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QUANTITATIVE ANALYSIS OF
MICROVASCULAR OSCILLATIONS
AND THEIR ALTERATIONS

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Chapter 1

Introduction

1.1 The microcirculation

The major role of the cardiovascular system is to continuously provide adequate blood perfusion levels to the organism, in order to ensure the proper delivery of nutrients and O_2 and, thus, meet its tissue metabolic demands. In this regard, the microcirculation plays a pivotal function, in that represents the ultimate level of the system where the blood solutes, carried by the convective action of the systemic arteries, are diffused to the tissue. From an anatomical standpoint, the microcirculation is generally defined as the blood vessels with intra-luminal diameter below $100\text{ }\mu\text{m}$ [1]. In specific, microvascular beds are composed of arterioles (luminal radius from 5 to $25\text{ }\mu\text{m}$), capillaries ($2\text{--}5\text{ }\mu\text{m}$), and venules ($5\text{--}25\text{ }\mu\text{m}$). Capillaries are made by a single layer of endothelial cells, which corresponds to the physiological interface where the exchange of nutrients and metabolic waste products takes place. On the other hand, the arterioles, from which the capillary beds branch, have also an internal tunica media including a layer of smooth muscle cells (SMCs). The latter represents, in fact, the physiological *effector* which modulates the cross-sectional area of the separate feeding arterioles, so as to regulate the access resistance to the downstream capillary networks and, thus, their local perfused density and flow rate. The local periodic adaptation of the microvascular tone is an indispensable physiological mechanism underlying the efficient distribution of the blood volumes pumped by the heart. As detailed in [2], neurotransmitters and circulating hormones interact with the vascular SMCs through a vast range of complex molecular signalling pathways, exert-

ing an inhibitory or excitatory effect on the activity of myosin light chains and, accordingly, on the contractile state of the microvascular walls. Furthermore, vascular SMCs exhibit also spontaneous oscillatory contractions, known as *vasomotion*. This physiological process, as thoroughly discussed in a recent review by Alkjaer et al. [3], is deemed to be caused by pacemaker Ca^{2+} currents, originating from the sarcoplasmatic reticulum; these, in turn, induce a periodic modification of the membrane potential, that is spread to neighbouring cells via low resistance gap junctions, producing a coordinated influx of Ca^{2+} through voltage-gated calcium channels and, thus, a synchronized oscillation in the vascular tone. Namely, vasomotion is thought to arise from the interaction between a *cytosolic* oscillator (the sarcoplasmatic reticulum) and a *membrane* oscillator (the depolarizing current) [3]. Moreover, vascular SMCs express specific Ca^{2+} -permeable, stretch-activated nonselective cation channels [4] which rapidly fuel the contraction of the arteriolar walls in response to increasing flow rates, producing an intrinsic autoregulatory mechanism of the precapillary resistance to variations in the local perfusion. Another physiological control mechanism is involved in the local adaptation of the microvascular perfusion. In fact, increasing or decreasing tissue metabolic rates determine consistent variations in the interstitial concentration of chemical factors, e.g. P_{O_2} , P_{CO_2} , ATP, ADP, adenosine; these, in turn, trigger the production of vasoactive substances via endothelial signal transduction pathways. In this regard, a potent vasodilator agent is nitric oxide (NO). This lipophilic gas is produced by the endothelium from the L-arginine amino acid, through the action of the enzyme endothelial nitric oxide synthase (eNOS). NO diffuses locally into the adjacent SMC layer, activating specific receptors expressed by vascular SMCs (i.e. the enzyme soluble guanylate cyclase [5]); this interaction, in turn, elicits a decline in the intracellular availability of Ca^{2+} and, thus, the relaxation of the tunica media. The metabolic control of the microvascular flow is reflected by the post-occlusive reactive hyperaemia (PORH) phenomenon, i.e. the characteristic increase in tissue perfusion over resting levels, observed after a transient blockage of the local blood supply [6]. The vascular endothelium is also known to play an important role in the cutaneous tissue thermoregulation: in fact, the local release of NO has been shown to be the fundamental mechanism underlying the sustained reactive hyperaemia of the microcirculation to localized warming stimuli, following a first transient increase in perfusion mediated by a fast-responding cholinergic neural pathway [7–9]

Various pathological conditions have been associated with alterations of the physiological vasomotion. In detail, Bernardi et al. have highlighted a specific attenuation of the sympathetic component of the microvascular flow in insulin-dependent diabetic subjects [10]. This decline might represent an early marker of sympathetic dysfunction, as later suggested by Meyer [11]. The above abnormality has been consistently identified by Bocchi et al. in type 2 diabetic patients suffering from severe dysautonomia [12]; interestingly, in the present study the authors show the possibility of restoring the neurogenic vasomotor activity by means of an innovative protocol of transcutaneous electric stimulation (*Frequency Rhythmic Electrical Modulation System* [13]). In type 2 diabetes, Wick et al. have reported a delayed onset of the cutaneous hyperaemic response to whole body heating, with a significantly higher internal threshold temperature with respect to control subjects [14]. An impairment of the cutaneous reactive hyperaemia to body heating has been also observed in essential hypertension [15]. Furthermore, in this pathological state, Rossi has demonstrated a reduction in the efficiency of the myogenic autoregulatory mechanism [16]. The same author has also shown an exhaustion of the endothelial, neurogenic and myogenic vasomotor activities during PORH in patients diagnosed with peripheral arterial obstructive disease (PAOD) [17]. Moreover, in a recent pilot study involving PAOD patients with critical limb ischaemia [18], Bagno et al. have highlighted a recovery of the cardiac flow pulsatility following the in-line revascularization of the pedal artery but, conversely, no parallel improvement in the local vasomotion. This outcome suggests the presence of an intrinsic derangement of the microvascular physiology, possibly due to atherosclerotic risk factors, despite the restoration of the arterial patency and the improved peripheral transmission of the cardiac stroke volume. In addition to the characteristic fluctuations produced by the local vasomotor mechanisms, the microvascular perfusion exhibits, in fact, extrinsic oscillations produced by the cardiac cycle and the respiratory activity [19]. The aforementioned studies are based on two cardinal pillars: the scientific evidence that the separate vasomotor mechanisms give rise to *flowmotion* components within distinct and established frequency bands; and, secondly, the concept that the cutaneous microvascular beds are a reliable model of the systemic microcirculation, thereby representing a convenient target for the application of non-invasive optical techniques. The following Section includes a concise review of the most relevant optical modalities currently applied in microvascular research.

1.2 Optical techniques for microvascular research: state of the art

1.2.1 Laser Doppler flowmetry

Laser Doppler flowmetry (LDF) is an optical method which enables the non-invasive monitoring of microvascular tissue perfusion. As indicated by the name, this technique relies on the Doppler effect, i.e. the change in frequency of a wave propagating from a source that is moving with respect to the observer, first described in 1842 by the Austrian physicist Christian Doppler [20].

The biomedical application to the measurement of tissue perfusion dates back to 1975, with the work of Stern et al. [21], whereas the first implementation in a portable instrument was reported two years later by Holloway and Watkins [22]. Afterwards, Nilsson and co-workers had a leading role in the advancement and validation of this laser-based technique and in the establishment of a thorough theoretical framework [23], paving the way for its clinical application. In the following, the theory underlying LDF is outlined, by referring to the early work by Nilsson and to a thorough review on the subject by Humeau et al. [24].



Figure 1.1: Periflux 5000 laser Doppler flowmeter (Perimed, Sweden) [25].

In general, a LDF system is comprised of a photodetector, a fibre optic probe including a transmitting and a receiving light guide and, finally, a coherent monochromatic light source (usually a red, $\lambda = 633 \text{ nm}$, or infrared, $\lambda = 780 \text{ nm}$, low-power laser). In LDF, the monochromatic light beam interacts with the superficial layers of the tissue under study (penetration depths are in the range of $\approx 1 \text{ mm}$, depending on the wavelength and the distance between the light guides), where it is either absorbed or backscattered by static and moving elements, i.e. the flowing erythrocytes. The incident photons can be modeled by means of the electric field vector, $\vec{E}_i$:

$$\vec{E}_i = E_{i,0} e^{i(\omega t - \vec{k}_i \cdot (\vec{v}t + \vec{r}_0))}, \quad (1.1)$$

where $\vec{v}t + \vec{r}_0$ identifies the position of the photon, whereas ω and $\vec{k}_i$ represent respectively its angular frequency and wave vector ($||\vec{k}_i|| = 2\pi/\lambda_i$). Accordingly, the photons scattered by the tissue medium can be expressed as follows:

$$\vec{E}_s = E_{s,0} e^{i(\omega t - \vec{k}_i \cdot (\vec{v}t + \vec{r}_0)) + \vec{k}_s \cdot \vec{v}t}, \quad (1.2)$$

where $\vec{k}_s$ is the wave vector of the scattered light. The above equation can be conveniently rearranged so as to express $\vec{E}_s$ as a function of the Bragg scattering vector, $\vec{q} = \vec{k}_i - \vec{k}_s$:

$$\vec{E}_s = E_{s,0} e^{i\omega t} e^{-i\phi} e^{-i\vec{q} \cdot \vec{v}t}, \quad (1.3)$$

where $\omega_D = \vec{q} \cdot \vec{v}$ is the Doppler angular frequency. The noise-less expression of the photocurrent signal, $i(t)$, is obtained from the integral of the instantaneous photocurrent $i(t, \vec{r})$, generated locally at $\vec{r}$ by the total field produced by static and moving scatterers, $\vec{E}_t(t, \vec{r})$:

$$i(t) = \int_A i(t, \vec{r}) d^2r. \quad (1.4)$$

In the above expression, A corresponds to the photosensitive area of the detector, whereas the infinitesimal contribution, $i(t, \vec{r})$, is given by:

$$i(t, \vec{r}) = k_p \vec{E}_t(t, \vec{r}) \vec{E}_t^*(t, \vec{r}), \quad (1.5)$$

where k_p is an instrumentation constant, related to the detector quantum efficiency. As detailed in [24], computing the autocorrelation function, or

intensity, of the photocurrent leads to the following expression:

$$\begin{aligned} \langle i(0)i(\tau) \rangle = & 2i_s \langle i_m \rangle + i_s^2 + \langle i_m \rangle^2 + i_s \langle i_m \rangle (\langle e^{i\vec{q} \cdot \vec{v}\tau} \rangle \langle e^{-i\vec{q} \cdot \vec{v}\tau} \rangle) + \\ & + \langle i_m \rangle^2 \left\langle \sum_{\substack{m=1 \\ m \neq n}}^M \sum_{n=1}^M e^{i(\vec{q}v_m - \vec{q}v_n)\tau} \right\rangle \end{aligned} \quad (1.6)$$

where $\langle \cdot \rangle$ indicates ensemble averaging; in detail, $i_m = M E_{m,0}^2$ represents the photocurrent component produced by M photon interactions with moving scatterers, whereas $i_s = S E_{s,0}^2$ is similarly related to interactions with static structures. The theoretical framework behind the LDF estimate of tissue perfusion assumes that the fraction of photons undergoing multiple Doppler shifts is negligible; thus, the last term related to the homodyne mixing of the light backscattered by moving elements is henceforth ignored. Instead, the information regarding the velocity field of the moving scatterers is contained in the fourth term of the equation, which models the heterodyne mixing of the Doppler-shifted and -unshifted electric fields (the first three, in fact, are time-invariant components). In detail, the LDF blood perfusion estimate is based on the analysis of the photocurrent power spectral density, $P(\omega)$; according to the Wiener-Khintchine theorem, the latter can be derived from the Fourier transform of the above autocorrelation function (if wide-sense stationarity is assumed). Neglecting the homodyne mixing term, this leads to the following proportionality relation [24, 26]:

$$\begin{aligned} P(\omega) \propto i_s^2 \langle i_m \rangle \int_{v=0}^{\infty} N(v) \int_{\alpha=0}^{\pi} \sin(\alpha) S(q(\alpha)) \\ \int_{\tau=-\infty}^{\infty} \frac{\sin(qv\tau)}{qv\tau} e^{i\omega\tau} d\tau d\alpha dv, \end{aligned} \quad (1.7)$$

where v and α are respectively the erythrocyte velocity and the exiting angle of the scattered photons, whereas the terms $N(v)$ and $S(q(\alpha))$ model the one-dimensional distributions of the erythrocyte speed and the Bragg scattering vectors, inside the effective scattering volume. According to the LDF theory, these statistical properties are hypothesized to remain stationary and spatially uniform during data sampling. In detail, the LDF signal of tissue perfusion is estimated from the integral of the ω -weighted power spectral density, $P(\omega)$; by referring to the expression of the Doppler angular frequency ($\omega_D = \vec{q} \cdot \vec{v} = \|\vec{q}\| \|\vec{v}\| \cos(\theta)$), and relying on the cosine theorem,

Nilsson has derived the following model:

$$\begin{aligned}
 P_{LDF} &\propto \int_0^\infty \omega P(\omega) d\omega \propto \\
 &\propto \int_{v=0}^{v_{max}} \langle i_m \rangle v N(v) \int_{x=0}^{4\pi/\lambda_t} \int_{q=x}^{4\pi/\lambda_t} S(q) dq dx dv \quad (1.8)
 \end{aligned}$$

where the information on the erythrocyte flow speed is specifically contained in the outer integral. However, the above model retains no information regarding the directionality of the microvascular flow. In fact, as discussed by Bonner and Nossal [27], the LDF theory also assumes that the incident light becomes diffusely scattered due to multiple interactions with the static tissue matrix and, secondly, that the velocity vectors of the moving erythrocytes exhibit no dominant direction on a space scale in the order of the mean scattering distance. Thus, LDF only provides a non-absolute measure of the microvascular blood flow (thus usually reported in arbitrary units). In practice, the resultant P_{LDF} signal depends on the total light intensity impinging on the detector (i.e. the static terms in the autocorrelation function); therefore, in LDF systems, the above perfusion estimate is generally normalized with respect to the squared dc component of the photocurrent. LDF is inherently affected by a number of technical limitations. First, the effective measurement volume cannot be rigorously determined and, secondly, it is directly influenced by the measurand, since it depends on the optical properties of the tissue medium. Moreover, the limited size of the sampling volume makes the LDF signal particularly prone to regional heterogeneities in the microvascular density and flow rate across different measurement sites, giving rise to potential intra-subject repeatability issues. Furthermore, depending on the relative motion of the scattering structures, LDF signals are subject to spurious artefacts produced by the unintentional displacement of the optical probe or, alternatively, by the deformation of the measurement volume. Despite the aforementioned drawbacks, LDF offers in general very high temporal resolution and, thus, the possibility to perform a real-time non-invasive assessment of the physiological vasomotor dynamics. However, due to the punctual nature of the measure, LDF does not allow the characterization of the spatial distribution and heterogeneity of the microvascular flow. Laser speckle contrast imaging, introduced in the following paragraph, has now emerged as a valuable technique in this regard.

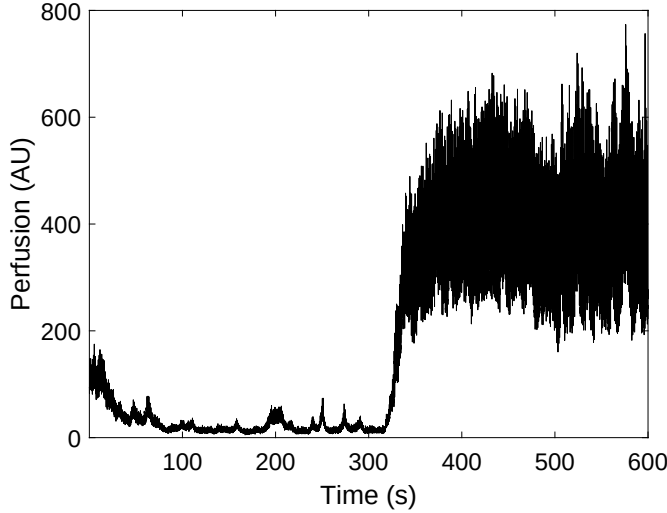


Figure 1.2: Sample laser Doppler flowmetry signal measured on the hallux.

1.2.2 Laser speckle contrast imaging

Laser speckle contrast imaging (LSCI) is a contactless optical modality, which has recently found widespread application in research and clinical settings for the non-invasive derivation of microvascular perfusion maps. This technique relies on the statistical analysis of the interference pattern produced by diffusely backscattered photons, which are emitted by a perfused tissue region illuminated by means of a low-power infrared laser beam ($\lambda = 785 \text{ nm}$) [28]. The present pattern is characterized by bright and dark areas (hence the name *speckle* imaging), that arise respectively from the constructive and destructive interference of the backscattered radiation. Under the assumptions of a stable optical setup and an ideal laser source (i.e. a noiseless, coherent, polarized, monochromatic light beam), if the target tissue surface is completely static, then the interference pattern will not change over time and the speckle map will be consistently static, that is *fully-developed*. Under these conditions, and assuming a perfectly diffusing rough surface, characterized by a Gaussian-distributed asperity, Goodman et al. have demonstrated that the mean and the standard deviation of the speckle intensities are equal [29]. Conversely, if the tissue medium under study is perfused, then the interference pattern will be dynamically modified because of the Doppler shifts induced by the microvascular blood flow;

therefore, due to the inherently finite integration times of the imaging system, the speckle maps will be locally blurred, leading to a reduction in the standard deviation of the intensities [30]. This evidence lays the basis for the adoption of a contrast parameter, defined as:

$$K = \frac{\sigma_s}{\langle I_s \rangle} = \frac{\sqrt{\langle I_s^2 \rangle - \langle I_s \rangle^2}}{\langle I_s \rangle}. \quad (1.9)$$

where I_s represents the speckle pattern intensity. According to the above discussion, K strictly depends on the decorrelation time of the interference pattern and, thus, is locally related to the underlying velocity of the moving scatterers (i.e. the erythrocytes). Specifically, the contrast is ideally 1 in perfectly static tissue media and progressively decreases with increasing perfusion levels.

