible of the FS parameters studied. Assessment of AF_{NADH} in the dorsal forearm in combination with a thermal functional test is the most promising method for clinical use for assessing energy metabolism in the skin.

1 | Introduction

Energy metabolism is a collective concept that includes a set of chemical reactions for energy production in the form of adenosine triphosphate (ATP) and other high-energy compounds, including glycolysis, the tricarboxylic acid cycle and oxidative phosphorylation processes in mitochondria. The energy of macroergic compounds is spent on various vital processes (maintenance of membrane potential, protein synthesis, muscle contraction, etc.). One of the results of these biochemical processes is the production of heat, the amount of which characterizes the basal metabolic rate [1, 2].

The gold standard for assessing basal metabolic rate is indirect respiratory calorimetry by measuring pulmonary gas exchange (volume of consumed O_2 and produced CO_2) [3]. Despite its value in assessing basal metabolic rate in intensive care [4], obesity and thyroid dysfunction [5, 6], the method is costly and difficult to use in clinical routine.

Fluorescence spectroscopy (FS) of endogenous fluorophores is one of the modern methods of assessing tissue metabolism. FS is based on measuring the intensity of autofluorescence of a number of substances (fluorophores) present in biological tissues [7]. In particular, this method is used to noninvasively

assess the fluorescence intensity of cellular respiratory cofactors, NADH (reduced nicotinamide adenine dinucleotide) and FAD^{++} (oxidized flavin adenine dinucleotide), in skin and other tissues [8, 9]. The excitation spectrum of NADH lies in the range of 300–400 nm with a peak at ~355 nm, the emission spectrum lies in the range of 400–600 nm with a maximum at ~470 nm, while for FAD^{++} the excitation spectrum lies in the range of 350–500 nm with two maxima—at 370 and 450 nm, the emission spectrum lies in the range of 500–600 nm with a maximum at ~525 nm [10].

NADH and FAD^{++} play important roles in a set of cellular oxidation–reduction reactions [11], including being electron carriers in the tricarboxylic acid cycle (Krebs cycle), thereby participating in ATP synthesis [9]. The quantitative analysis of the fluorescence intensity of these coenzymes allows an indirect assessment of the tissue's metabolic activity, which determines the diagnostic value of this method in different pathological conditions [12].

To date, FS is widely used in a number of medical fields [13], such as for noninvasive or minimally invasive tissue assessment in oncology [14–16], transplantation [17], general surgery [18, 19], dermatology [20, 21], and so forth. In addition, NADH and FAD^{++} fluorescence parameters measured by FS in the skin (in vivo and in situ) have applications in socially important systemic diseases such as coronary heart disease (CHD) [22], hypertension [23], diabetes mellitus (DM) [24], including chronic kidney disease [25].

In hypoxia, NADH is not oxidized to nicotinamide adenine dinucleotide (NAD^{+}), leading to a cellular accumulation of NADH [26]. Cellular NADH has been shown to increase in patients with CHD and aortic stenosis [27], newly diagnosed primary hypertension [23], and chronic obstructive pulmonary disease [28]. NADH fluorescence has also been found to be significantly higher in DM compared with healthy volunteers [29].

However, FS has a number of limitations that should be considered when using this method for diagnostic purposes. Biological tissues, and skin in particular, are complex heterogeneous media and therefore it is almost impossible to isolate the contribution of signals from different fluorophores (NADH, FAD^{++}, porphyrin, melanin, collagen, etc.). To overcome this limitation, several signal processing algorithms have been developed to evaluate the influence of individual fluorophores on the fluorescence intensity of biological tissue [30] or to determine the percentage contribution of components to the overall signal [19, 31, 32]. Environmental factors (ambient temperature and light intensity) and biological characteristics of the skin (color and heterogeneity of the microvascular bed) can cause significant variability in FS parameters [33, 34].

The variability of physiological parameters largely determines the reproducibility of measurement results and the reliability of the diagnostic test [35]. Unsatisfactory reproducibility of physiological measurements reduces the reliability of the diagnostic test.

The use of functional tests allows to obtain additional diagnostic information and detect pathological processes that are compensated at rest [29]. In particular, temperature functional tests (local heating and local cooling) are used to assess skin microcirculatory reactivity [36]. It is known that cooling slows down the metabolic rate and leads to a decrease in the production and utilization of ATP, and a decrease in the utilization of NADH, leading to a transient accumulation of NADH in cells [37]. Conversely, heating increases the metabolic rate by promoting NADH utilization and decreasing its intracellular concentration [2]. Thus, the combination of FS with temperature tests is a promising method for assessing energy metabolism in the skin in clinical medicine [29]. Quantitative assessment of the reproducibility of FS of NADH and FAD^{++} in skin with temperature tests is necessary for subsequent validation of their use in various clinical applications.

The primary objective of the study was to evaluate the interday reproducibility of NADH and FAD^{++} fluorescence intensity measurements in the skin of healthy volunteers assessed by the FS at rest and during local temperature tests. We also compared the investigated parameters on different sites of the forearm.