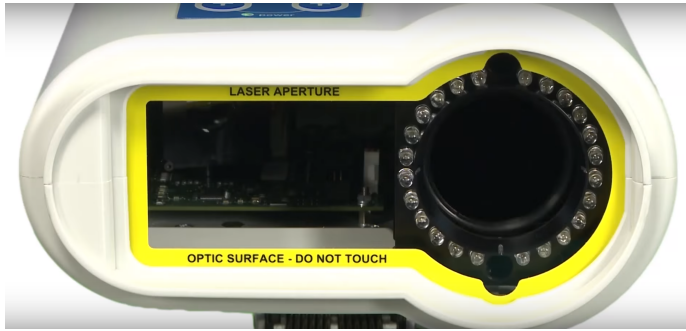


Figure 1.3: FLPI-2 laser speckle contrast imager (Moor Instruments, UK).

Two main strategies can be adopted for computing the speckle contrast. Namely, K can be determined in the spatial domain by applying the above equation inside adjacent blocks of $n \times n$ pixels, as performed in the Laser Speckle Contrast Analysis (LASCA), proposed by Briers and Richards [31]; LASCA typically relies on 5×5 or, alternatively, 7×7 pixel masks, in order to achieve an adequate trade-off between statistical robustness and spatial resolution. The latter can be improved (at the expense of temporal resolution) by computing the speckle contrast at pixel level over subsequent frames, i.e. in the time domain, as originally described in [32]. Temporal and spatial processing modes are concurrently available in state-of-the-art commercial speckle imagers [33].

The theoretical framework which relates the measurable speckle contrast to the velocity field of the moving scatterers has been thoroughly covered by Vaz et al. in a recent methodological review [34]. Conceptually, the LSCI theory is based on the autocorrelation function of the intensity, $I_s(t)$, whose temporal evolution carries information on the dynamics of the moving scatterers:

$$R_I(t) = \frac{\langle I(t) \cdot I(t + \tau) \rangle}{\langle I(t) \rangle^2}. \quad (1.10)$$

The above second-order intensity autocorrelation can be related to the first-order autocorrelation of the backscattered electric field, $\vec{E}(t)$, by means of the Siegert equation:

$$R_I(\tau) = 1 + \beta |R_E(\tau)|^2 \quad (1.11)$$

where β is a correction factor, required to account for the nonideality of the optical setup.

As reported in [34], Bandyopadhyay et al. [35] have identified a model relating the contrast parameter, K , with the field autocorrelation function, $R_E(\tau)$, and the exposure time of the speckle imager, T :

$$K^2(T) = \frac{2\beta}{T} \int_0^T |R_E(\tau)|^2 \left(1 - \frac{\tau}{T}\right) d\tau. \quad (1.12)$$

In LSCI techniques, the moving scatterers are commonly assumed to follow a Lorentzian velocity distribution; this is associated with the following field autocorrelation:

$$R_{I,l}(\tau) = e^{-\tau/\tau_d}, \quad (1.13)$$

where τ_d is the decorrelation time of the backscattered light. Substituting $R_{I,l}$ in Eq. 9.19 leads to:

$$K_l^2(T) = \beta \left[\frac{\tau_d}{T} + \frac{\tau_d^2}{2T^2} \left(e^{-2T/\tau_d} - 1 \right) \right]. \quad (1.14)$$

However, the above assumption is valid only if the dynamics of the light scatterers mimics a random Brownian motion. In order to account for the spatially-ordered nature of the microvascular flow, a Gaussian profile can be alternatively adopted:

$$R_{I,g}(\tau) = e^{-(\tau/\tau_d)^2}; \quad (1.15)$$

in this case, the model of the speckle contrast becomes:

$$K_g^2(T) = \beta \left[\frac{\tau_d}{T} \sqrt{\frac{\pi}{2}} \operatorname{erf} \left(\frac{\sqrt{2}T}{\tau_d} \right) + \frac{\tau_d^2}{2T^2} \left(e^{-2T^2/\tau_d^2} - 1 \right) \right]. \quad (1.16)$$

Ramirez-San-Juan et al. [36] have thoroughly addressed the ultimate impact of this fundamental hypothesis on the LSCI perfusion estimates. Nevertheless, the following relation holds for both zero-mean Lorentzian and Gaussian probability distributions:

$$v = \frac{\lambda}{2\pi\tau_d}. \quad (1.17)$$

Therefore, notwithstanding the adopted velocity distribution, Eq. 1.14 and 1.16 both constitute a theoretically sound approach for evaluating the light scatterer velocities, and thus mapping the microvascular perfusion of superficial tissue layers, through a straightforward analysis of the local contrast of dynamic interference patterns (assessed either in the spatial or time domain). Despite being not covered in this summary introduction to LSCI, more complex and complete models, which take into consideration the contribution of static scattering structures, have been also proposed [34].

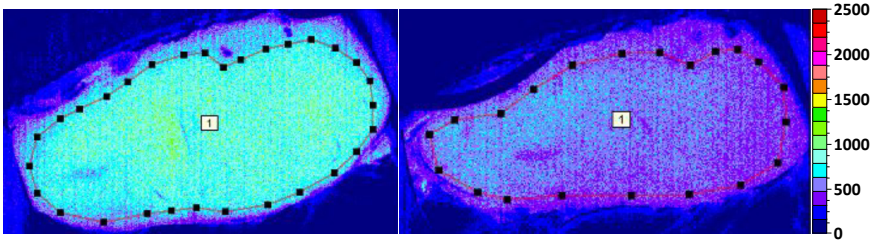


Figure 1.4: LSCI perfusion of the porcine renal cortex in basal conditions (left) and during distributive septic shock (right).

1.2.3 Portable incident dark field microscopy

Portable incident dark field (IDF) microscopy is an optical technique for the real-time visualization of the microcirculation of superficial semi-transparent tissue. This modality enables the imaging of the individual erythrocytes, which flow through the microvascular networks, and the evaluation of the leukocyte rolling and extravasation dynamics in post-capillary venules. In general, portable IDF is applied *in vivo* in order to assess the local functional capillary density and flow rate of the sublingual mucosa, directly at the patient bedside.

Its origin is traced back to 1971, when Sherman et al. [37] reported the development of a new IDF illumination method for conducting microvascular studies on the surface of major organs (namely lungs, kidneys, liver

and brain). The technique was praised as a valuable alternative to transillumination, which had been extensively used for observing organic tissue samples but, however, could not be satisfactorily applied *in vivo*, especially to moving organs such as the lungs. In the microscopy setup implemented by Sherman and co-workers, the dark field illumination was produced by delivering a light beam through a prismatic lens surrounding the objective. In this way a halo could be created around and beyond the focal point of the optical system, generating an unusual depth of field and conferring “a tri-dimensional quality to the tissue observed” [37].

Inspired by this concept, in 2007 Ince introduced the Sidestream Dark Field (SDF) imaging modality [38]. SDF devices are hand-held microscopes, in which the IDF illumination is provided by a ring of pulsed LEDs, emitting green light within a narrow range of wavelengths centred on $\lambda = 530$ nm. The latter coincides with the isosbestic point of oxygenated and de-oxygenated haemoglobin, thus producing a selective contrast between perfused microvessels and perivascular areas. The stroboscopic epi-illumination of the field of view improves the resolution of the capillary flow, and reduces the blurring due to the relative motion between the probe and the region of interest. Furthermore, the SDF microscope is designed so as to passively isolate the optical system from the outer illuminating ring, preventing the fraction of light directly reflected at the tissue interface from being collected by the detector. This technical solution, which circumvents the use of a polarizer-analyzer couple, reduces the weight of the device and, moreover, allows the adoption of low-power sources (in that a larger percentage of the emitted radiation contributes to the image generation), thus relaxing the requirement for an external power supply and improving the overall portability of the system.

The CytoCam system (Braedius Medical, the Netherlands) is the latest generation of commercial hand-held IDF microscopes [39], and can be regarded as a technical evolution of the earlier SDF concept. Similarly to SDF, this pen-like microscopy device (length: 220 mm, diameter: 23 mm) implements the Sherman’s IDF technique by means of a ring of 12 LEDs, which provide a stroboscopic monochromatic illumination in the green region of the visible spectrum. Nevertheless, CytoCam exhibits a range of significant technical improvements. It is made mostly by titanium and aluminium so as to contain its overall weight (120 g against the 350 g of SDF devices), and reduce in turn the risk of pressure artefacts during video capture.

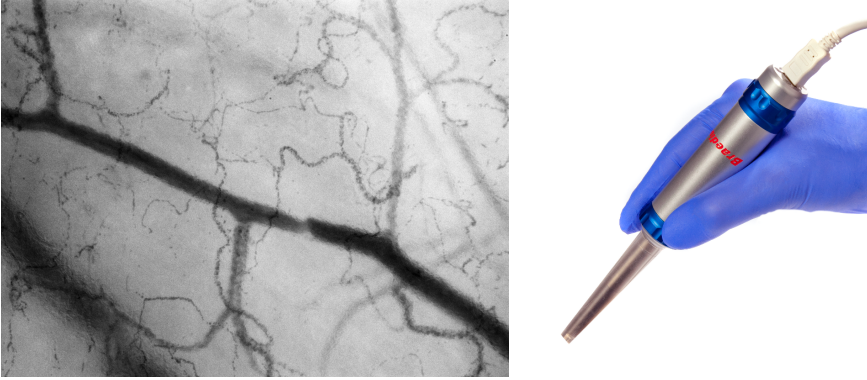


Figure 1.5: Sublingual microvascular flow (left), captured with the CytoCam IDF microcirculation camera (Braedius Medical, the Netherlands) [40].

Furthermore, the CytoCam features very short illumination pulse times of 2 ms which produce a better definition of the microvascular structures, compared to the 16-ms light pulses emitted in the earlier SDF design. Moreover, the analogue CCD included in the previous generation has been substituted for a 14 Megapixel CMOS optical sensor, with a $1.4\text{ }\mu\text{m}$ pixel size. The CMOS detector is used in binning mode so as to achieve improved SNR levels, thus producing an effective image size of 2208×1648 pixel. This, combined with a 4x objective lens, leads to an overall field of view of 1.55×1.16 mm and to an optical resolution higher than 320 line pairs/mm. These technical specifications significantly exceed the ones offered by the SDF microscopes (namely a 0.43 Megapixel CCD with a $6.25\text{ }\mu\text{m}$ pixel size; a 1.12×0.75 mm field of view; and an optical resolution of 220 line pairs/mm), leading to a substantial improvement in the quality of the captured videos. Fig. 1.5 shows a frame of the sublingual microcirculation, imaged at the bedside with the CytoCam IDF system.

1.3 Objective and outline of the thesis

The main objective of the present thesis is the development of signal processing techniques for the analysis of biomedical data of microvascular perfusion, acquired non-invasively on the skin by means of established optical modalities, first and foremost LDF. In specific, the thesis explores the possibility of deriving clinically relevant information on the control mechanisms involved in the microvascular *vasomotion* and, moreover, on the mechanical properties of the arterial walls, from the decomposition and the quantitative characterization of the oscillatory dynamics of the cutaneous perfusion. In this regard, the thesis considers the implementation and application of data modeling and time-frequency methods for the assessment of known physiological components identified in the literature: Chapter 2 presents a study on their spatial heterogeneity across measurement sites typically adopted in microvascular research. The core of the present work is formed by the implementation of a model-based decomposition algorithm for the analysis of peripheral pulse wave contours, and the non-invasive extraction of indirect markers of vascular stiffness. The details of this novel *pulse decomposition algorithm* (PDA) are discussed in Chapter 3, whereas Chapter 4 covers the validation of a *support vector machine* classifier of vascular ageing, trained over a dataset of morphological features of the LDF pulse, derived through the above PDA method. The following Chapters 5 and 6 deal respectively with the application of the above methodological framework to the non-invasive assessment of respiratory-vascular coupling mechanisms and to the automatic analysis of the transient neurogenic vasoconstriction to a deep inspiration. The thesis continues with the validation of the above non-invasive markers of vascular stiffness against established parameters of central and distal arterial compliance, derived from a Windkessel model of the circulatory system (Chapter 7). Afterwards, Chapter 8 reports on a clinical case-control study, involving a group of 47 subjects affected by type 1 diabetes, aimed to identify abnormal microvascular parameters and to highlight a potential correlation with the long-term glycemic control. Finally, Chapter 9 includes two related studies, focused respectively on: the improvement of a video-tracking algorithm for the automatic quantification of capillary flow speeds from IDF videos of the microcirculation; and the development of a novel method for assessing the spatial distribution of the microvascular flow, through the textural analysis of pulse propagation delay maps obtained by means of a prototype imaging photoplethysmography system.

Chapter 2

Spatial heterogeneity of microvascular perfusion

*Pathological alterations of the microcirculatory system can be identified through the evaluation of temporal and spectral properties of laser Doppler flowmetry (LDF) signals of cutaneous perfusion, and by modeling their response dynamics following the application of non-invasive stimuli. However, a wide range of stimulation protocols and measurement sites is found in literature and a solid consensus has yet to be reached. Researchers often adopt tests of post-occlusive reactive hyperaemia, or protocols involving whole-body or local cooling/heating. However, recent clinical investigations have showed that different anatomical sites might present dissimilar response patterns in blood perfusion after the application of standardized stimuli. Therefore, studies based on the direct comparison of LDF data, obtained from various anatomical locations, might be methodologically inaccurate. In microvascular research, the forearm, fingers, and toes are typically adopted as stimulation/recording sites. This chapter presents a study on the quantitative characterization of the physiological heterogeneity in the spectral and vasodilatory properties of the cutaneous microcirculation, at these latter anatomical locations.*¹

¹This chapter has been published as “Spatial heterogeneity in the time and frequency properties of skin perfusion” in *Physiological Measurement*, vol. 38, pp. 860-876, 2017.

2.1 Introduction

As reported in the introductory chapter, the blood microcirculation is a complex, distributed system, on which several biological oscillatory processes and physiological control pathways, of central and local origin, act in order to tailor the local rates of blood flow and, in turn, meet the metabolic and thermoregulatory demands of the organism. Thus, the disruption of the normal microvascular physiology is believed to play a pivotal role in the development of metabolic, neurologic and cardiovascular diseases. Due to its accessibility, the skin microcirculation is deemed as a convenient *window* on the general state of the microvascular districts of the circulatory system [41]. Therefore, pathological alterations of the microcirculation could be assessed through the spectral and temporal properties of signals of cutaneous perfusion. These can be acquired non-invasively by means of the optical modalities presented in Chapter 1, either in basal conditions or after the application of non-hazardous stimuli, in the framework of stimulus-response experiments. Nevertheless, a wide range of stimulation patterns (e.g. occlusive, thermal, postural, respiratory, and pharmacological) and measurement locations (glabrous vs. non-glabrous skin, upper vs. lower limbs, proximal vs. distal anatomical locations etc.) are reported in literature. Because of the complexity and non-linearity of the mechanisms underlying the regulation of peripheral blood perfusion, a large dependency on the experimental protocol is thus expected. Indeed, recent clinical investigations have demonstrated that different locations, undergoing a standardized stimulation protocol, can exhibit dissimilar physiological responses [42]. Therefore, studies involving data of microvascular perfusion acquired at various anatomical locations, and thus potentially subject to the heterogeneity of microvascular beds, might be methodologically inaccurate and unreliable. Furthermore, in a recent review on the relationship between microvascular biomarkers and cardiovascular disease [43], Hellmann et al. have emphasized the current lack of a shared and structured methodology, which might effectively guide clinicians through the treatment of pathological conditions involving microangiopathy. Indeed, nowadays, no solid consensus has been reached upon the optimal measurement location, the stimulation pattern, nor the physiological parameters of interest.

Based on this background, the aim of the present study was to derive a quantitative characterization of the spatial heterogeneity in the properties of microvascular perfusion signals, measured at the forearm, the pulp of the index

finger, and the plantar side of the hallux, in order to support the identification and adoption of a standardized measurement protocol in microvascular research. Local skin heating above $\approx 42^\circ\text{C}$ evokes a local composite response by the microvascular beds [9]. This includes an early transient vasodilation effected by a neurogenic *axon reflex*, followed by a prolonged hyperaemic *plateau*, that is sustained by the local release of nitric oxide (NO) by the vascular endothelium. In the present work, a parametric model-based representation of this physiological response was adopted so as to quantify its spatial heterogeneity. Moreover, since skin blood flow is in general a highly dynamical process which, even in basal conditions, exhibits known physiological oscillatory components fluctuating at specific time scales, a wavelet transform analysis (WTA) was carried out, in parallel to the former time-domain approach, so as to assess the presence of potential spatial heterogeneities in the power spectra of these biosignals.

2.2 Experimental setup and protocol

Signals of cutaneous microvascular perfusion were recorded with the 5010 Laser Doppler Perfusion Monitoring (LDPM) unit of a Periflux 5000 LDF System (Perimed, Sweden). The 5010 LDPM module houses a low-power laser diode ($\lambda = 633\text{ nm}$), which offers a penetration depth of around 1 mm, thus ensuring the selective assessment of the superficial microvessels. Furthermore, the 5020 Temperature unit of this medical device allows for a precise thermal stimulation of the skin area under evaluation, with a maximum error of 0.1°C , as reported by the instrument manufacturer. A single small-angled thermostatic probe (model 457) was used to provide this focused thermal stimulus and to simultaneously record non-absolute signals of local blood perfusion. The probe was secured to the underlying skin with medical tape, in order to limit relative displacements and deformations of the effective volume of measurement, which would induce spurious spikes in the recorded signals. Moreover, the recruited participants were invited to relax and sit in a comfortable position throughout the measurements. LDF perfusion (expressed in Arbitrary Units, AU) and temperature signals were respectively digitized at a sampling frequency of 40 Hz and 10 Hz. The measurement sessions were conducted in controlled thermal conditions, at an ambient temperature of about 21°C . Furthermore, before the start of the experimentation, the LDF system was properly calibrated according to the

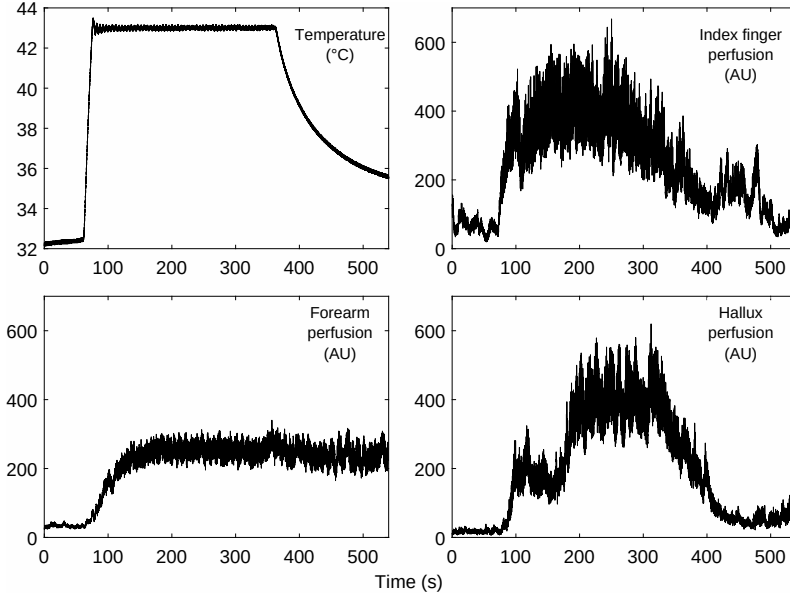


Figure 2.1: Temperature curve of the LDF thermostatic probe, corresponding to the adopted stimulation protocol (top left), and sample LDF signals, measured at the index finger (top right), forearm (bottom left) and hallux (bottom right).

manufacturer's specifications, by means of the Motility Standard and the Zeroing Disc provided with the instrument. Device calibration was checked approximately every two weeks, as required. Nevertheless, the obtained values were always within the specified tolerance ranges (i.e. 250 ± 15 AU in Motility Standard, and 0 ± 1 AU on the Zeroing Disc); thus, no additional action was actually needed.

A preliminary evaluation was performed among the participating institutions for identifying a common stimulation protocol, used to conduct investigations on the physiological mechanisms and the microvascular responses, involved in the local regulation of skin temperature, with the aim of maximizing the size of the experimental dataset. Thus, the standard protocol identified for this study consisted in a 60-s baseline phase, followed by 5 min of local thermal stimulation at 43°C and, finally, by a recovery phase of 3 min, where the skin returned to its thermal equilibrium, as shown in Fig. 2.1.

Nevertheless, the above evaluation brought out that the available duration of the baseline phase could not allow for a reliable characterization of the slower oscillatory components of the biological *vasomotion*, associated with the spontaneous and neurogenic periodical change in the tone of vascular smooth muscle cells, and with the activity of the vascular endothelium. Hence, at this preliminary stage, it was agreed to integrate the pre-existing dataset of LDF signals with new acquisitions including a baseline of 15 min.

2.3 Dataset and power analysis

Measurements of microvascular skin perfusion were obtained from 41 healthy volunteers, at three different anatomical sites, namely: the volar surface of the right forearm; the pulp of the right index finger; the pulp of the right hallux. All the recruited subjects signed an informed consent form, agreeing to take part to the study after having received comprehensive information on the purpose of the research and the involved procedures. Data were collected and treated anonymously in accordance with privacy regulations. The present study involved the analysis of LDF signals recorded at three separate institutions: the Clinic of Occupational Diseases of the University Hospital St. Ivan Rilsky (Sofia, Bulgaria), the Institute of Continuous Media Mechanics (Perm, Russian Federation), and the Laboratory of Biomedical Engineering of the University of Florence (Florence, Italy). Among the enrolled volunteers, 6 were evaluated only at the hallux, whereas 5 were measured only at the forefinger, resulting in an overall dataset of 36, 35, and 30 acquisitions collected respectively for the hallux, index finger and forearm data groups. Furthermore, due to the constrained time of the measurement sessions, a 15-min baseline required to capture the slow dynamics of cutaneous perfusion was recorded in 10 cases only, for each anatomical location. Nevertheless, these latter sub-groups were properly matched according to age, sex, and smoking condition.

A preliminary sensitivity analysis [44] was conducted in order to assess the theoretical population effect size which, on the basis of the available sample sizes, was required to identify the presence of statistically significant differences between the compared skin sites (at a level of significance $\alpha = 0.05$), with a statistical power of 80%. The outcome, summarized in Table 2.1, highlighted the possibility to detect moderate to strong correlations between the parameters of the hyperaemic response and the compared locations; on

the other hand, the smaller sample size available for the assessment of a potential spatial heterogeneity in the spectral content of cutaneous perfusion signals, limited the scope of this latter analysis to the observation of large effects.

Table 2.1: Sensitivity analysis: minimum effect size required to achieve a statistical power of 80%, given the available sample sizes.

PAIRS	Pearson's r ($\alpha = 0.05$, $1 - \beta = 0.80$)	
	Vasodilation	WTA
Index finger vs Forearm	0.34	0.56
Index finger vs Hallux	0.33	0.56
Forearm vs Hallux	0.34	0.56

2.4 Modeling of local thermal hyperaemia

The model-based analysis of the vasodilatory response to skin heating relied on signals of LDF perfusion, including a 60-s baseline and a time window of 5 minutes associated with the localized thermal stimulation at 43 °C. Considering the interval which preceded the start of the stimulus was functional to the integration of an offset into the model of thermal hyperaemia, which could properly represent the average basal levels of cutaneous perfusion. Reliable and reproducible reference boundaries of the latter heating phase were manually identified on the synchronous temperature trace of the thermostatic probe.

Raw LDF data were preprocessed with a 10-s median filter, in order to attenuate the cardiac oscillatory components and to extract the trend of the hyperaemic response. Mirror padding by a span equal to half the filter's support (i.e. 5 s) was preliminarily applied so as to limit the generation of boundary artefacts. Finally, the trend produced by the median filter was further smoothed through a local regression (performed again over non-overlapping 10-s intervals), based on a second degree polynomial model and a weighted linear least squares approach.

The data model of thermal hyperaemia was formulated in order to represent the two physiological components that can be identified in the skin vasodilatory response to the local thermal stimulus, as shown in Fig. 2.2.

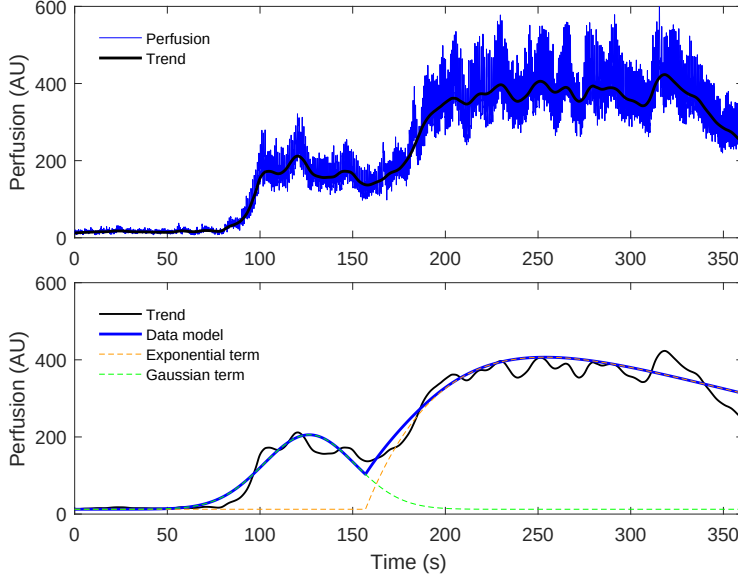


Figure 2.2: Perfusion trend extraction (top) and data fitting with the adopted data model (bottom).

Accordingly, its analytical expression, $F(t)$, is composed of two main terms, namely: a first Gaussian curve for describing the fast, transient response linked to the activation of the *axon reflex* and, secondly, a sum of exponentials for the sustained hyperaemic plateau mediated by the local release of NO [45]:

$$F(t) = k + a_{ar} \cdot e^{-\frac{(t-t_{ar})^2}{2 \cdot \sigma_{ar}^2}} + a_p \cdot \frac{(1 + \text{sign}(t - t_p))}{2} \cdot (e^{-b_p(t-t_p)} - e^{-c_p(t-t_p)}). \quad (2.1)$$

In Eq. 2.1, the constant term k represents the basal skin perfusion; t_{ar} and a_{ar} respectively describe the time delay of the axon reflex activation, and the extent of its vasodilatory effect, whereas the standard deviation σ_{ar} is related to its duration. Moreover, the coefficient a_p is linked to the absolute excursion of the hyperaemic plateau, whereas b_p and c_p are the rate constants of its descent and ascent phases, respectively; finally, the time delay t_p was introduced in the model, in combination with the *sign* term, so as to consider the delay of the NO-mediated response. The identification of these eight model parameters was performed with a *trust-region* algorithm for non-

linear least squares problems. Table 2.2 summarizes the configuration of the optimization algorithm, with the assigned constraints and initial conditions.

Table 2.2: Configuration of the trust-region optimization algorithm.

PARAMETER	k	a_{ar}	t_{ar}	σ_r	a_p	b_p	c_p	t_p
Lower boundary	0	0	60	0	0	0	0	60
Upper boundary	1000	1000	300	60	9999	1	1	300
Initial value	50	500	100	10	500	0.001	0.01	200

2.5 Wavelet transform analysis

Peripheral perfusion is modulated by the complex, and inter-dependent activity of neurogenic, myogenic and endothelial physiological mechanisms. This composite regulatory system manifests itself through periodic oscillations which can be non-invasively monitored with LDF at the level of the cutaneous microcirculation. Previous studies, based on the WTA of LDF signals, have identified six frequency ranges, specifically related to these separate physiological oscillatory processes [46–48] (listed in Table 2.3).

Table 2.3: Physiological frequency intervals of cutaneous blood perfusion.

INTERVAL	SOURCE
0.6 - 2 Hz	cardiac rhythm
0.145 - 0.6 Hz	breathing
0.052 - 0.145 Hz	vascular smooth muscle cells
0.021 - 0.052 Hz	sympathetic nervous system
0.0095 - 0.021 Hz	NO-dependent endothelial activity
0.005 - 0.0095 Hz	NO-independent endothelial activity

Therefore, a preliminary evaluation was required to define the lowest range that could be reliably assessed with a WTA, on the basis of the available observation window of 15 min. As indicated in a recent, comprehensive review on time-frequency representation techniques by Iatsenko et al. [49], in order

to derive a statistically robust characterization of a stationary process, the acquisition time should last at least *five* oscillation periods. In our case, this criterion would identify a minimum threshold frequency equal to:

$$f_{min} = \frac{5}{T_{bsl}} \approx 0.006 \text{ Hz}, \quad (2.2)$$

where T_{bsl} is the duration of the baseline phase. However, in practical scenarios, the finite support of the LDF signals would lead to boundary distortions of the wavelet coefficients, since the theoretical definition of the transform would require an infinite integration time. Therefore, it is generally advised to only consider the region of the wavelet scalograms where these detrimental effects are negligible, i.e. the so-called *cone of influence* (COI) [49]. In practice, the edges of the COI can be identified on the time-frequency plane by setting a proper accuracy threshold on the wavelet coefficients. A conventional relative error tolerance of 0.1% was adopted in this regard. The actual number of cycles available for different acquisition times, after the extraction of the 0.1%-COI, is displayed in Fig. 2.3: it is effectively shown that, for our protocol, the aforementioned requirement is met only at 0.012 Hz, i.e. inside the frequency interval associated with NO-dependent endothelial oscillations. Nevertheless, given the availability of two cycles at 0.0095 Hz, it was finally decided to include also this latter range in our analysis. Prior to WTA, the raw LDF signals were preprocessed so as to remove the components below the minimum frequency of interest (i.e. 0.0095 Hz), and above the nominal upper threshold of the cardiac range (i.e. 2 Hz). The slow trend and the *unphysiological* high frequencies of the raw data of skin perfusion were removed with a bandpass FIR filter, designed with the window method for FIR filters. In this regard, the Hamming window was selected, due to its good side-lobe attenuation performance. Furthermore, the order of the FIR filter was tailored so as to obtain transition bands as wide as the spectral resolution of the WTA. However, since the visual inspection of the original LDF signals included in our dataset did not reveal any significant motion-induced spike, automatic peak-trimming solutions were not applied at this preprocessing stage, to avoid any potential undesired distortion. The complex Morlet's mother wavelet was used for the WTA, as generally done in studies involving the time-frequency decomposition of LDF perfusion signals [16, 50–54],

$$\psi(t) = \frac{1}{\sqrt{2\pi}} \left(e^{i2\pi f_0 t} - e^{-\frac{(2\pi f_0)^2}{2}} \right) e^{-t^2/2}, \quad (2.3)$$

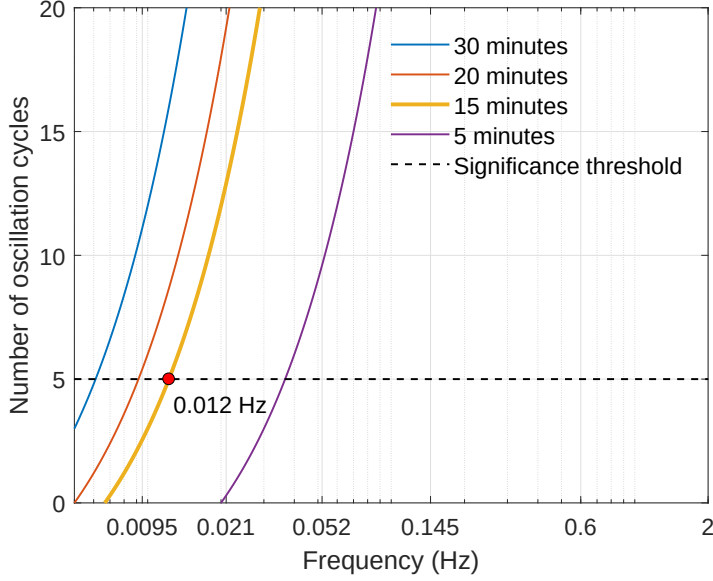


Figure 2.3: Number of oscillation cycles available inside the physiological frequency range of cutaneous perfusion, after the extraction of the 0.1%-COI, for LDF signals of 5, 15, 20, and 30 minutes.

where f_0 represents the central frequency of the mother wavelet [47]. In time-frequency representation techniques relying on the wavelet transform, the mother wavelet is stretched and scaled so as to generate a family of basis functions, able to capture the dynamics of the analyzed signal at different time scales (i.e. frequencies); the temporal localization of the signal's spectral content is instead obtained by shifting this family of windows along the temporal domain:

$$\psi_{s,t}(\tau) = \frac{1}{\sqrt{s}} \psi\left(\frac{\tau - t}{s}\right). \quad (2.4)$$

$\psi_{s,t}(\tau)$ defines the wavelet of scale s , at the time point t ; $\frac{1}{\sqrt{s}}$ is a correcting term, introduced to normalize the energy of the time-frequency windows at different scales [55]. The scale s can be related to frequency by the following expression:

$$f = \frac{f_0}{(s \cdot \Delta t_s)}, \quad (2.5)$$

where Δt_s represents the sampling period. It can be recognized how tuning the central frequency f_0 of the Morlet's wavelet constitutes a trade-off between spectral and time resolution; in the present work, a $f_0 = 1$ was selected in agreement with the standard configuration used by other authors, for the analysis of LDF signals [46–48, 56, 57].

The discretization of the frequency domain was performed with a density of 32 voices/octave, which allowed for an adequate resolution inside the physiological range of interest, at acceptable computing times. With these settings, the continuous WTA, producing the time-frequency representation of the perfusion signal $p(t)$, was computed as:

$$w(t, s) = \frac{1}{\sqrt{s}} \int_{-\infty}^{+\infty} \psi\left(\frac{\tau - t}{s}\right) p(\tau) d\tau. \quad (2.6)$$

Afterwards, the time-averaged power spectral density was determined as follows:

$$\wp(f) = \frac{1}{\Delta t_f} \int_{t_{lb}(f)}^{t_{ub}(f)} |w(t, f)|^2 dt, \quad (2.7)$$

where $t_{lb}(f)$ and $t_{ub}(f)$ are respectively the lower and upper time boundaries of the COI, at the frequency f . A sample wavelet spectrogram, and the corresponding time-averaged power spectrum, are displayed in Fig. 2.4

Next, the absolute basal power of the aforementioned physiological processes was computed integrating $\wp(f)$ over the corresponding frequency intervals (Table 2.3). Finally, relative estimates were obtained by normalization with respect to the total power, associated with the entire (0.0095, 2) Hz range.

2.6 Statistical analysis

Differences arising between the three investigated locations were tested for statistical significance with the IBM SPSS Statistics 21 software, by means of the non-parametric Kruskal-Wallis ANOVA test. Whenever a significant dissimilarity emerged from the analyzed data ($p < 0.05$), a post-hoc Mann-Whitney U test was applied so as to identify the specific significant pairwise differences. At this stage, taking into account the Bonferroni's correction, a $p < 0.017$ was considered as statistically significant. Finally, in order to obtain a standardized measure of the spatial heterogeneity observed between the estimated parameters, approximate effect sizes were derived by converting the statistical z-scores into Pearson's r correlation coefficients, as described in [58].