2 | Patients and Methods

2.1 | Ethical Approval

The study was conducted at the Department of Endocrinology, University Clinical Hospital No. 2, I.M. Sechenov First Moscow State Medical University, Moscow, Russia. The study protocol was approved by the local Ethics Committee (protocol No. 02-23, of January 26, 2023). All participants provided written informed consent.

2.2 | Study Population

Fifty-one healthy volunteers (26 ± 4 years, 35 females, and 16 males) were included in the study. Persons with a history of cardiovascular disease (hypertension, CHD, valvular heart disease, and heart failure), endocrine disease (type 1 and 2 DM, obesity, thyroid, and adrenal disease), skin disease (scleroderma, systemic lupus erythematosus, trauma, and scarring of the skin in the measurement area), anemia, and severe systemic disease (e.g., cancer, autoimmune disease) were not included. Participants with fever, symptoms of acute viral respiratory infection, and vigorous exercise on study day, and those taking medications or supplements that alter vascular function were excluded. Smoking and time of last meal and caffeinated drinks were recorded on the case report form.

2.3 | Study Design

This prospective experimental clinical study involved two visits 3–7 days apart. NADH and FAD^{++} fluorescence intensity signals were assessed in the skin of the dominant forearm sequentially on the dorsal and ventral sides at baseline and during local heating and cooling on the first (D1) and next day (D2).

Before recording the FS parameters, the main anthropometric and physiological parameters were assessed: systolic, diastolic, and mean arterial pressure using an automated

sphygmomanometer «OMRON M2 Classic» (OMRON HEALTHCARE Co., Ltd., Japan); heart rate (HR); arterial blood oxygen saturation (S_pO_2) by a ChoiceMMed finger pulse oximeter (Beijing Choice Electronic Technology Co., Ltd., China); axillary body temperature by an electronic thermometer «OMRON Eco Temp Basic» (OMRON HEALTHCARE Co., Ltd., Japan); body mass index (BMI) according to the Quetelet formula. All measurements were performed in the first half of the day (09:00 a.m.–12:00 p.m.) in a room with thermoneutral conditions (22–26°C) in a seated position after a 15-min acclimatization period.

The «LAZMA ST» system (SPE LAZMA Ltd., Russia) and its software version 3.2.0.475 were used to register NADH and FAD^{++} fluorescence intensity signals and to perform functional local temperature tests. The 3-mm optic probe combined with the temperature probe of the «LASMA ST» system was placed on the dorsal and ventral sides of the dominant forearm (in the wristwatch area) and fixed to the skin with a tape, avoiding excessive pressure on the investigated area. Light sources (laser LEDs) with emission wavelengths of 365 and 450 nm were used to excite NADH and FAD^{++} fluorescence, respectively. A multioptical fiber probe was used to guide exciting light and collect backscattered light from the tissue. The diameters of the probing and receiving fibers are 400 μm. Skin fluorescence amplitudes at wavelengths of 460 nm (taken as NADH fluorescence—AF_{NADH}) and 515 nm (taken as FAD^{++} fluorescence—$AF_{FAD^{++}}$) were measured and normalized to the amplitudes of backscattered light at wavelengths 365 and 450 nm, respectively. AF_{NADH} and $AF_{FAD^{++}}$ are dimensionless quantities.

The diagnostic protocol described in the «LAZMA ST» system user manual was used in this study. Each examination included measurement of baseline values of parameters AF_{NADH} and $AF_{FAD^{++}}$ on the dorsum of the forearm (4 min), followed by measurement of these variables during local cooling (10°C for 1 min) and heating of the skin (35°C for 4 min). After this, the same measurements were taken on the ventral side of the same forearm. AF_{NADH} and $AF_{FAD^{++}}$ values were recorded at the end of the baseline and local temperature tests measurements. These are referred to in the text with the corresponding protocol stage indices: AF^{base}_{NADH}–NADH fluorescence amplitude at baseline; $AF^{base}_{FAD^{++}}$–FAD^{++} fluorescence amplitude at baseline; $AF^{10^{\circ}C}_{NADH}$–NADH fluorescence amplitude after cooling to 10°C; $AF^{10^{\circ}C}_{FAD^{++}}$–$FAD^{++}$ fluorescence amplitude after cooling to 10°C; $AF^{35^{\circ}C}_{NADH}$–NADH fluorescence amplitude after heating to 35°C; $AF^{35^{\circ}C}_{FAD^{++}}$–$FAD^{++}$ fluorescence amplitude after heating to 35°C. The study flow diagram is shown in Figure 1.

2.4 | Statistical Analysis

Statistical analysis of data was carried out using IBM SPSS Statistics software (IBM SPSS Statistics for Windows, Version 27.0.1 Armonk, NY: IBM Corp) and Statistica 12 (StatSoft, USA). The type of data distribution was assessed using the Shapiro–Wilk test. Since most of the studied variables had a distribution other than normal, nonparametric statistics methods were used. Data are expressed as the median and interquartile range. The difference between males and females was estimated by the Mann–Whitney test. Comparison of values on the dorsal and ventral sides of the forearm, as well as assessment of the dynamics of fluorescence amplitudes during local temperature tests were carried out using the Wilcoxon matched pairs test. A level of $p < 0.05$ was considered significant.