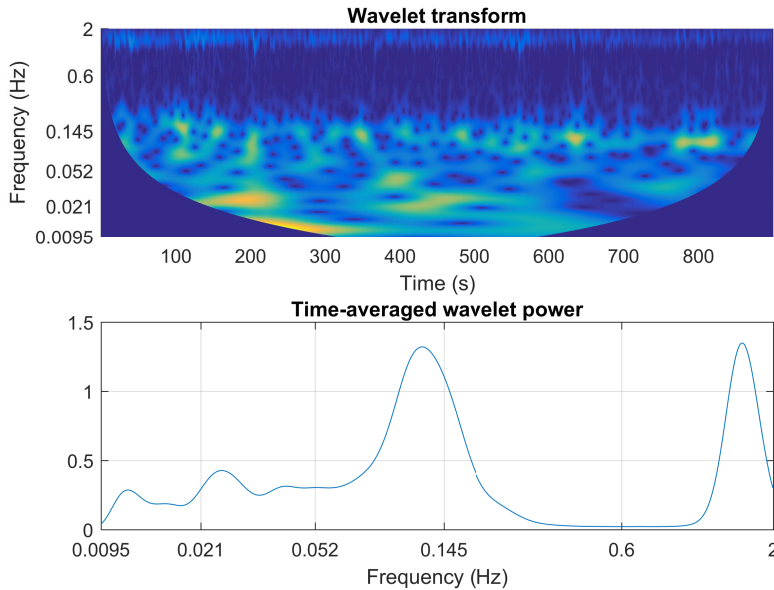


Figure 2.4: False-colour spectrogram (top) and time-averaged power spectrum (bottom) obtained through the WTA of a 15-min perfusion measurement, taken on the hallux.

2.7 Experimental results and discussion

Due to the saturation of the LDF signals during the phase of thermal stimulation, a number of acquisitions had to be excluded from the investigation of the heterogeneity in the thermal hyperaemic response, thus reducing the actual size of the data groups pertaining to the index finger and the hallux to 31 and 24 samples, respectively. Furthermore, because of the very low values obtained for the descent rate of the NO-mediated plateau b_p (with respect to the duration of the thermal stimulus), this parameter was excluded from further consideration, due to its relatively poor reliability.

The modeling of the vasodilatory response to the local heating stimulus at 43 °C highlighted several quantitative differences between the index, forearm, and hallux locations. Table 2.4 reports the median and interquartile ranges of the identified model parameters, whereas the results of their statistical analysis are summarized in Table 2.5. Superscripts mark statistically signif-

Table 2.4: Hyperaemic response to local heating: descriptive statistics.

PARAMETERS		INDEX		FOREARM		HALLUX	
		Median	IQR	Median	IQR	Median	IQR
a_{ar}	(AU)	230	270	180	130	250	320
σ_{ar}	(s)	27	26	42	18	33	17
t_{ar}	(s)	51	29	74	29	55	15
a_p	(AU)	990	3950	280	170	1000	4170
b_p	(s^{-1})	0.0076	0.011	0.0004	0.0016	0.0040	0.0072
c_p	(s^{-1})	0.026	0.018	0.029	0.028	0.024	0.026
t_p	(s)	64	43	83	33	65	19

IQR: interquartile range.

icant differences, detected with the non-parametric post-hoc Mann-Whitney test. Regarding the reactivity of the cutaneous microcirculation to the local steep rise in temperature, a slower reaction time and a milder hyperaemic plateau were observed at the forearm, compared to the other sites. In detail, at this location, the median time delay of the transient axon reflex component, $\bar{t}_{ar}$, was 74 ± 15 s, against the 51 ± 15 s of the index finger ($p < 0.001$, $r = 0.53$) and the 55 ± 7 s estimated for the hallux ($p < 0.001$, $r = 0.52$), where variability is represented by the median absolute deviation. Furthermore, σ_{ar} , which is linked to the duration of the latter transient response, resulted to be higher for the forearm ($\bar{\sigma}_{ar} = 42 \pm 11$ s), with respect to the index ($\bar{\sigma}_{ar} = 27 \pm 13$ s) and the hallux ($\bar{\sigma}_{ar} = 33 \pm 9$ s), despite statistical significance being reached only for the latter pairwise comparison ($p = 0.003$, $r = 0.38$). Consistently, the forearm exhibited also a delayed NO-mediated hyperaemic plateau ($\bar{t}_p = 83 \pm 17$ s), compared to the index finger ($\bar{t}_p = 64 \pm 20$ s, $p = 0.012$, $r = 0.32$) and the hallux ($\bar{t}_p = 65 \pm 10$ s, $p = 0.015$, $r = 0.33$). Moreover, at the forearm, the values of the a_p coefficient ($\bar{a}_p = 280 \pm 80$ AU), associated with the maximum relative excursion of the secondary plateau from the basal perfusion levels, appeared to be lower than the estimates obtained for the index finger ($\bar{a}_p = 990 \pm 718$ AU, $p < 0.001$, $r = 0.57$), and the hallux ($\bar{a}_p = 1000 \pm 781$ AU, $p < 0.001$, $r = 0.54$). This latter result might be indicative of a lower vasodilatory reserve, potentially due to a lower vascular density of the skin microcirculation at this anatomical location.

Table 2.5: Hyperaemic response to local heating: statistical tests.

COMPARISONS ($\alpha = 0.05$)		All	Index vs Forearm $N_1=31$ $N_2=30$		Index vs Hallux $N_1=31$ $N_2=24$		Forearm vs Hallux $N_1=30$ $N_2=24$	
		KW	MW		MW		MW	
		p	p	r	p	r	p	r
a_{ar}	(AU)	0.11	0.17	0.18	0.45	0.10	0.043	0.28
σ_r	(s)	0.008 [†]	0.003 [†]	0.38	0.38	0.11	0.030	0.30
t_{ar}	(s)	< 0.001 [†]	< 0.001 [†]	0.53	0.32	0.14	< 0.001 [†]	0.52
a_p	(AU)	< 0.001 [†]	< 0.001 [†]	0.57	0.93	0.01	< 0.001 [†]	0.54
c_p	(s^{-1})	0.43	0.35	0.12	0.61	0.07	0.24	0.16
t_p	(s)	0.015 [†]	0.012 [†]	0.32	0.77	0.04	0.015 [†]	0.33

[†] Statistically significant, KW: Kruskal-Wallis test, MW: Mann-Whitney test, N_i : sample size.

Regarding the WTA of cutaneous perfusion in basal conditions, a marked spatial heterogeneity could be appreciated in the absolute power estimates, obtained for the separate physiological intervals at the compared sites. However, as emphasized in a recent study by Mizeva et al. [59], the relative amplitude of the LDF oscillatory components, associated with the aforementioned physiological bands, is proportional to the average perfusion levels. Therefore, differences in absolute power might not imply an actual anatomical variability of the physiological mechanisms which adapt the microvascular flow rates but, in fact, be the effect of an underlying inter-subject variability in the basal cutaneous perfusion. For this reason, the present evaluation was only focused on estimates normalized to the total power, inside the (0.0095, 2) Hz frequency range. In detail, the WTA highlighted significant spatial differences in the relative power of the respiratory oscillations, estimated from the nominal interval (0.145, 0.6) Hz: the index finger exhibited the lowest median value ($3.2 \pm 0.4\%$) compared to the forearm ($21 \pm 8\%$, $p < 0.001$, $r = 0.81$), and the pulp of the hallux ($6.8 \pm 3.3\%$, $p = 0.006$, $r = 0.62$); also the difference emerged between these latter sites is statistically significant ($p = 0.013$, $r = 0.56$). With respect to the normalized power spectra, significant spatial differences were detected within the respiratory frequency band (0.145, 0.6) Hz, where the index finger exhibited the lowest median value ($3.2 \pm 0.4\%$) compared to the forearm ($21 \pm 8\%$, $p < 0.001$, $r = 0.81$), and the hallux ($6.8 \pm 3.3\%$, $p = 0.006$, $r = 0.62$); the difference observed between these latter sites is statistically significant as well ($p = 0.013$, $r = 0.56$). Although no significant spatial heterogeneity could be demonstrated for the other four physiological ranges, considered in the present investigation, it

Table 2.6: WT normalized power (%): descriptive statistics.

INTERVAL	INDEX		FOREARM		HALLUX	
	Median	IQR	Median	IQR	Median	IQR
0.0095 - 0.021 Hz	15.6	10.4	10.8	4.5	16.0	6.8
0.021 - 0.052 Hz	25.1	20.3	16.8	12.2	26.5	14.9
0.052 - 0.145 Hz	22.9	5.2	25.5	7.6	21.8	9.2
0.145 - 0.6 Hz	3.2	1.3	21.0	14.7	6.8	9.7
0.6 - 2 Hz	24.7	22.5	16.4	13.8	22.3	16.3

IQR: interquartile range.

Table 2.7: WT normalized power: statistical tests.

COMPARISONS ($\alpha = 0.05$)			All KW	Index vs Forearm MW*		Index vs Hallux MW*		Forearm vs Hallux MW*	
			p	p	r	p	r	p	r
0.0095 - 0.021	Hz		0.24	0.23	0.27	1	0	0.10	0.37
0.021 - 0.052	Hz		0.33	0.11	0.36	0.71	0.09	0.41	0.19
0.052 - 0.145	Hz		0.32	0.15	0.32	0.82	0.05	0.24	0.26
0.145 - 0.6	Hz	< 0.001†	< 0.001†	< 0.001†	0.81	0.006†	0.62	0.013†	0.56
0.6 - 2	Hz		0.67	0.71	0.09	0.88	0.03	0.29	0.24

† Statistically significant; KW: Kruskal-Wallis test; MW: Mann-Whitney test;

* $N_1 = N_2 = 10$.

is important to recall how the available sample size did not allow to reveal subtle dissimilarities. In this regard, expanding the current dataset will be crucial for achieving improved levels of statistical sensitivity. On the other hand, the large effect sizes observed for the breathing activity indicate that its relative contribution to the modulation of LDF signals of cutaneous perfusion is particularly location-sensitive. Descriptive statistics and statistical tests are reported in Tables 2.6 and 2.7, where significant differences are properly annotated.

Thanks to its non-invasiveness, ease of use, low cost and high temporal resolution, LDF represents a valuable technique for assessing the ability of the microcirculation to recruit unperfused arterioles and capillary beds, in response to external stimuli and physiological requirements [60]; this is especially valuable in conditions of a global derangement in peripheral perfusion (e.g. during haemorrhagic circulatory shock), where the skin microcirculation provides a reliable *window* on the perfusion state of inaccessible body districts.

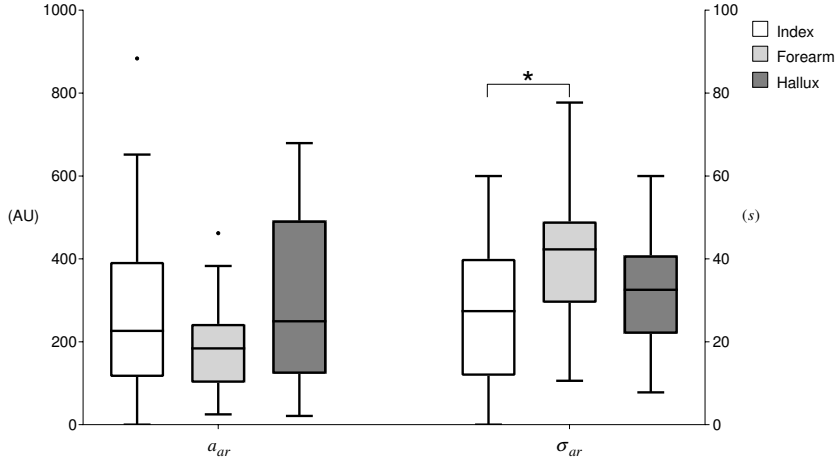


Figure 2.5: Box plot of the Gaussian a_{ar} and σ_r parameters of the axon reflex-mediated vasodilation, identified for the index finger, forearm, and hallux ($\star p = 0.003$).

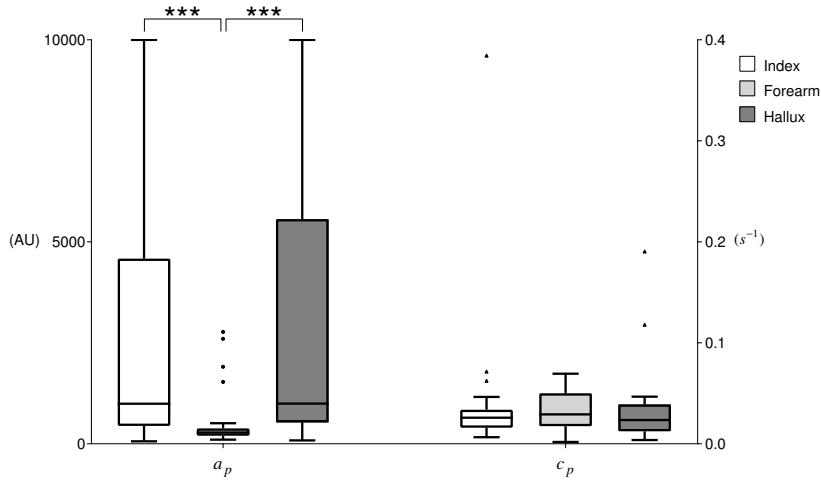


Figure 2.6: Box plot of the amplitude and ascent rate parameters, a_p and c_p , associated with the NO-mediated hyperaemic plateau at the index finger, forearm, and hallux ($\star \star \star p < 0.001$).

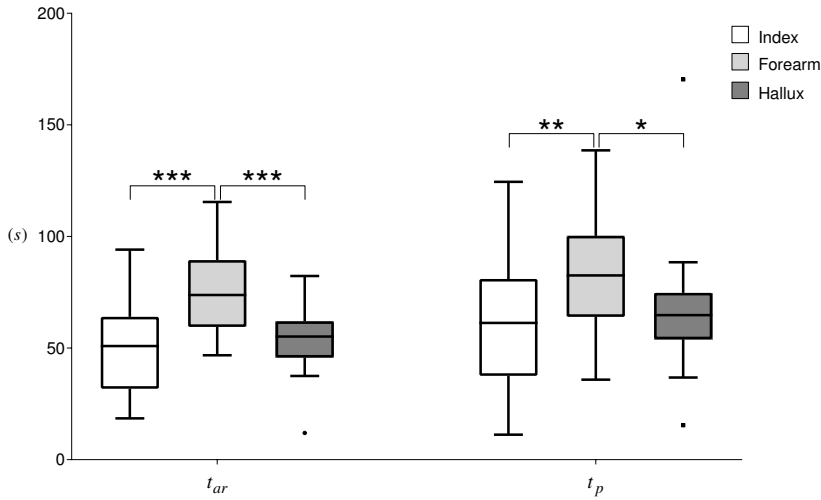


Figure 2.7: Box plot of the t_{ar} and t_p time delays, identified for the axon reflex and the hyperaemic plateau, at the index finger, forearm, and hallux locations (*** $p < 0.001$; ** $p = 0.012$; * $p = 0.015$).

Thermal, occlusive or pharmacological tests are often used in conjunction with LDF in order to probe the microvascular reactivity and assess the potential deterioration of the blood microcirculatory system. Direct, local skin heating provokes a focused reactive vasodilation which, in addition to the specific properties of the measurement site (e.g. microvessel density) is in general affected by several variables, namely: ambient temperature, probe-skin interface, warming rate and target temperature of the thermostatic probe. One of the shortcomings of past investigations, involving the evaluation of the absolute values or the power spectra of LDF signals of skin perfusion, was the lack of a standardized approach to cope with these sources of variability, allowing for a meaningful comparison with experimental results obtained in different studies. This limitation is exacerbated by the absence of a thorough consensus on the optimal anatomical location of measurement.

Against this background, the present study was designed so as to ensure a thorough uniformity of the measurement protocol and, in turn, to limit to the highest degree the presence of undesired sources of variability apart from the measurement location. The finger pad, the hallux, and the volar side of the forearm were selected, in that they represent the most frequently adopted

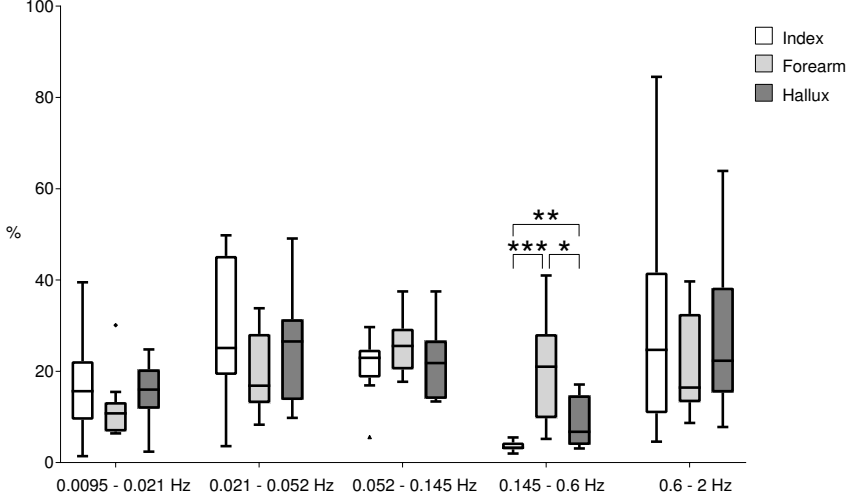


Figure 2.8: Box plot of the normalized wavelet power estimates, obtained in basal conditions for the cardiac (0.6, 2) Hz, respiratory (0.145, 0.6) Hz, myogenic (0.052, 0.145) Hz, neurogenic (0.021, 0.052) Hz and NO-dependent endothelial (0.0095, 0.021) Hz oscillatory processes (*** $p < 0.001$; ** $p = 0.006$; * $p = 0.013$).

anatomical sites in microvascular research. Model 2.1 was used to derive a quantitative characterization of the heterogeneity in the reactive hyperaemia at these sites, highlighting significant dissimilarities. The perfusion curves generated from the median values of the model parameters, identified at the index finger (yellow), forearm (blue) and hallux (red), are shown in Fig. 2.9. It can be observed that the biphasic vasodilatory response to a local skin stimulus, successfully evoked at all the investigated locations, is correctly represented by the proposed data model. The rate and level of the *axon reflex*-related vasodilation are very similar at the index and the hallux. The main difference between these sites is observed, on the other hand, during the NO-mediated plateau, where the hallux exhibits a faster vasodilation, reaching higher absolute perfusion levels. This result is in accordance with the work of Hodges et al. [61], where the authors have found that the NO-mediated vasodilation is more pronounced in the cutaneous microcirculation of the lower limbs. The forearm skin is associated with an inferior vasodila-

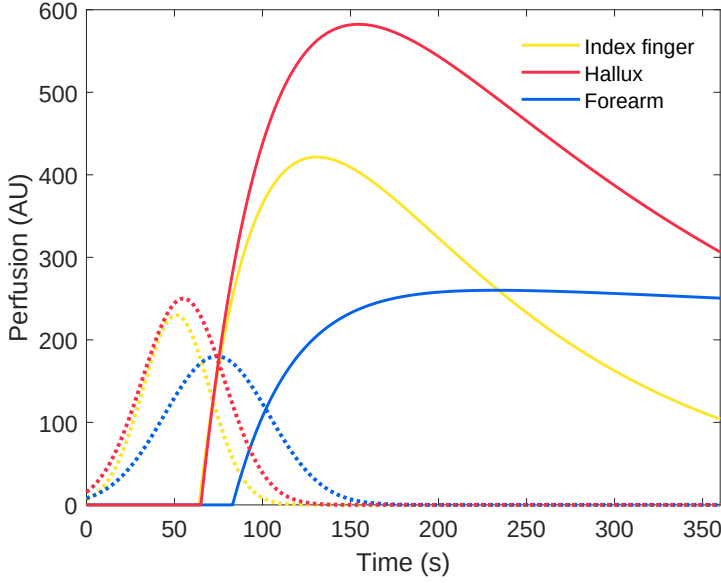


Figure 2.9: Simulated cutaneous vasodilatory response at each investigated location (Table 2.4), based on the median parameters estimated for model 2.1. Axon reflex and NO-mediated components are displayed with dotted and solid lines, respectively.

tory reserve, showing lower a_{ar} and a_p amplitudes with respect to the index finger and the hallux. Moreover, the reaction time to the steep rise to 43 °C appears to be significantly longer on the volar aspect of the forearm, as can be clearly recognized in Fig. 2.9 from the position of the Gaussian peak and the local minimum at the onset of the plateau. Furthermore, it is relevant to emphasize the marked heterogeneity in the amplitude ratio a_p/a_{ar} , observed between the non-glabrous skin of the forearm, where $a_p/a_{ar} = 1.4 \pm 0.3$, and the glabrous skin of index and hallux (where this ratio respectively increases to 3.3 ± 2.5 and 3.0 ± 1.8). These data suggest a difference in the relative contribution of the *axon reflex* and the NO-mediated vasodilatory components to the overall hyperaemic response. Glabrous skin has a high density of capillaries and arteriovenous shunts, it is highly innervated and actively participates to the body thermoregulation. This might explain why its response to the thermal stimulus is faster and more intense. However, the vasodilatory parameters associated with the glabrous skin of index and

hallux were characterized by a higher degree of inter-subject variability, especially as regards the relative amplitude of the hyperaemic reaction. At these locations, the IQR of the a_p and a_{ar} estimates was larger than the corresponding median value, reaching approximately 400% for the NO-mediated plateau (compared to 70% on the forearm). Also, it must be recalled how, in the present study, signal saturation issues during the heating phase occurred in 11% and 33% of the measurements taken respectively on index and hallux, whereas no saturation was detected on the forearm. Therefore, as regards the reproducibility of these tests of thermal hyperaemia, the skin of the forearm appears to be the preferential choice for taking LDF measurements.

Perfusion signals, recorded in basal conditions at the three compared sites, demonstrated a similar spectral content. It must be emphasized that the frequency domain analysis of microvascular oscillatory processes is complicated by their dependence on the average perfusion levels [59]. However, this study focused only on the *relative* contribution of the different mechanisms, involved in the modulation of the local skin perfusion; in this respect, the normalization of the power estimates played a key role in obtaining a robust, and meaningful comparison. Based on these data, the oscillations in blood flow, produced by the respiratory rhythm, were the only to exhibit a significant degree of spatial heterogeneity, with the skin of the forearm showing a significantly larger normalized spectral power. This result agrees with the observation made by Krupatkin [62], which has reported that breathing-related oscillations in skin perfusion are especially pronounced over areas lacking arteriovenous anastomoses, such as the volar aspect of the forearm. Although the exact physiological mechanism underlying this difference is still unclear, these data should be taken into account in the design of future LDF measurement protocols.

2.8 Conclusion

For the first time, a quantitative comparison of time and spectral properties of LDF signals of skin perfusion has been concurrently made at three locations frequently used in microvascular research: the index finger, the forearm, and the hallux. A biphasic vasodilatory response to local heating at 43 °C was observed in the time domain, independently of the examined location. Nevertheless, on the volar aspect of the forearm, this reactive hyperaemia was significantly milder and delayed, with respect to the other sites.

The wavelet time-frequency representation of the LDF signals, measured at the three investigated locations, demonstrated a significant dissimilarity of the basal modulation of the cutaneous perfusion, related to the respiratory activity. However, the moderate sample size available for this analysis might have limited the ability to highlight potential differences in the other physiological ranges with the required sensitivity.

In conclusion, these results strongly indicate that a direct comparison of LDF data acquired at different anatomical locations should be avoided, without a proper consideration of the inherent heterogeneity in the temporal and spectral properties of the skin microvascular perfusion.

Chapter 3

Pulse decomposition analysis

*The shape of arterial pulse waveforms is intimately related to the physical properties of the vascular system, and might carry valuable information for the diagnosis and management of cardiovascular disease. In general, these pulse waves can be monitored continuously and non-invasively with optical techniques, at the level of the cutaneous microvascular beds. This Chapter covers the development of a new decomposition algorithm devoted to the automatic reconstruction of LDF pulse waveforms, and to the extraction of physiologically relevant information via motivated morphological features.*¹

¹A preliminary version of this chapter has been published as “Detecting vascular age using the analysis of peripheral pulse” in *IEEE Transactions on Biomedical Engineering*, 2018.

3.1 Introduction

The physical properties of the circulatory system affect the propagation of arterial pulse waves inside the vascular tree, exerting a complex influence on their shape which, in general, undergoes consistent spatio-temporal modifications. This propagation phenomenon can be investigated via one-dimensional biomechanical models simulating the systemic blood flow in visco-elastic arteries. The present approach is effective for probing the specific role of mechanical properties which are not directly accessible for measurement in clinical scenarios, e.g. total arterial compliance, local visco-elasticity of vascular walls, total peripheral flow resistance; conversely, the same methodological framework could be applied in combination with haemodynamic data, available *in vivo*, in order to derive a theoretical estimate of these physical quantities. However, as elegantly described in Alastruey et al. [63], the computation of local visco-elastic properties necessitates the availability of simultaneous measurements of wall thickness, intra-luminal area and pressure at a specific location of the arterial tree. Hence, despite the unquestionable relevance of these patient-specific parameters in the assessment of cardiovascular function, the clinical applicability of these models is extremely challenging, as they must rely on invasive pressure monitoring and MR imaging.

Nevertheless, since the morphology of systolic pulse waves carries implicit information on the blood vessels in which they propagate, the quantitative characterization of peripheral pulse contours, detected with optical techniques at the level of the cutaneous microcirculation, has gained interest as a potentially alternative non-invasive approach for the diagnosis of vascular abnormalities. More specifically, the analysis of the second derivative of the photoplethysmographic digital volume pulse (DVP), defined as *acceleration photoplethysmogram*, has been proposed as Takazawa et al. as a straightforward method for deriving empirical markers of vascular ageing and arteriosclerosis, based on the relative amplitude of five characteristic peaks [64–67]. However, despite its simplicity, these features are not founded on solid physiological grounds and, thus, are not subject to clear interpretation; furthermore, the reliance on the second derivative of the DVP makes them considerably susceptible to the presence of noise or movement artefacts.

Pulse decomposition algorithms (PDA) represent another class of recent techniques for the characterization of peripheral waveforms, and the non-

invasive extraction of physiologically relevant features. Conceptually, PDAs are based on the generally accepted hypothesis that the contour of arterial pulse waves is shaped by multiple wave reflections which occur inside the circulatory system, due to local inhomogeneities in the effective vascular impedance. Hence, pulse waveforms can be seen as the linear superposition of a forward-travelling systolic pulse and counter-propagating reflected components. Accordingly, in PDAs, peripheral pulse waves are decomposed into a varying number of basic terms (i.e. three to five Gaussian, log-normal, Rayleigh or gamma functions), where each secondary component is theoretically meant to reproduce a reflection wave originating from a major arterial junction [68–70].

This Chapter presents the implementation and the validation of a new PDA, which exploits a four-order multi-Gaussian model in order to derive an accurate representation of peripheral pulse waves, extracted from LDF signals of microvascular perfusion.

3.2 Pulse wave reconstruction

The multi-Gaussian PDA developed for the analysis of peripheral LDF pulse waves is comprised of two main building blocks: a first detection stage, which addresses the automatic recognition of the separate cardiac cycles, and the identification of systolic, dicrotic, and diastolic reference points on the LDF pulse contour; and a second modeling stage, where each peripheral pulse waveform is accurately reconstructed by means of four Gaussian components. The following paragraphs present the details of these fundamental stages.

3.2.1 Detection

The proposed multi-Gaussian PDA includes a few preprocessing operations, which are required for improving the performance of the following detection and modeling functional blocks. First, the LDF signals of microvascular perfusion, recorded at a sampling frequency of 32 Hz by means of a custom data acquisition software, are upsampled to 128 Hz, where each value of the expanded signal is generated using 8 samples of the original recording; next, a low-pass interpolation FIR filter (length: 33 samples; normalized cut-off frequency: 0.5) is applied so as to minimize the degree of spectral distortion introduced by this operation.

For the automatic segmentation of the pulse waves, a maxima detector module is initially included in order to identify the systolic local peaks of the perfusion signals; to this end, a maximum theoretical threshold of 150 bpm is imposed on the instantaneous heart rate (assuming resting conditions), thus limiting the occurrence of false positive pulse waves, due to the secondary ripples which usually characterize their diastolic phase. Next, the PDA detects the absolute minima between these systolic references so as to locate the end-diastolic troughs of the waveforms, and obtain a preliminary windowing of the distinct cardiac cycles. These latter references are then employed for the recognition and exclusion of *false* instances, based on the *a priori* observation that the relative amplitude of the cardiac pulsations is generally not subject to sudden cycle-to-cycle changes; specifically, only candidate pulse waves whose valley-to-peak excursion exceeds 50% of the previous ones' are preserved at this stage. However, since the *true* location of primary systolic waves and end-diastolic depressions does not always correspond to the absolute maxima and minima of the blood perfusion signal, inside the separate cardiac cycles, a further refinement mechanism is applied to improve the preliminary solutions. In detail, regarding the correction of the systolic peaks, the algorithm searches for any local maxima preceding the original, whose local prominence with respect to the reference valley does not fall below 80% of the former amplitude; on the other hand, the starting end-diastolic references are changed for possible local minima inside the original trough-to-peak time interval, if their amplitude is lower than 20% of the overall pulse excursion.

Furthermore, the proposed pulse reconstruction method requires the identification of an *incisura* on the descending limb of the pulse, in addition to the above feature points; the purpose is to separate the forward-travelling systolic pulse from the diastolic phase of the cardiac cycle, which is generally associated with delayed secondary waves, generated along the arterial tree due to vascular impedance inhomogeneities. As a preliminary step, signals are detrended by subtracting a cubic spline interpolant fitted to the end-diastolic detections, thus allowing for the accurate extraction of the cardiac-related oscillatory component of the microvascular perfusion. Then, the pulse contour's first derivative, $p'(t)$, is computed by means of a 3-point differentiator, as follows:

$$p'(t) = \frac{p(t+1) - p(t-1)}{2} \quad (3.1)$$

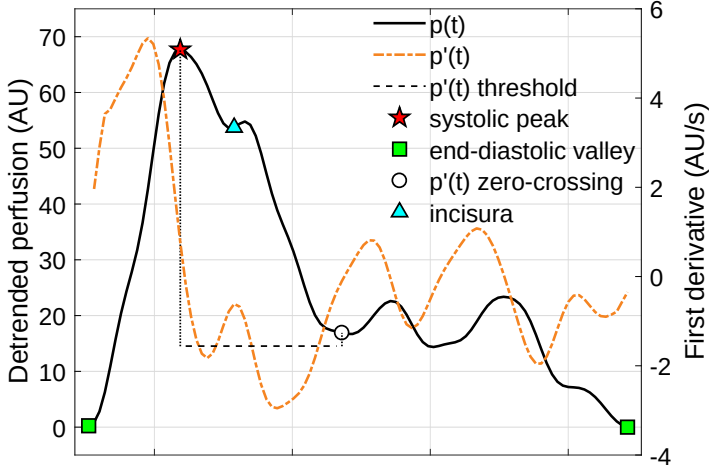


Figure 3.1: LDF pulse windowing and reference points identification.

where $p(t)$ represents the LDF perfusion signal at discrete time t . Next, a 7-point moving average filter is applied to $p'(t)$, so as to attenuate the undesired effect of local spurious fluctuations. Fig. 3.1 schematizes the approach followed for the determination of this reference incisura: inside each cardiac cycle, the algorithm first identifies the earliest negative-to-positive zero crossing of $p'(t)$ after the systolic reference (if none are detected, the end-diastolic trough is selected instead); then, the time span between this point and the former peak is analyzed for the presence of local maxima in $p'(t)$ which exceed the local average slope of the waveform: if detected, the earliest of them is adopted as reference incisura, otherwise the former zero crossing is chosen. This strategy proved to be effective for isolating the primary systolic pulse even when the temporal superposition with secondary diastolic components produces only a slight notch in the pulse contour, but still without introducing an excessive sensitivity to minor variations in the $p'(t)$ signal.

3.2.2 Modeling

Following the detection stage and the identification of these reference points, a four-Gaussian model is iteratively fitted to each cardiac cycle's pulse wave, in order to derive a parametric representation of its waveform:

$$G_{pulse}(t) = \sum_{i=1}^4 g_i = \sum_{i=1}^4 \alpha_i \cdot e^{-\frac{(t-\mu_i)^2}{2\sigma_i^2}}. \quad (3.2)$$

In general, the developed PDA is designed so as to use only g_1 for reproducing the primary systolic component to the left of the dicrotic *incisura*, and the remaining g_2 , g_3 , and g_4 terms for the complex profile of the diastolic phase, which generally exhibits a number of secondary ripples arising from multiple wave reflections inside the vascular system. Curve fitting is performed with a trust region algorithm for nonlinear least-squares problems. The configuration options specifically adopted for this application are hereby summarized: maximum and minimum allowed changes in the model parameters respectively equal to 0.1 and 10^{-8} ; maximum number of objective function evaluations: 600; maximum number of iterations: 10^6 ; termination tolerance on objective function and model parameters: 10^{-6} . Furthermore, upon identification, the initial and boundary values assigned to the twelve parameters of the adopted model are properly tuned with respect to the specific properties of each pulse waveform to be separately reconstructed; this is performed in order to place the starting point of the optimization problem close to the *ideal* solution, limit the search hypervolume, and improve the overall stability of the parameter identification process. As detailed in Table 3.1, the amplitude α_i of the four Gaussian components is tuned according to the maximum excursion of the pulse wave, Δp , whereas their standard deviations σ_i are constrained by limiting the corresponding full width at half maximum (FWHM) with respect to the span of the systolic and diastolic phases, using the following relation:

$$\text{FWHM} = 2\sigma\sqrt{2\log(2)} \quad \Rightarrow \quad \sigma = \frac{\text{FWHM}}{2\sqrt{2\log(2)}}. \quad (3.3)$$

It must be emphasized that, despite initializing the means μ_i of the three *reflection* Gaussians so as to be progressively distributed along the diastolic portion of the pulse waves, their original order is not necessarily retained after optimization. Therefore, at this stage, the algorithm must rearrange the identified Gaussian components, in order to keep the inequality constraint $\mu_{2,i} < \mu_{3,i} < \mu_{4,i}$ always satisfied for each cardiac cycle, i .

After completing the reconstruction of the whole set of pulse waves, extracted from the analyzed LDF signal, a series of exclusion criteria is imposed on the

Table 3.1: Configuration of the trust-region optimization algorithm.