Interday reproducibility was expressed in intersubject coefficients of variation (CV) and intraclass correlation coefficients (ICC) [38]. The CV values were calculated using the root mean square method. CV values less than 25% were considered acceptable. ICC values were assessed using a two-factor mixed approach (model—alpha, type—absolute agreement), with ICC values less than 0.40, 0.40 to 0.75, and more than 0.75 considered unsatisfactory, fair-to-good and excellent, respectively [39, 40]. This parameter characterizes the repeatability of measurements for each individual.

3 | Results

The general characteristics of the study participants according to the main anthropometric and physiological parameters are shown in Table 1. During the study, six subjects were excluded from subsequent analysis due to significant deviations from the study protocol (measurements not taken on D2, unexpected illness, and technical reasons).

Males and females differed in weight, height, BMI, and blood pressure values, which were within the normal range (Table 2). No statistically significant differences were found in other physiological variables and FS results (Table 3).

Therefore, in subsequent data analysis, males and females were combined into one sample.

Spatial variability of FS parameters was assessed by comparing normalized fluorescence amplitudes (AF_{NADH} and AF_{FAD++}) in the skin of the dorsal and ventral sides of the forearm. The

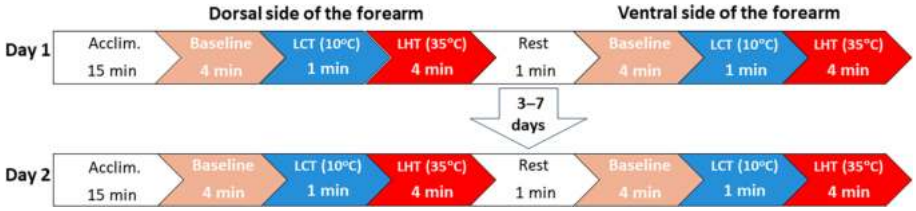

FIGURE 1 | Flow diagram of the study: Day 1—the first day of the study; Day 2—the next day of the study after 3–7 days. Acclim., acclimatization; LCT, local cooling test; LHT, local heating test.

values of AF_{NADH} and AF_{FAD++} were higher on the ventral side of the forearm than on the dorsal side, both in the baseline and during local cooling and heating tests (Figure 2).

After local cooling, the amplitude of NADH fluorescence increased compared with baseline values only on the ventral side of the forearm ($p = 0.005$), while on the dorsal side, the changes in this parameter were not statistically significant ($p = 0.35$) (Figure 2). After local heating, the amplitudes of NADH fluorescence decreased on both sides of the forearm compared with baseline values ($p < 0.001$) (Figure 2).

The amplitudes of FAD^{++} fluorescence, both after local cooling and after heating, were not statistically significantly different from the values in the baseline on the dorsal and ventral sides of the forearm ($p > 0.05$) (Figure 3).

TABLE 1 | Characteristics of the study group of healthy volunteers on the first day of the study (D1).

Variable	Value
Gender (male/female, number of participants)	16/35
Age (years)	25 [24; 26]
Body weight (kg)	64 [54; 76]
Height (m)	1.69 [1.62; 1.76]
BMI (kg/m^2)	23 [19; 25]
Systolic blood pressure (mmHg)	116 [107; 124]
Diastolic blood pressure (mmHg)	69 [64; 76]
Mean blood pressure (mmHg)	84 [79; 92]
Heart rate (1/min)	78 [74; 84]
S_pO_2 (%)	98 [97; 98]
Axillary body temperature (°C)	36.3 [36; 36.6]

Note: Data are expressed as the median and interquartile range.
Abbreviations: BMI, body mass index; S_pO_2, oxygen saturation of arterial blood.

Thus, a regular dynamics of NADH fluorescence was revealed during temperature tests: an increase during cooling and a decrease during heating compared with the baseline. However, as can be seen in Figure 4, in some subjects, the fluorescence amplitude at the excitation wavelength of 365 nm changed during temperature tests insignificantly or paradoxically (relative to the expected dynamics of the NADH coenzyme), for example, it decreased after cooling.

The studied FS parameters had different individual interday reproducibility of measurement results. Baseline NADH fluorescence amplitude on the dorsal and ventral forearm, NADH fluorescence amplitude during local cooling on the dorsal forearm, and FAD^{++} fluorescence amplitude during local heating on the dorsal forearm had the best reproducibility because they met two statistical conditions (CV < 25% and ICC > 0.4) (Table 4). At the same time, most of the studied FS parameters had acceptable reproducibility, assessed only by the criterion of CV < 25%. Additionally, absolute and relative differences (Δ and Δ%, respectively) between the values of AF_{NADH} and AF_{FAD++} during local cooling/heating and baseline values of these parameters were calculated (Table 4). These parameters had acceptable reproducibility according to the CV < 25% criterion, but had unsatisfactory reproducibility according to the ICC > 0.4 criterion on both sides of the forearm (Table 4).

4 | Discussion

Despite the widespread use of FS in research practice and the availability of various devices that implement this method and are recommended for medical diagnostics, there are still a number of unsolved problems, including the reproducibility of the data obtained and their interpretation.

When using skin FS in combination with functional tests, most studies evaluate the dynamics of NADH fluorescence amplitude during an occlusion test [23, 25, 28, 39]. In this study, we for the first time assessed the individual interday reproducibility of NADH and FAD^{++} fluorescence amplitudes in the skin of the forearm, measured by the FS method supplemented with local temperature tests. The choice of these tests

TABLE 2 | Anthropometric and physiological parameters of males and females on the first day (D1).

Variable	Females ($n = 31$)	Males ($n = 14$)	p
Body weight (kg)	59 [51; 65]	80 [77; 99]	**<0.001**
Height (m)	1.65 [1.60; 1.70]	1.83 [1.76; 1.93]	**<0.001**
BMI (kg/m^2)	22 [19; 24]	24 [23; 26]	**0.046**
Systolic blood pressure (mmHg)	112 [108; 120]	129 [120; 134]	**<0.001**
Diastolic blood pressure (mmHg)	66 [62; 72]	74 [70; 76]	**<0.001**
Heart rate (1/min)	75 [68; 80]	76 [64; 86]	0.856
S_pO_2 (%)	98 [97; 98]	98 [97; 98]	0.274
Axillary body temperature (°C)	36.4 [36.2; 36.6]	36.5 [36.1; 36.8]	0.421
Skin temperature at the FS measurement point (°C)	28 [27; 29]	28 [27; 31]	0.670

Note: p values are reported for males versus females (the Mann–Whitney test).
Abbreviations: BMI, body mass index; S_pO_2, oxygen saturation of arterial blood.

TABLE 3 | FS parameters of males and females on the first day (D1).

Variable	Females ($n=31$)	Males ($n=14$)	p
Dorsal side of the forearm			
AF^{base}_{NADH} (a.u.)	0.435 [0.390; 0.485]	0.432 [0.377; 0.485]	0.791
$AF^{10^\circ C}_{NADH}$ (a.u.)	0.437 [0.377; 0.476]	0.417 [0.391; 0.514]	0.419
$AF^{35^\circ C}_{NADH}$ (a.u.)	0.402 [0.358; 0.460]	0.379 [0.363; 0.476]	0.952
$AF^{base}_{FAD^{++}}$ (a.u.)	0,389 [0.351; 0.445]	0.487 [0.405; 0.536]	0.077
$AF^{10^\circ C}_{FAD^{++}}$ (a.u.)	0.860 [0.350; 0.429]	0.479 [0.390; 0.522]	0.112
$AF^{35^\circ C}_{FAD^{++}}$ (a.u.)	0.375 [0.340; 0.435]	0.447 [0.378; 0.509]	0.076
Ventral side of the forearm			
AF^{base}_{NADH} (a.u.)	0.480 [0.450; 0.543]	0.447 [0.403; 0.532]	0.181
$AF^{10^\circ C}_{NADH}$ (a.u.)	0.500 [0.448; 0.553]	0.459 [0.399; 0.553]	0.112
$AF^{35^\circ C}_{NADH}$ (a.u.)	0.457 [0.430; 0.511]	0.438 [0.379; 0.547]	0.763
$AF^{base}_{FAD^{++}}$ (a.u.)	0.542 [0.449; 0.652]	0.539 [0.446; 0.613]	1
$AF^{10^\circ C}_{FAD^{++}}$ (a.u.)	0.513 [0.445; 0.632]	0.472 [0.438; 0.656]	0.140
$AF^{35^\circ C}_{FAD^{++}}$ (a.u.)	0.500 [0.443; 0.670]	0.545 [0.416; 0.662]	0.637

Note: AF^{base}_{NADH}–NADH fluorescence amplitude at baseline; $AF^{base}_{FAD^{++}}$–FAD^{++} fluorescence amplitude at baseline; $AF^{10^\circ C}_{NADH}$–NADH fluorescence amplitude after cooling to 10°C; $AF^{10^\circ C}_{FAD^{++}}$–$FAD^{++}$ fluorescence amplitude after cooling to 10°C; $AF^{35^\circ C}_{NADH}$–NADH fluorescence amplitude after heating to 35°C; $AF^{35^\circ C}_{FAD^{++}}$–$FAD^{++}$ fluorescence amplitude after heating to 35°C. Data are expressed as the median and interquartile range. p values are reported for males versus females (the Mann–Whitney test).

was based on their noninvasiveness, relative ease of implementation, and frequent use in medical research to diagnose microvascular disorders [29, 36, 41]. Local heating can induce an increase in cutaneous blood flow, and cooling can cause a decrease in the blood flow compared with the resting state [36, 42], thereby influencing metabolism [43]. It is known that the rate of enzymatic reactions, including redox processes, is in a certain dependence on tissue temperature: the rate of biochemical processes increases by two times with an increase in temperature by every 10°C and slows down by two times with a decrease in temperature by 10°C [2].