	LOWER BOUNDARY	STARTING POINT	UPPER BOUNDARY
α_1	$0.5 \cdot \Delta p$	$0.8 \cdot \Delta p$	Δp
μ_1	$t_{P \text{ start}}$	$t_{P \text{ start}} + 0.5 \cdot \Delta t_{sys}$	t_{inc}
σ_1	$0.5 \cdot \Delta t_{sys} / 2\sqrt{2 \log(2)}$	$\Delta t_{sys} / 2\sqrt{2 \log(2)}$	$1.5 \cdot \Delta t_{sys} / 2\sqrt{2 \log(2)}$
α_2	0	$0.4 \cdot \Delta p$	$0.6 \cdot \Delta p$
μ_2	t_{inc}	t_{inc}	$t_{P \text{ end}}$
σ_2	$0.1 \cdot \Delta t_{dia} / 2\sqrt{2 \log(2)}$	$0.25 \cdot \Delta t_{dia} / 2\sqrt{2 \log(2)}$	$0.33 \cdot \Delta t_{dia} / 2\sqrt{2 \log(2)}$
α_3	0	$0.4 \cdot \Delta p$	$0.6 \cdot \Delta p$
μ_3	t_{inc}	$t_{inc} + 0.33 \cdot \Delta t_{dia}$	$t_{P \text{ end}}$
σ_3	$0.1 \cdot \Delta t_{dia} / 2\sqrt{2 \log(2)}$	$0.25 \cdot \Delta t_{dia} / 2\sqrt{2 \log(2)}$	$0.33 \cdot \Delta t_{dia} / 2\sqrt{2 \log(2)}$
α_4	0	$0.4 \cdot \Delta p$	$0.6 \cdot \Delta p$
μ_4	t_{inc}	$t_{inc} + 0.67 \cdot \Delta t_{dia}$	$t_{P \text{ end}}$
σ_4	$0.1 \cdot \Delta t_{dia} / 2\sqrt{2 \log(2)}$	$0.25 \cdot \Delta t_{dia} / 2\sqrt{2 \log(2)}$	$0.33 \cdot \Delta t_{dia} / 2\sqrt{2 \log(2)}$

$t_{P \text{ start}}, t_{P \text{ end}}$: left and right boundaries of the pulse (corresponding to the end-diastolic valleys);

t_{inc} : incisura;

$\Delta t_{sys}, \Delta t_{dia}$: systolic and diastolic time spans;

Δp : maximum amplitude excursion of the pulse.

identified parameters, in order to detect residual *false* cardiac cycles and, secondly, to remove possibly misfitted models from further consideration, hence reducing their impact on the outcome of the following analyses. In detail, the PDA is designed so as to cope with the detrimental effect of motion-induced artefact spikes by setting a $3\text{-}\sigma$ upper limit on the systolic α_1 amplitude of the identified models of pulse wave. Furthermore, consecutive cardiac cycles which have been spuriously merged are recognized and removed by enforcing an analogous upper boundary on the pulse duration, T_p ; on the other hand, the algorithm associates concurrently small $3\text{-}\sigma$ outlier values of T_p and α_1 with the possible presence of *split* pulse waves. Finally, the conjoint detection of large σ_1 and R^2 outliers is adopted for identifying the potential mis-reconstruction of the waveforms' primary systolic component, which might arise from a sub-optimal detection of the diastolic incisura. The qualitative performance of the multi-Gaussian modeling algorithm is displayed in Fig. 3.2.

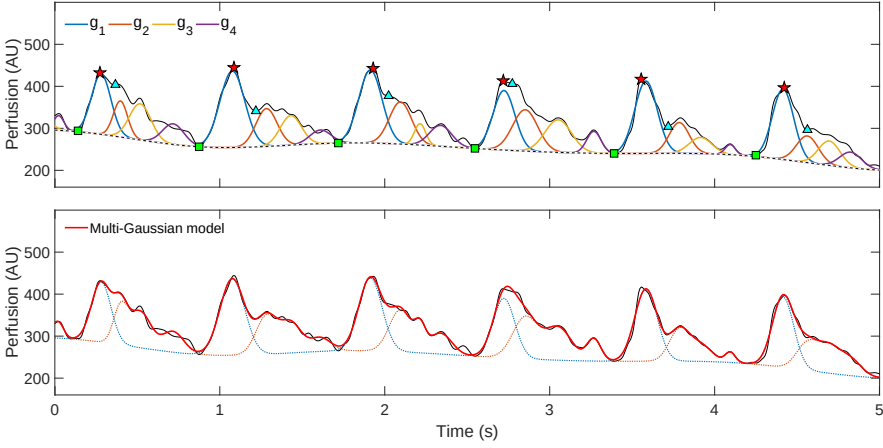


Figure 3.2: Multi-Gaussian decomposition of the LDF pulse wave: single components (top) and general model (bottom).

3.3 Feature extraction

After the modeling stage, the developed PDA performs a detailed characterization of the identified waveform models, through a set of quantitative shape descriptors. The definition of these morphological features relied in part on recent literature concerning the analysis of digital volume pulse waves (DVP), recorded with photoplethysmographic sensors. In this regard, Chowienczyk et al. [71] have effectively shown that the time interval separating the systolic and diastolic peaks of DVP waves, ΔT_{DVP} , is correlated with the transit time of arterial pressure waves between the root of the subclavian artery to the aortic bifurcation (Pearson's $r = 0.75$, $p < 0.001$); this, in turn, is known to hold an inverse relation to vascular stiffness, in accordance with the Moens-Korteweg equation [72]. Consistently, in a subsequent work [73], the same research group has highlighted a similar significant correlation with respect to the aorto-femoral transit time ($r = 0.70$, $p < 0.001$), and thus proposed an arterial *Stiffness Index*, SI_{DVP} , defined as the ratio of the height of the measured subject to the former ΔT_{DVP} delay. As expected, the latter index has been successfully related to the carotid-femoral pulse wave velocity ($r = 0.65$, $p < 0.001$) and ageing ($r = 0.63$, $p < 0.001$) [74], which is known to be associated with a progressive reduction in the compliance of vascular walls.

In addition to the SI_{DVP} , which is derived from the relative timing of systolic and diastolic wave components of peripheral pulse waves, a second physiologically-motivated index of pressure wave reflection, i.e. the *Reflection Index* (RI_{DVP}), has been introduced in the aforementioned studies [74]. This feature is generally defined as the relative amplitude of the early diastolic peak to the direct forward-travelling systolic component of the waveform. Accordingly, this parameter was originally designated as IP_{DVP} , in that its estimation was specifically based on the amplitude of the *inflection point*, IP, where the first derivative of the DVP waveform was closest to zero. The RI_{DVP} has exhibited a positive correlation with the tone of small peripheral *resistance* arteries, showing consistent dose-dependent changes in response to the administration of vasoactive drugs (angiotensin II and glyceryl trinitrate) [74].

However, it must be here emphasized that, despite the understandable attempt to overcome the unclear physiological significance of the “acceleration plethysmogram” method [64] (which, as stated in the introduction, involves the analysis of five distinct waves that generally characterize the second derivative d^2DVP/dt^2 of the pulse), both the SI_{DVP} and RI_{DVP} still directly depend on the accurate detection of the inflection reference along the $dDVP/dt$ signal, and hence may lack adequate reliability in the case of seriously damped or noisy photoplethysmographic signals.

Nevertheless, by integrating these features into the framework of the proposed PDA, their derivation could now benefit from the improved robustness and reliability offered by the multi-Gaussian parametric representation of the pulse waveform and, secondly, by the suite of control mechanisms which are set up after the reconstruction stage, in order to exclude unreliable models. Accordingly, the PDA estimates a modified version of the SI parameter from the time span between the primary systolic component and the centroid of the three diastolic Gaussians, as shown in Fig. 3.3a; this interval is then normalized to the overall duration of the pulse wave (SI_{norm}), in order to correct for its inherent dependence on the instantaneous heart rate of the analyzed subjects. On the other hand, the original principle of the RI_{DVP} was translated and adapted to the proposed decomposition analysis, by computing this marker of peripheral vascular tone as the ratio of the areas beneath the diastolic (A_d) and systolic (A_s) phases of the pulse model (Fig. 3.3a).

Furthermore, in one of the earliest studies involving the quantitative analysis of photoplethysmographic pulse contours (1941), Dillon and Hertzman

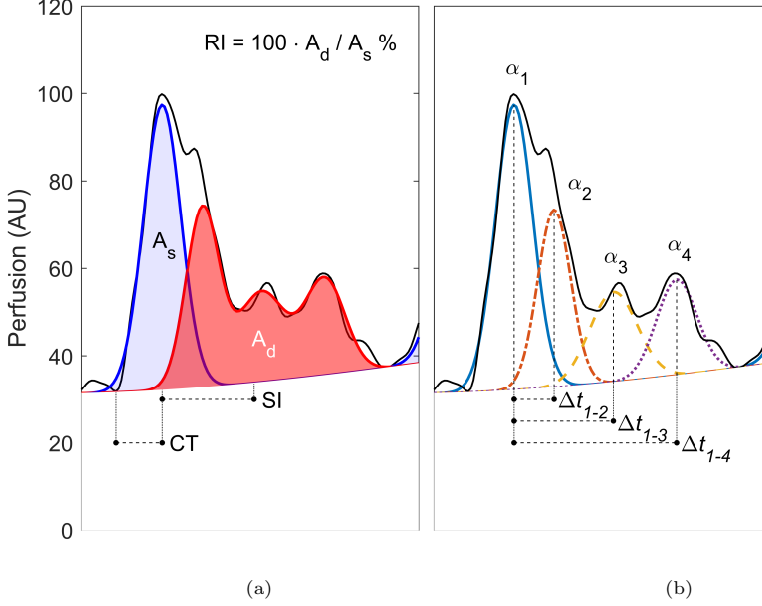


Figure 3.3: Extraction of the morphological feature vector for the detailed characterization of the LDF waveform models.

already revealed that the interval from the foot of the DVP to its systolic peak, defined as *Crest Time* (CT), was increased in the elderly population and in patients suffering from arteriosclerosis or chronic hypertension [75]. Hence, at this stage, the PDA also estimates the rise time of the systolic Gaussian component, using its mean, μ_1 , and the preceding end-diastolic point as time references; then, as performed for the SI, this latter time is divided by the estimated duration of each cardiac cycle, resulting in a normalized, adimensional feature, i.e. CT_{norm} .

Additionally, in order to derive a thorough morphological characterization of the LDF pulse waves, these physiologically motivated properties were integrated with the relative delays Δt_{1-2} , Δt_{1-3} , and Δt_{1-4} of the secondary Gaussian components and, secondly, with their percentage amplitude ratios α_2/α_1 , α_3/α_1 , α_4/α_1 (Fig. 3.3b). The sole adoption of relative amplitude values greatly reduces the sensitivity of these descriptors to the effective microvessel density characterizing the measured location (which is subject to a relevant inter- and intra-subject variability), hence improving their overall reproducibility [76].

In summary, the quantitative characterization of each multi-Gaussian model of LDF pulse waveform relies on the following 9-element feature vector, $\mathbf{x}_i$:

$$\mathbf{x}_i = \left(\text{SI}_{\text{norm},i}, \text{RI}_i, \text{CT}_{\text{norm},i}, \frac{\alpha_{2,i}}{\alpha_{1,i}}, \frac{\alpha_{3,i}}{\alpha_{1,i}}, \frac{\alpha_{4,i}}{\alpha_{1,i}}, \Delta t_{1-2,i}, \Delta t_{1-3,i}, \Delta t_{1-4,i} \right). \quad (3.4)$$

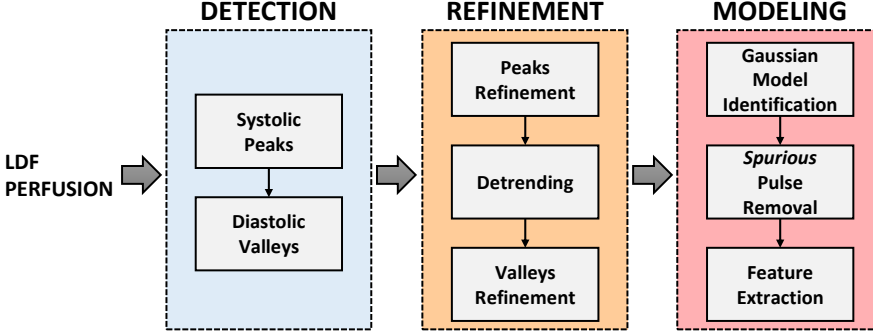


Figure 3.4: Multi-Gaussian pulse decomposition algorithm: block diagram.

3.4 Performance

The assessment of the PDA performance involved the evaluation of the goodness of fit exhibited by the adopted multi-Gaussian model, and the sensitivity to the initial conditions assigned to its twelve parameters. The present analysis focused on $N_p = 20935$ models of LDF waveform, extracted from a set of 54 LDF signals of microvascular perfusion, acquired in basal conditions from healthy control volunteers.

3.4.1 Goodness of fit

In order to quantify how successful the multi-Gaussian fit is in reproducing the actual profile of the LDF waveforms, the coefficient of determination R^2 was estimated for each of the available N_p pulse waves. This statistic corresponds to the square of the linear correlation between the model-predicted values and the original LDF data, increasing (up to a value of 1) when the model is able to account for a higher proportion of the data variance. Over the whole set of N_p test waveforms, the developed PDA showed a R^2 of 0.98 ± 0.03 (mean $\pm \sigma$), which strongly indicates a consistently high goodness of fit.

Moreover, for each analyzed LDF signal, a normalized metric of goodness of fit was evaluated as:

$$\text{GF}_{\text{norm}} = 1 - \frac{\|p(t) - G_{\text{pulse}}(t)\|}{\|p(t) - \bar{p}\|} \quad (3.5)$$

where $p(t)$ and $G_{\text{pulse}}(t)$ represent, respectively, the original signal and its multi-Gaussian model (whereas the overline indicates time averaging). Based on this formulation, a perfect data fit would correspond to a theoretical $\text{GF}_{\text{norm}} = 1$ whereas, on the other hand, a value of 0 would imply that the multi-Gaussian model does not perform better than fitting the LDF signal with its average line. In the available dataset of 54 perfusion signals, a mean GF_{norm} of 0.90 ± 0.03 was observed. The results of the present goodness-of-fit analysis are summarized below, in Table 3.2:

Table 3.2: Goodness-of-fit metrics.

TEST DATASET: $N_p = 20935$		Mean	σ
Coefficient of determination	R^2	0.98	0.03
Modeling error	GF_{norm}	0.90	0.03

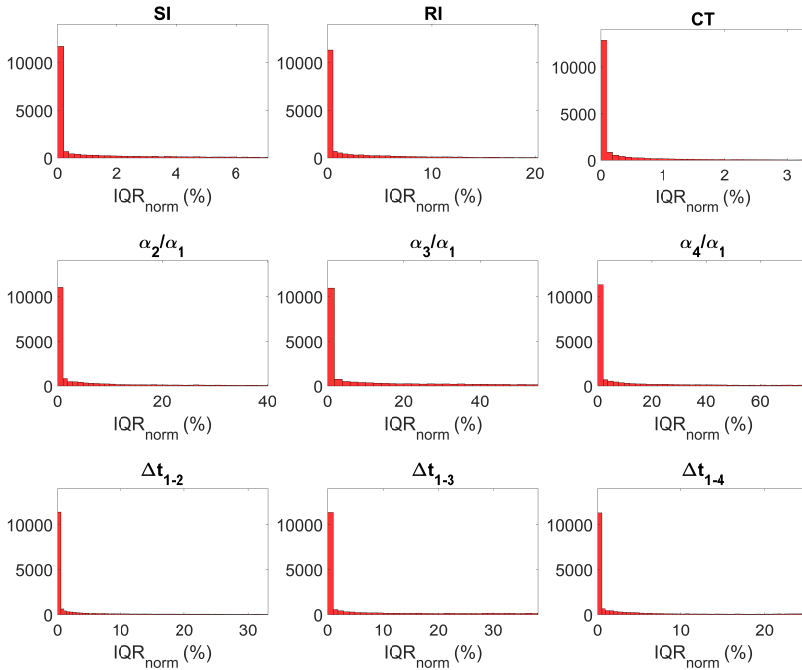
3.4.2 Stability

In order to probe the stability of the multi-Gaussian data fit, with respect to the initial conditions assigned to the optimization algorithm, a sensitivity analysis was run on the N_p pulse waves composing the test dataset. In detail, the fitting procedure was repeated over 100 iterations, setting the starting values of the model parameters according to a uniform probability distribution, inside the respective lower and upper boundaries listed in Table 3.1. Then, for each test pulse wave, the normalized interquartile range of the nine morphological features, IQR_{norm} (defined as the interquartile range of the data distribution, divided by the corresponding median value), was estimated so as to assess their degree of variability. The results of this simulation (expressed as median and IQR) are reported in Table 3.3.

The median IQR_{norm} values, obtained for the adopted morphological features, highlight the numerical stability of the multi-Gaussian PDA. On the other hand, regarding the relatively wide IQR specifically associated with the single diastolic components of the LDF pulse, it is relevant to recall how

Table 3.3: Multi-Gaussian pulse fitting: sensitivity analysis.

FEATURES	IQR _{norm}	
	Median	IQR
SI _{norm}	0.21%	(0.00%, 7.81%)
RI	0.05%	(0.00%, 3.15%)
CT _{norm}	0.01%	(0.00%, 0.84%)
α_2/α_1	0.58%	(0.00%, 17.64%)
α_3/α_1	1.16%	(0.00%, 31.30%)
α_4/α_1	0.73%	(0.01%, 35.28%)
$\Delta t_{1-2,i}$	0.14%	(0.00%, 13.74%)
$\Delta t_{1-3,i}$	0.29%	(0.00%, 22.60%)
$\Delta t_{1-4,i}$	0.17%	(0.00%, 10.73%)

Figure 3.5: Sensitivity analysis: distributions of the IQR_{norm}, estimated for the $N_p = 20935$ test feature vectors, over 100 simulation runs.

the original identification procedure exploited fine-tuned initial conditions, thoroughly assigned according to the specific characteristics of each detected waveform. However, in the present random simulation, parameter initialization was not fully driven by *a priori* information (on the reference points, identified along the pulse contour at the detection stage of the PDA), and this might have had a detrimental influence on the overall performance of the PDA. Nevertheless, this non-negligible variability does not appear to deteriorate the reconstruction of the diastolic phase of the waveforms, as implied by the narrow IQR associated with the SI_{norm} and RI features. The histograms of the IQR_{norm} are shown in Fig. 3.5.

3.5 Conclusion

This Chapter presented the details of a new decomposition algorithm for the accurate reconstruction and analysis of peripheral pulse waveforms, extracted from non-invasive measurements of cutaneous perfusion. The main stages of the technique, involving the automatic detection, modeling, and physiological characterization of LDF pulse waves, have been covered in detail. Furthermore, the evaluation of the multi-Gaussian modeling algorithm, in terms of goodness-of-fit metrics and numerical stability, resulted in a consistently high performance over the reference test set, comprised of 20935 LDF waveforms.