In our study, the spatial variability of FS parameters was manifested by a higher intensity of NADH and FAD^{++} fluorescence in the skin of the ventral side of the forearm compared with the dorsal side. However, it is unlikely that the identified differences are due to real differences in the rate of metabolic processes in different parts of the forearm. A more likely reason for the differences in fluorescence intensity is the different optical properties of the skin of the dorsal and ventral sides of the forearm (including the thickness of the dermis, hair density, content of melanin, and other pigments). With the same diagnostic volume, the smaller thickness of the dermis on the ventral side of the forearm can lead to the involvement of subcutaneous layers in the studied tissue volume [44]. The influence of skin melanin levels on FS results was studied by Dremin and Dunaev [30], showing that the fluorescence amplitudes of NADH and FAD^{++} decreased with increasing levels of this pigment. Therefore, when using the method clinically to compare FS parameters

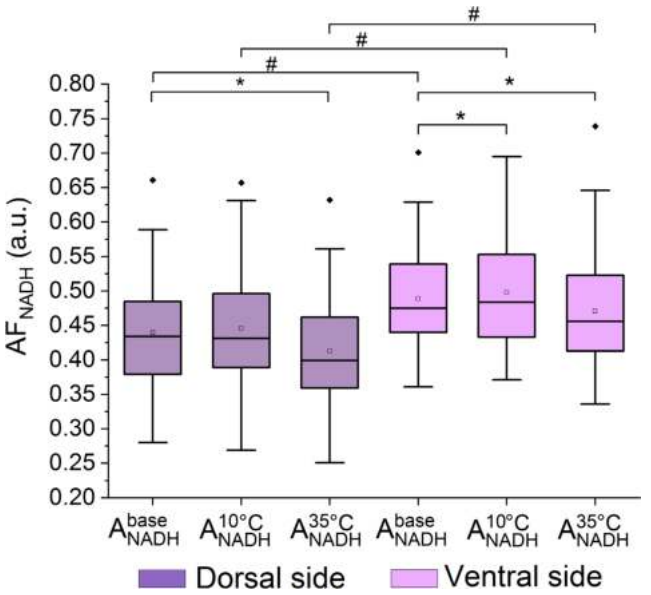

FIGURE 2 | Dynamics of NADH fluorescence amplitudes during temperature tests on the dorsal and ventral sides of the forearm on the first day (D1). AF^{base}_{NADH}–NADH fluorescence amplitude at baseline; $AF^{10^\circ C}_{NADH}$–NADH fluorescence amplitude after cooling to 10°C; $AF^{35^\circ C}_{NADH}$–NADH fluorescence amplitude after heating to 35°C. $^*p<0.05$ versus baseline (the Wilcoxon matched pairs test). $^\#p<0.001$ for dorsal side vs ventral side (the Wilcoxon matched pairs test). In each box, the central line is the median of the group, while the edges are the 25th and 75th percentiles.

over time in an individual, it is necessary to carry out measurements in the same area of the body.

In this study, it was shown that skin FS parameters AF^{base}_{NADH}, $AF^{10^\circ C}_{NADH}$, and $AF^{35^\circ C}_{FAD^{++}}$ on the dorsum of the forearm and AF^{base}_{NADH} on the ventral side had the best reproducibility, since they met both reproducibility criteria (CV and ICC). However, given the reliability of changes in fluorescence intensity during temperature testing, the most promising approach for clinical use is to measure the normalized amplitude of NADH fluorescence in forearm skin at rest and during a local heating test. Statistically significant changes in the AF_{NADH} during the cold test were detected only in the skin of the ventral side of the forearm. Depending on the rate and extent of local cooling, transient vasodilation and increased skin perfusion may occur [42], possibly counteracting the expected reduction in NADH utilization during cooling. At the same time, we did not detect statistically significant changes in the AF_{FAD++} in the skin of the forearm during both cold and heat tests, which indicates the low suitability of this parameter for assessing energy metabolism in clinical functional diagnostics.

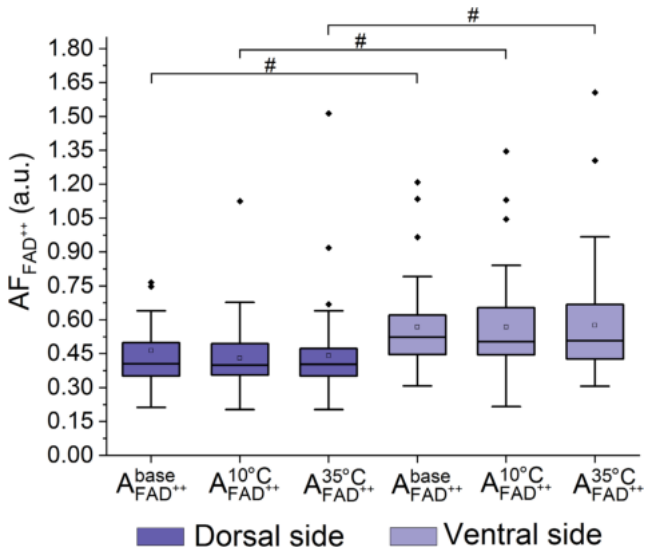

FIGURE 3 | Dynamics of FAD^{++} fluorescence amplitudes during temperature tests on the dorsal and ventral sides of the forearm on the first day (D1). $AF^{base}_{FAD^{++}}$–FAD^{++} fluorescence amplitude at baseline; $AF^{10^\circ C}_{FAD^{++}}$–$FAD^{++}$ fluorescence amplitude after cooling to 10°C; $AF^{35^\circ C}_{FAD^{++}}$–$FAD^{++}$ fluorescence amplitude after heating to 35°C. $^{\#}p < 0.001$ for dorsal side versus ventral side (the Wilcoxon matched pairs test). In each box, the central line is the median of the group, while the edges are the 25th and 75th percentiles.

A large number of publications from various research groups [45–47] indicate that there is continued interest in finding ways to monitor the levels of metabolic coenzymes, and especially NADH, in biological tissues (particularly skin). Mayevsky and Rogatsky showed that the influence of blood on FAD^{++} monitoring is much greater than on NADH assessment [45]. It has also been shown that, in addition to oxidative phosphorylation, FAD^{++} is involved in a number of other biochemical reactions [48, 49], which does not allow us to separate the contributions of FAD^{++} to energy and general cell metabolism. Probably, these reasons also became the source of uncertainty in the FS results in the channel with an excitation source of 450 nm.

Although NADH fluorescence signals are not calibrated in absolute units, monitoring their changes in functional tests is important for the interpretation of physiological or pathological conditions. However, as has been rightly noted previously [33, 34, 45], for the correct interpretation of FS results, it is important to consider a number of factors: motion artifacts, the contribution of other fluorophores, and blood supply in the area of interest. In our study, the optical probe was securely fixed to the test site, minimizing the possibility of movement. We also used normalization of tissue reflectance to excitation wavelength, which has been repeatedly shown to compensate for the effect of blood on NADH fluorescence [45, 50]. A promising way to improve data would be to use an additional channel of diffuse reflectance spectroscopy (multimodal approach) [51], with which it is possible to calculate tissue blood supply and tissue oxygen saturation to consider their influence on the result obtained.

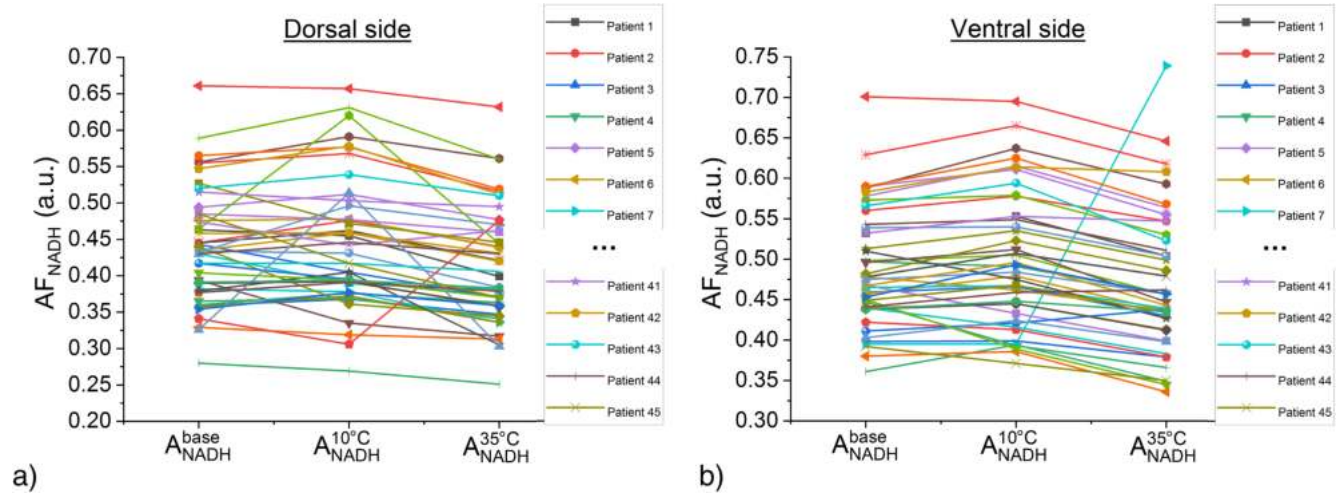

FIGURE 4 | Line graphs of the dynamics of NADH fluorescence amplitude in the skin for individual observations on the dorsal (a) and ventral (b) sides of the forearm on the first day (D1). AF^{base}_{NADH}–NADH fluorescence amplitude at baseline; $AF^{10^\circ C}_{NADH}$–NADH fluorescence amplitude after cooling to 10°C; $AF^{35^\circ C}_{NADH}$–NADH fluorescence amplitude after heating to 35°C.