The next Chapter describes a supervised classification method, based on this new multi-Gaussian PDA, which exploits the morphological features introduced in Section 3.3, in order to detect vascular ageing patterns and perform an automatic recognition of young and elderly subjects.

Chapter 4

Vascular age recognition through pulse decomposition analysis

*Ageing is accompanied by a progressive stiffening of arterial walls and by the dysfunction of the vascular endothelium, and is regarded as a primary factor which independently favours the development of cardiovascular disease. The profile of peripheral pulse waves is shaped by the biomechanical modifications of the circulatory system; hence, it is relevant to investigate the feasibility of an automatic, non-invasive detection of vascular ageing, which is uniquely based on the decomposition of LDF pulse waves, and the analysis of their morphology. Accordingly, the present Chapter covers the implementation and the validation of a SVM classifier of vascular ageing, trained over the dataset of feature vectors, extracted by means of the novel multi-Gaussian PDA, introduced in Chapter 3.*¹

¹A preliminary version of this chapter has been published as “Detecting vascular age using the analysis of peripheral pulse” in *IEEE Transactions on Biomedical Engineering*, 2018.

4.1 Ageing of the cardiovascular system

Ageing is deemed a major non-reversible risk factor for cardiovascular disease, contributing per se to the development of vascular dysfunction and to the incidence of hypertension, stroke, coronary heart disease, and heart failure [77]. The ageing of the vascular system is characterized by a gradual reduction in arterial compliance and has been independently correlated with endothelial dysfunction [78]. In this regard, the progressive accumulation of reactive oxygen species (ROS) has been identified as an important underlying mechanism, since it functionally inactivates the endothelial production of nitric oxide (NO), hence impairing the physiological NO-dependent regulation of the vascular muscle cells tone [79]. Moreover, the ageing-related dysfunction of the vascular endothelium is fuelled by the upregulation of inflammatory cytokines, which promote a chronic state of low-grade inflammation, thus favouring endothelial leakage, and further contributing to the production of ROS [80]. Conversely, the increased vascular stiffness in the elderly population has been associated with augmented levels of collagen cross-linking (fuelled by non-enzymatic glycosylation) which, in turn, lead to the formation of vessel walls characterized by a thicker tunica media and higher collagen-to-elastin ratios [80].

In accordance with the Moens-Korteweg equation [72], vascular stiffening manifests itself through an increase in pulse wave velocity (PWV); this is usually evaluated transcutaneously by measuring the *foot-to-foot* transit time of arterial pulse waves between the common carotid artery and the common femoral artery, where the foot is identified with the instant which immediately precedes the steep pressure rise during early systole. PWV is generally regarded as a robust, and reproducible marker of arterial stiffness and has been related with adverse prognosis [81]. Nevertheless, as considered in Chapter 3, the biomechanical properties of the circulatory system does not solely modify the propagation velocity of the arterial pulse wave, but also affect its characteristic shape. Thus, in recent years, the development of new techniques for the extraction of clinically relevant information, based on the quantitative analysis of peripheral pulse waveforms, has gained the attention of many investigators [69,70,82–84]. In this regard, Pulse Decomposition Analysis (Chapter 3) has emerged as a particularly valuable tool. A few authors have recently applied machine learning techniques in order to classify differently aged individuals [85] or detect high values of PWV [86], relying uniquely on features extracted from the waveform of LDF and PPG

signals. This Chapter reports on the implementation of a *support vector machine* supervised classifier which learns from morphological features of LDF pulse waves (extracted by means of the new multi-Gaussian PDA, presented in Chapter 3), for the automatic recognition of elderly subjects.

4.2 Experimental setup

54 healthy, non-smoker volunteers (age: 35 ± 27 years) with no established history of cardiovascular disease were recruited. Their age distribution is shown in Fig. 4.1. Research activities were carried out in accordance with the guidelines of the Declaration of Helsinki of the World Medical Association. All the enrolled participants received exhaustive information on the measurement protocol and the purpose of the research activity, before signing an informed consent form. Data were collected and treated anonymously in accordance with privacy regulations.

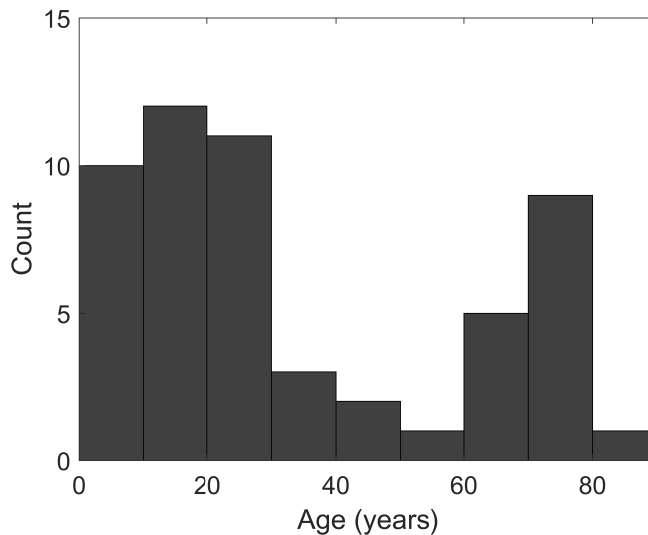


Figure 4.1: Age distribution of the subjects.

A Periflux 5000 LDF system (Perimed, Sweden) was used to acquire signals of microvascular perfusion from the plantar side of the right hallux. As reported in Chapter 2, before the initiation of the study, the device was

properly calibrated conforming to the manufacturer’s specifications. The measurement sessions were performed in resting conditions, in a thermally stable environment ($T \approx 22^\circ\text{C}$), following a 10-minute period of acclimatization. Subjects lay supine in a comfortable position and were thoroughly instructed to refrain from any abrupt movement during the measurements, so that the risk of motion-induced artefacts was minimized. Signals were acquired with a 32 Hz sampling frequency; moreover, the time constant of the instrument output low-pass filter was set at 0.03 s, in order to prevent the distortion of the cardiac frequency components. Nevertheless, during the measurements, the quality of the recorded pulse waveforms was visually inspected on the GUI of the data acquisition software, before including the related signals into the study dataset.

4.3 Pulse wave characterization

The input observations of the SVM correspond to the $N_p = 20935$ pulse waves, used for testing the performance of the multi-Gaussian PDA, as discussed in Chapter 3. Accordingly, the study dataset included N_p vectors $\mathbf{x}_i$ of the form reported in Eq. 3.4: The accuracy and precision of these morphological features is supported by the results of the goodness-of-fit analysis, available in Tables 3.2 and 3.3.

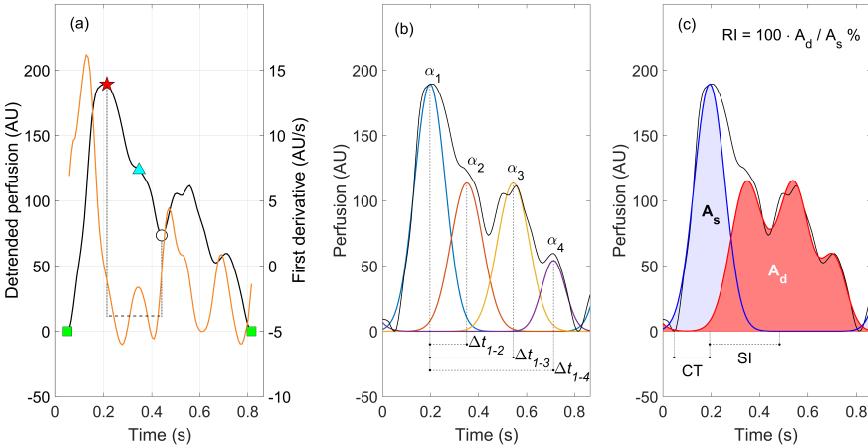


Figure 4.2: Multi-Gaussian PDA. (a) Detection stage; (b) relative amplitudes and delays; (c) physiologically-motivated features.

4.4 Support Vector Machines for binary classification

Support vector machines (SVM) are efficient linear supervised learning systems for binary classification problems, which were first introduced by Vapnik in 1992 [87]. The following section covers a brief overview of the mathematical theory behind SVMs, before moving on to introduce the details of implementation of the vascular ageing classifier. For an exhaustive treatment, please refer to [88, 89].

In a two-class learning problem, the training dataset is comprised, in general, of a number of *example* vectors $\mathbf{x}_i$, each associated to a label $y_i \in (-1, +1)$ which identifies the corresponding class. Conceptually, a linear classifier is based on the definition of a discriminant function of form:

$$f(\mathbf{x}) = \mathbf{w}^T \mathbf{x} + b, \quad (4.1)$$

where $\mathbf{x} : f(\mathbf{x}) = 0$ identify a *decision* hyperplane, which virtually divides the input space I into two separate parts. In these two regions $f(\mathbf{x})$, which is proportional to the signed distance to this hyperplane, is either positive or negative; therefore, a classification rule can be directly derived from:

$$R(\mathbf{x}) = \text{sign}(\mathbf{w}^T \mathbf{x} + b). \quad (4.2)$$

The simplest formulation of a SVM model is the so-called *maximal margin classifier*, i.e. the classifier which maximizes the geometric margin between two linearly separable classes. Considering the points $\mathbf{x}_+$ and $\mathbf{x}_-$ which are closest to a given hyperplane $f(\mathbf{x}) = 0$, inside the *positive* and *negative* regions, and assuming they are equidistant from the decision boundary; then, the following relations hold:

$$\begin{aligned} f(\mathbf{x}_+) &= \mathbf{w}^T \mathbf{x}_+ + b = c, \\ f(\mathbf{x}_-) &= \mathbf{w}^T \mathbf{x}_- + b = -c. \end{aligned} \quad (4.3)$$

Accordingly, the mathematical expression of the geometric margin, M_f , can be derived from:

$$\begin{aligned} \mathbf{w}^T \mathbf{x}_+ + b - c &= 0, \\ \mathbf{w}^T \mathbf{x}_- + b + c &= 0, \end{aligned} \quad (4.4)$$

which define the corresponding hyperplanes, parallel to the decision boundary, whose reciprocal distance (i.e. the margin) is given by:

$$M_f = \frac{2c}{\|\mathbf{w}\|}. \quad (4.5)$$

Based on this formulation, the hyperplane which determines the widest separation between the classes can be obtained by maximizing $1/\|\mathbf{w}\|$ or, equivalently, by minimizing $\frac{1}{2}\|\mathbf{w}\|^2$, thus leading to the following optimization problem:

$$\begin{aligned} & \min_{\mathbf{w}, b} \frac{1}{2} \|\mathbf{w}\|^2 \\ & \text{subject to } y_i(\mathbf{w}^T \mathbf{x} + b) \geq 1 \quad i = 1, \dots, N. \end{aligned} \quad (4.6)$$

However, in real-world classification problems, datasets cannot often be separated by a linear decision boundary, as classes may significantly overlap inside the input space domain. This observation is the basis for the formulation of the *soft* margin classifier, in which the constraint of the maximal *hard* margin classifier (Eq. 4.6) is relaxed so as to allow the presence of misclassified instances:

$$\begin{aligned} & \min_{\mathbf{w}, b} \frac{1}{2} \|\mathbf{w}\|^2 + C \sum_{i=1}^N \xi_i \\ & \text{subject to } y_i(\mathbf{w}^T \mathbf{x} + b) \geq 1 - \xi_i \quad i = 1, \dots, N, \end{aligned} \quad (4.7)$$

where $\xi \in R_0^+$ are *slack* variables, associated with a number of observations that are allowed to fall either inside the margin ($0 \leq \xi \leq 1$) or on the wrong side of the decision boundary ($\xi > 1$). The cost term C ($C \in R_0^+$) represents a key *hyperparameter* of the soft margin SVM, in that it determines the relative importance given to the training accuracy of the classifier and maximization of the geometric margin, separating the predicted classes inside the feature space. As explained later on, C must be properly tuned.

The above optimization problem is generally solved using the dual formulation obtained through the method of Lagrange multipliers:

$$\max_{\alpha} \sum_{i=1}^N \alpha_i - \frac{1}{2} \sum_{i=1}^N \sum_{j=1}^N y_i y_j \alpha_i \alpha_j \mathbf{x}_i^T \mathbf{x}_j$$

$$\text{subject to } \begin{cases} \sum_{i=1}^N y_i \alpha_i = 0 & i = 1, \dots, N \\ 0 \leq \alpha_i \leq C \end{cases} \quad (4.8)$$

The dual optimization problem leads to boundary weight vectors of the following form:

$$\hat{\mathbf{w}} = \sum_{i=1}^N \hat{\alpha}_i y_i \mathbf{x}_i, \quad (4.9)$$

where only those input examples $\mathbf{x}_i$ associated to non-zero $\hat{\alpha}_i$ coefficients lie either on or inside the soft margin. These instances are the so-called *support vectors*, as the solution $\hat{\mathbf{w}}$ depends only on them. In the present work, the above optimization problem was solved with Platt's Sequential Minimal Optimization algorithm for fast SVM training [90].

In general, SVM classifiers can achieve higher flexibility and training accuracy if the original input space I is mapped into a new *feature* space $F = \{\phi(\mathbf{x}) | \mathbf{x} \in I\}$ according to a non-linear transformation, so as to allow the linear learning system to generate complex non-linear decision boundaries. As a result, the form of the discriminant hyperplane becomes:

$$f(\mathbf{x}) = \hat{\mathbf{w}}^T \phi(\mathbf{x}) + b = \sum_{i=1}^N \hat{\alpha}_i y_i \langle \phi(\mathbf{x}_i) \cdot \phi(\mathbf{x}) \rangle + b, \quad (4.10)$$

while the Lagrange dual function gets the form:

$$L = \sum_{i=1}^N \alpha_i - \frac{1}{2} \sum_{i=1}^N \sum_{j=1}^N y_i y_j \langle \phi(\mathbf{x}_i) \cdot \phi(\mathbf{x}_j) \rangle. \quad (4.11)$$

In the above formulation, the feature map $\phi : I \rightarrow F$ appears only through inner products; as a consequence, the knowledge of the *kernel* function:

$$K(\mathbf{x}_i, \mathbf{x}_j) = \langle \phi(\mathbf{x}_i) \cdot \phi(\mathbf{x}_j) \rangle \quad (4.12)$$

is sufficient to train the SVM in F , without an explicit calculation of the underlying feature map $\phi(\mathbf{x})$. In the present study, the widely used *radial basis function* (RBF) kernel was adopted:

$$K(\mathbf{x}_i, \mathbf{x}_j) = e^{-\gamma \|\mathbf{x}_i - \mathbf{x}_j\|^2}, \quad (4.13)$$

where the *kernel scale* γ represents, in addition to the soft margin constant C , a hyperparameter which directly affects the flexibility of the classifier

and, accordingly, needs careful configuration. More specifically, for “small” values of γ , a given example $\mathbf{x}_i$ will be associated to a non-zero $K(\mathbf{x}_i, \mathbf{x}_j)$ with respect to any other input data, which will thus affect the $f(\mathbf{x})$ solution, favouring the generation of a smooth classification boundary. On the other hand, “high” values will make the kernel function decay faster to a value of 0 for increasing $\|\mathbf{x}_i - \mathbf{x}_j\|$ distances; therefore, the discriminant function $f(\mathbf{x})$ will be influenced only by the closest training examples, leading to a higher degree of curvature and boundary flexibility. Analogous considerations can be made for the C hyperparameter, whose role is to weight the penalty assigned to misclassified examples: in this regard, “high” values will discourage the admission of misclassified instances during *training*, thus generating narrow, sinuous margins, whereas “small” constants will in turn lead to lower $1/\|\mathbf{w}\|$ values (Eq. 4.7) and then to a wider separation.

4.5 Vascular ageing classification

In the present study, the feature data extracted from the set of N_p LDF waveforms were standardized (by subtracting their mean and dividing by their standard deviation) so as to scale them to a comparable range. The binary classes of the SVM model were defined on the basis of a cut-off age of 40 years: each property vector $\mathbf{x}_i$ associated with the LDF signal of a given subject was labelled accordingly. In the remainder of this Chapter, the *old* group (i.e. age > 40 years), which accounted for 33.3% of the subjects, will be referred to as the *positive class*. Fig. 4.3 shows the separate distributions of the morphological features related to the young (i.e. age < 40 years) and *old* (i.e. age ≥ 40 years) groups.

In order to assess the performance of the SVM, a K-fold cross-validation framework was implemented; i.e. the classifier was trained and validated over K balanced random subgroups of the original input dataset, and the metrics computed for each partition were properly combined so as to derive a global evaluation of performance [89]. In detail, the dataset partition was conducted so as not to place part of the $\mathbf{x}_i$ vectors of a given subject into both the training and validation subsets of each fold, thus avoiding any potential bias due to intra-subject self-similarity. Accordingly, a rounded 10% of the subjects (with their associated $\mathbf{x}_i$ feature vectors) was sequentially placed into the validation set, resulting in 11 folds which included mutually exclusive validation sets of 5 subjects (reduced to 4 in the last fold).

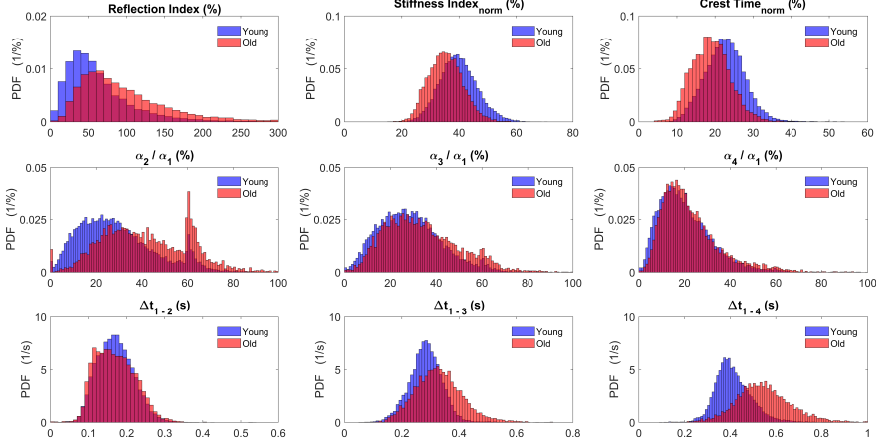


Figure 4.3: Microvascular feature probability density functions (PDF) for the *young* (i.e. age < 40 years) and *old* (i.e. age $\geq$ 40 years) classes.