TABLE 4 | Interday reproducibility of NADH (460 nm) and FAD^{++} (515 nm) fluorescence amplitudes at baseline and during temperature tests in the skin of healthy volunteers.

Variable	Forearm side	D1	D2	CV	ICC
NADH					
AF^{base}_{NADH} (a.u.)	Dorsal	0.434 [0.379; 0.485]	0.463 [0.393; 0.505]	**4.5**	**0.771**
	Ventral	0.475 [0.440; 0.539]	0.473 [0.437; 0.541]	**9.31**	**0.851**
$AF^{10°C}_{NADH}$ (a.u.)	Dorsal	0.431 [0.389; 0.496]	0.464 [0.382; 0.521]	**6.11**	**0.566**
	Ventral	0.484 [0.433; 0.553]	0.526 [0.443; 0.565]	7.69	0.028
$AF^{35°C}_{NADH}$ (a.u.)	Dorsal	0.399 [0.359; 0.462]	0.424 [0.369; 0.499]	4.44	0.158
	Ventral	0.456 [0.413; 0.523]	0.453 [0.413; 0.507]	23.09	0.212
$\Delta AF^{10°C}_{NADH}$ (a.u.)	Dorsal	0.003 [−0.01; 0.018]	0.005 [−0.006; 0.019]	31.87	0.31
	Ventral	0.010 [0.001; 0.022]	0.013 [−0.003; 0.027]	6.82	0.034
$\Delta AF^{10°C}_{NADH}$ (%)	Dorsal	0.932 [−2.757; 4.323]	1.484 [−1.277; 4.453]	11 018	0.191
	Ventral	2.358 [0.218; 5.079]	2.707 [−0.922; 5.437]	6.76	0.034
$\Delta AF^{35°C}_{NADH}$ (a.u.)	Dorsal	−0.024 [−0.046; −0.012]	−0.025 [−0.036; 0.015]	5.65	0.025
	Ventral	−0.026 [−0.042; −0.011]	−0.026 [−0.036; 0]	14.4	0.185
$\Delta AF^{35°C}_{NADH}$ (%)	Dorsal	−5.638 [−10.694; 2.99]	−5.668 [−7.583; 3.527]	5.6	0.026
	Ventral	−5.263 [−8.048; −1.749]	−5.275 [−7.914; 1.124]	19.74	0.211
FAD^{++}					
$AF^{base}_{FAD^{++}}$ (a.u.)	Dorsal	0.406 [0.352; 0.499]	0.393 [0.344; 0.489]	7.33	0.149
	Ventral	0.524 [0.446; 0.621]	0.494 [0.418; 0.644]	20.42	0.347
$AF^{10°C}_{FAD^{++}}$ (a.u.)	Dorsal	0.401 [0.356; 0.495]	0.387 [0.322; 0.468]	37.9	0.35
	Ventral	0.503 [0.445; 0.654]	0.552 [0.438; 0.662]	8.78	0.014
$AF^{35°C}_{FAD^{++}}$ (a.u.)	Dorsal	0.403 [0.352; 0.473]	0.389 [0.329; 0.487]	**18.38**	**0.509**
	Ventral	0.508 [0.427; 0.668]	0.499 [0.418; 0.641]	33.66	0.09
$\Delta AF^{10°C}_{FAD^{++}}$ (a.u.)	Dorsal	−0.002 [−0.015; 0.01]	−0.005 [0.014; 0.009]	21.97	0.152
	Ventral	0.005 [−0.007; 0.017]	0.011 [−0.002; 0.026]	6.27	0.05
$\Delta AF^{10°C}_{FAD^{++}}$ (%)	Dorsal	−0.43 [−3.704; 2.593]	−1.529 [−2.905; 2.419]	14.9	0.156
	Ventral	1.018 [−1.33; 3.837]	2.279 [−0.419; 5.025]	6.45	0.130
$\Delta AF^{35°C}_{FAD^{++}}$ (a.u.)	Dorsal	−0.004 [−0.025; 0.011]	−0.008 [−0.028; 0.009]	10.01	0.033
	Ventral	−0.003 [−0.022; 0.019]	−0.004 [−0.022; 0.012]	7.9	0.079
$\Delta AF^{35°C}_{FAD^{++}}$ (%)	Dorsal	−1.282 [−6.845; 2.729]	−1.980 [−5.684; 3.185]	22.6	0.14
	Ventral	−0.716 [−4.255; 3.226]	−1.672 [−4.566; 3.353]	9.83	0.238

Note: AF^{base}_{NADH} is NADH fluorescence amplitude at baseline. $AF^{10°C}_{NADH}$ is NADH fluorescence amplitude after cooling to 10°C. $AF^{35°C}_{NADH}$ is NADH fluorescence amplitude after heating to 35°C. $\Delta AF^{10°C}_{NADH}$ is the difference between NADH fluorescence amplitude after cooling to 10°C and the baseline. $\Delta AF^{10°C}_{NADH}$ (%) is the relative difference (in percentage) between NADH fluorescence amplitude after cooling to 10°C and the baseline. $\Delta AF^{35°C}_{NADH}$ is the difference between NADH fluorescence amplitude after heating to 35°C and the baseline. $\Delta AF^{35°C}_{NADH}$ (%) is the relative difference (in percentage) between NADH fluorescence amplitude after heating to 35°C and the baseline. $AF^{base}_{FAD^{++}}$ is FAD^{++} fluorescence amplitude at baseline. $AF^{10°C}_{FAD^{++}}$ is FAD^{++} fluorescence amplitude after cooling to 10°C. $AF^{35°C}_{FAD^{++}}$ is FAD^{++} fluorescence amplitude after heating to 35°C. $\Delta AF^{10°C}_{FAD^{++}}$ is the difference between FAD^{++} fluorescence amplitude after cooling to 10°C and the baseline. $\Delta AF^{10°C}_{FAD^{++}}$ (%) is the relative difference (in percentage) between FAD^{++} fluorescence amplitude after cooling to 10°C and the baseline. $\Delta AF^{35°C}_{FAD^{++}}$ is the difference between FAD^{++} fluorescence amplitude after heating to 35°C and the baseline. $\Delta AF^{35°C}_{FAD^{++}}$ (%) is the relative difference (in percentage) between FAD^{++} fluorescence amplitude after heating to 35°C and the baseline. Data are expressed as the median and interquartile range. Reproducibility is expressed as CV and intraclass correlation coefficients (ICC) between the first (D1) and next (3 to 7) days (D2). CV < 25% were considered acceptable. ICC values of <0.40, 0.40 to 0.75, and >0.75 represent poor, fair-to-good, and excellent agreements, respectively. Results appearing in bold fulfill both conditions at once (CV < 25% and ICC > 0.40).

An important limitation of the study is that when using light sources at wavelengths of 365 and 450 nm, the device also detects fluorescence from other skin fluorophores. The use of dynamic fluorescence variables ($\Delta AF^{10^\circ C}_{NADH}$ and $\Delta AF^{35^\circ C}_{NADH}$) during temperature testing allows the fluorescence signal derived from NADH to be distinguished to a certain extent from that of other fluorophores (e.g., skin collagen). However, in our study, these dynamic variables had relatively poor reproducibility on both sides of the forearm (ICC was less than 0.4).

In our study, the skin area in which measurements were taken was not marked with a marker, due to the impracticality of this approach in clinical functional diagnostics. The relatively poor interday reproducibility of FS parameters (when assessed simultaneously by two statistical criteria CV and ICC) may also be due to this factor. The results could also be influenced by the phase of the menstrual cycle in women. A study by Zhang et al. demonstrated changes in basal metabolic rate depending on the phase of the menstrual cycle, with an increase of 6.9% in the luteal phase relative to the follicular phase [52]. However, to date, there are no studies on the effect of the phase of the menstrual cycle on the intensity of NADH and FAD^{++} fluorescence in the skin, which may be the subject of further research.

Currently, there is no generally accepted protocol for conducting temperature tests for clinical monitoring of metabolic processes in the skin using the FS method. Taking into account our results for the thermal test, we can recommend the use of a higher heating temperature (38–42°C) to obtain the maximum difference in NADH fluorescence amplitude relative to the baseline values. In this temperature range, the rate of enzymatic reactions is maximum, but protein denaturation has not yet occurred [2]. On the other hand, this temperature range is close to the temperature of 42°C, at which maximum vasodilation and increased cutaneous perfusion occur [36]. The potential impact of increased perfusion on skin FS parameters requires experimental verification of the feasibility of greater tissue heating during a thermal test.

5 | Conclusion

The normalized amplitude of NADH fluorescence in the skin of the forearm is the most reproducible of the studied FS parameters. Among temperature test parameters, NADH fluorescence amplitude during cooling and FAD^{++} fluorescence amplitude during heating had the best interday reproducibility, but only on the dorsum of the forearm. Most other FS parameters had acceptable reproducibility when assessed only by the between-subject CV. Assessment of the amplitude of NADH fluorescence in the dorsal forearm in combination with a thermal functional test is the most promising method for clinical use for assessing energy metabolism in the skin. The results of this study may improve the reliability of diagnosing metabolic disorders in situ and in vivo by FS and may also be used to calculate the sample size of future clinical studies using FS.

Author Contributions

Ekaterina Ryzhkova: conceptualization, data curation, formal analysis, investigation, methodology, project administration, visualization, writing – original draft. **Tatyana Morgunova:** project administration, resources, supervision, writing – review and editing. **Elena Potapova:** methodology, visualization, writing – review and editing. **Ivan Ryzhkov:** conceptualization, formal analysis, methodology, writing – review and editing. **Valentin Fadeyev:** resources, supervision, writing – review and editing. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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