The following set of scores was adopted for characterizing the classification performance of the SVM model: *sensitivity* or true positive rate (TPR); *specificity* or true negative rate (TNR); the F_1 measure (i.e. the harmonic mean of sensitivity and precision, ranging from 0 to 1 [91]); and, finally, the Matthews Correlation Coefficient (MCC) [92], which reaches a theoretical value of +1 in case of a perfect classification, decreasing to 0 when the SVM performance is not superior to a random guess, and falling further to -1 when there is a complete disagreement between true classes and predicted age labels. These metrics were averaged over the 11 folds, so as to derive a global evaluation of the classifier. For the sake of completeness, their definitions are reported below:

$$\begin{aligned}
 \text{TPR} &= \frac{\text{TP}}{\text{TP} + \text{FN}}, \\
 \text{TNR} &= \frac{\text{TN}}{\text{TN} + \text{FP}}, \\
 F_1 &= \frac{2 \cdot \text{TP}}{2 \cdot \text{TP} + \text{FP} + \text{FN}}, \\
 \text{MCC} &= \frac{\text{TP} \cdot \text{TN} - \text{FP} \cdot \text{FN}}{\sqrt{(\text{TP} + \text{FP}) \cdot (\text{TP} + \text{FN}) \cdot (\text{TN} + \text{FP}) \cdot (\text{TN} + \text{FN})}},
 \end{aligned} \tag{4.14}$$

where TP and FP namely indicate true and false positive classifications, whereas TN and FN respectively represent true and false negative instances. As anticipated in Section 4.4, the *soft margin constant* C and the scale γ of the RBF kernel are crucial hyperparameters, as they have a significant impact on the final behaviour of the SVM model; hence, they require proper tuning. In the present implementation, this *model selection* was performed by means of a 3-fold validation nested in each of the 11 training sets; in detail, the optimal pair of hyperparameters was selected through a grid search over the domain $C_{grid} \in (10^1, 10^{1.5}, 10^2)$ and $\gamma_{grid} \in (10^0, 10^{0.5}, 10^1)$, aimed at maximizing the fold-wise average geometric mean of TPR and TNR. This latter parameter was adopted as objective function due to the unbalance of the available dataset and, specifically, because of the considerable lower number of *old* examples; in this context, the utilization of the overall accuracy would have led to a *majority* classifier with limited sensitivity with respect to the positive class (i.e. age > 40). Higher values of the hyperparameters were not systematically searched, since they were consistently associated with over-fitting regimes, during preliminary testing. Finally, following the classification of the separate pulse waveforms, the rate of input instances assigned to the *old* class by the SVM, hereby termed *Old Pulse Rate* (OPR), was adopted as a decision criterion for the age-wise categorization of each subject.

4.6 Experimental results and discussion

The evaluation of the SVM pulse classifier, according to the approach enunciated in Section 4.5, was repeated over 30 sequential experiments, so as to account for the potential variability in performance, resulting from a different partitioning of the input dataset.

Regarding the correct association of each modeled pulse wave to the respective age class of the corresponding subject, the analysis returned the following fold-wise average scores: TNR and TPR (95% CI) respectively equal to 0.902 (0.896, 0.907) and 0.646 (0.636, 0.657); a mean F_1 measure of 0.678 (0.668, 0.688); and, finally, a MCC of 0.550 (0.539, 0.561). The corresponding box plots are shown in Fig. 4.4.

Despite the non-negligible proportion of false negative instances highlighted by these figures, with respect to the predefined arbitrary threshold of 40

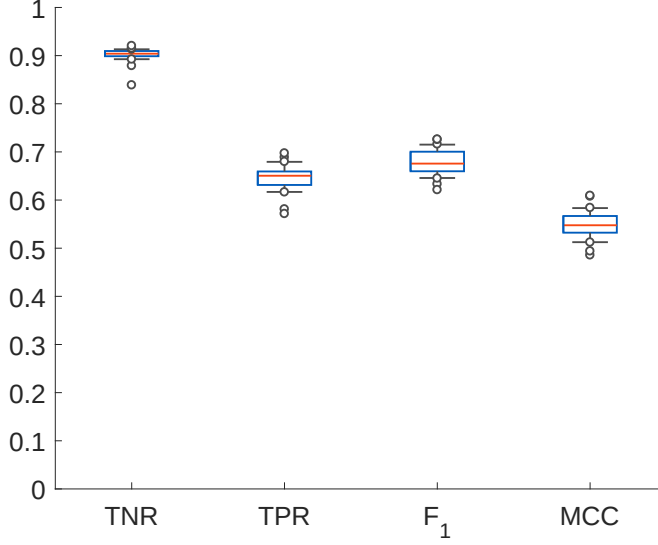
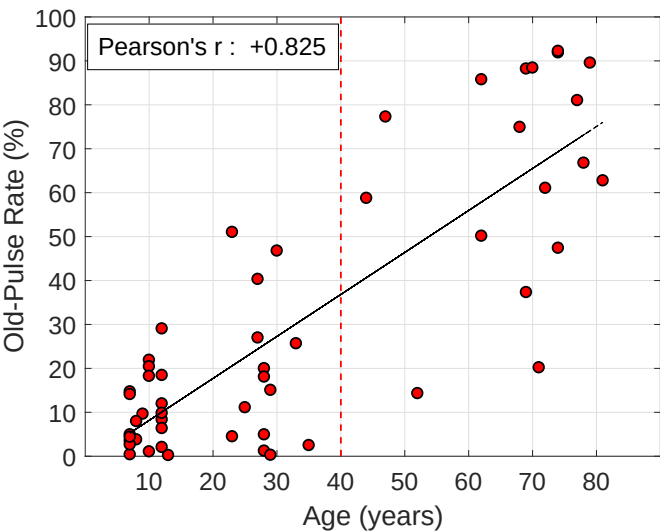


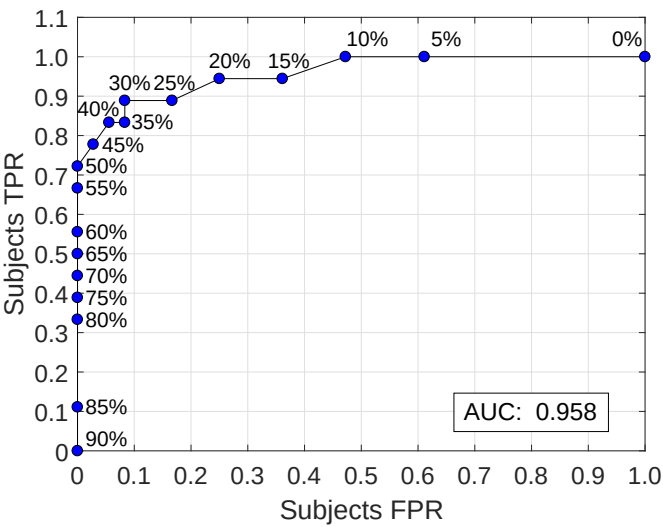
Figure 4.4: Pulse ageing classification: box plots of the fold-wise mean performance scores achieved over the 30 cross-validation experiments.

years, the classification of the separate subjects based on the OPR criterion revealed a very good performance (Figs. 4.5 and 4.6). Indeed, a high correlation between the OPR values and the actual age of the subjects was observed, reflected by a mean Pearson's correlation coefficient of 0.808 (0.803, 0.812). Furthermore, for each of the 30 experimental runs, a receiver operating characteristic (ROC) curve was generated by shifting the OPR threshold, and evaluating the subject true/false positive predictions over the 11 folds. Overall, from the entire set of experiments, an average area under the ROC curves (AUC) of 0.953 (0.951, 0.956) was obtained, which thus demonstrates a very good discrimination capability. Table 4.1 summarizes the overall results of the study.

In order to derive an indication on the qualitative aspect of the *old* and *young* LDF pulse waves, as predicted by the SVM classifier, the input feature vectors corresponding to the centroids of the *true positive* and *true negative* clusters were identified. These samples were then adopted for reconstructing two representative pulse models (normalized to the amplitude of the systolic component, α_1 , for the sake of comparison). Their shape, shown in Fig. 4.7, is in accordance with the observations made by Millasseau et al. [73], which



(a)



(b)

Figure 4.5: Subject ageing classification: (a) sample correlation plot between the age of the subjects and the *Old Pulse Rate* (OPR), corresponding to the maximum Pearson's r achieved in the experiments; (b) sample ROC curve obtained by shifting the OPR threshold (displayed next to the points).

Table 4.1: Age-wise classification of LDF pulse waves and subjects: performance metrics.

CLASSIFICATION	METRIC	MEAN	95% CI
LDF Pulse	TNR	0.902	(0.896, 0.907)
	TPR	0.646	(0.636, 0.657)
	F ₁	0.678	(0.668, 0.688)
	MCC	0.550	(0.539, 0.561)
Subjects	Pearson's r	0.808	(0.803, 0.812)
	ROC AUC	0.953	(0.951, 0.956)

have reported a reduction in the *incisura* of the plethysmographic DVP with ageing. These authors contextually referred to the findings of Dawber and Lax, which had previously recognized that DVP waveforms with no inflection along the descent of the systolic component or without any *dicrotic* local minima are more prevalent in healthy elderly individuals or in subjects with cardiovascular disease [93], or arteriosclerosis [94]. The general physiological interpretation is that the ageing-related stiffening of the large conduit arteries determines a faster back-propagation of the pressure waves reflected by the peripheral circulation and, thus, a higher degree of temporal superposition with the forward-travelling systolic pulse. This hypothesis is indeed reflected by the lower SI_{norm} of the *old* waveform model, and by its higher systolic peak value. Other salient evidence is the larger RI (107.7%), compared to the *young* model (65.1%): this may be indicative of an elevated impedance mismatch at the level of peripheral *resistance* vessels, due to a modification of the structural properties of the tunica media or to a disturbed regulation of the tone of smooth muscle cells.

Nevertheless, it must be emphasized that caution should be used while interpreting the LDF waveform and its quantitative descriptors through the same theoretical framework established for the analysis of the plethysmographic pulse, as these techniques have different underlying measurands. Specifically, LDF provides a non-absolute measurement of tissue perfusion which, under a series of theoretical assumptions, scales with the local concentration of moving erythrocytes and their average velocity [24]. On the other hand, photoplethysmography is sensible to local blood volumetric changes; under pulsatile conditions these are indirectly related to the local perfusion, as the

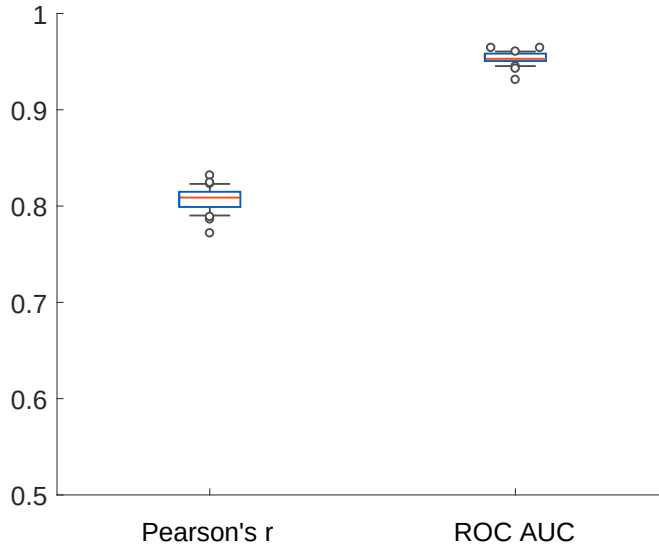
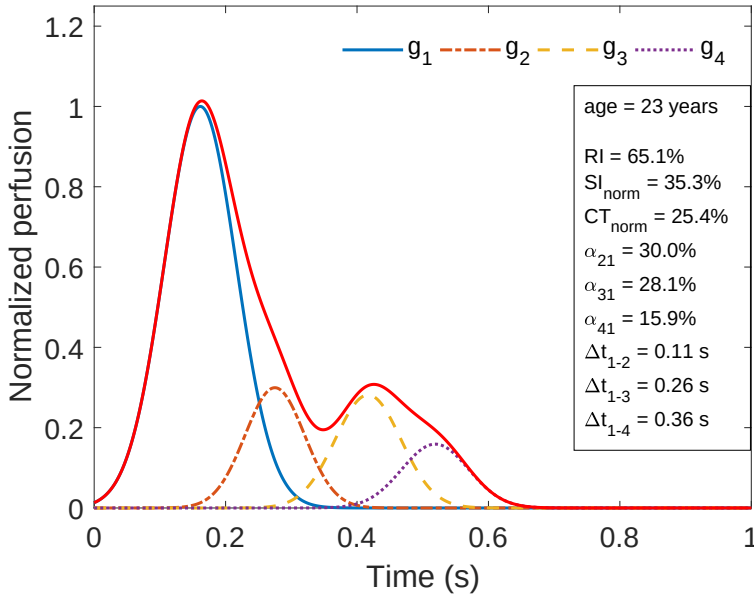


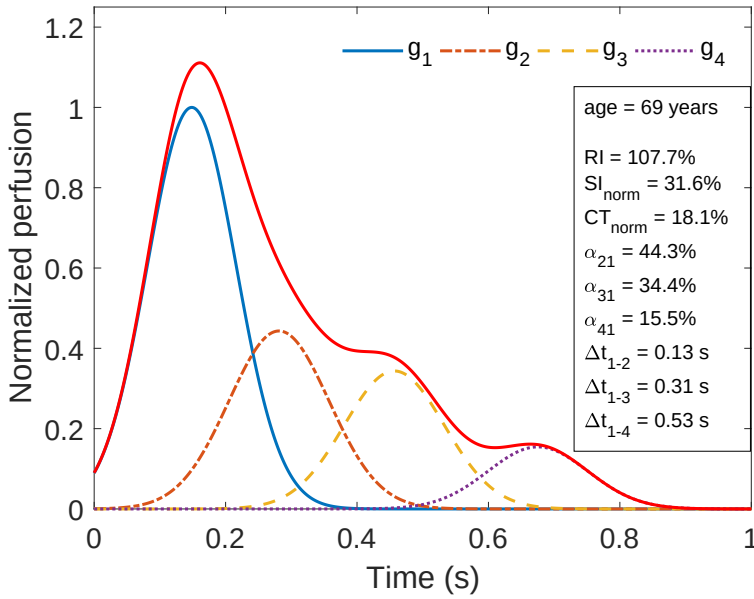
Figure 4.6: Subject ageing classification: box plots of the Pearson's correlation coefficients and the ROC AUC estimates obtained from the 30 experimental iterations.

variation in blood concentration is determined by the difference between the inflow and outflow of blood from the measurement volume. Mizeva et al. have recently demonstrated a significant correlation between the oscillations of digital plethysmographic and LDF signals inside the low frequency intervals associated with endothelial, myogenic and neurogenic vasomotion mechanisms [95]. However, to the author's knowledge a thorough comparison of DVP and LDF microvascular pulse contours is still lacking and further work is needed for the characterization of the LDF waveform changes in response to physiological and pharmacological stimuli, or in specific pathological conditions. This would be highly relevant for the identification of specific LDF pulse descriptors which are suitable for physiological interpretation.

Furthermore, the performance of the vascular ageing classifier may be generally improved through the inclusion of a systematic scheme of feature selection, aimed at the removal of redundant input variables with limited discriminant capability in this specific classification task. To this end, an approach such as the minimum-redundancy-maximum-relevance algorithm proposed by Peng et al. [96] could be adopted in future implementation of the SVM classifier.



(a)



(b)

Figure 4.7: LDF pulse models generated from the centroids of the *true negative* (top) and *true positive* (bottom) feature clusters.

4.7 Conclusion

This Chapter presented an approach for the automatic assessment of vascular ageing, based on the decomposition analysis of LDF pulse waveforms, introduced in Chapter 3, and a fine-tuned SVM classifier. Overall, the SVM exhibited encouraging performances with respect to the association of the separate pulse waves to the respective age class of the subjects. However, a relevant fraction of false negatives was returned (i.e. nominally *old* waveforms classified as *young*). This might be due to the imbalance of the binary classes available for this classification problem, and namely to the lower number of *old* observation vectors (partly counterbalanced by setting a relatively low threshold of 40 years). Moreover, it must be emphasized that the chronological age of the subjects, used for labelling the related models of LDF pulse, does not necessarily hold a linear relationship with the biological ageing of their vascular system, as other confounding factors (e.g. linked to their lifestyle) might have played a relevant role throughout the life-span of the recruited volunteers. In this regard, it could be speculated that higher performance levels might be attained through a better physiological characterization of the included subjects or, also, by translating this method to the automatic discrimination of matched groups of control and pathological subjects, e.g. affected by chronic hypertension.

Nevertheless, a high correlation between the OPR, i.e. the rate of pulse waves classified as *old*, and the underlying age of the test subjects was observed, and very high ROC AUC values were obtained when adopting this rate as a criterion for discriminating differently aged individuals. Therefore, the proposed methodology appears to be able to detect the progressive ageing of the vascular system.

Furthermore, since diabetes is deemed a model of accelerated ageing, being associated to comparable vascular alterations [97], this approach might be valuable for the early detection of diabetic angiopathy. Thus, it will be relevant to investigate the suitability of this supervised learning methodology for the automatic recognition of diabetic and control pulse waves.

Chapter 5

Non-invasive assessment of respiratory-vascular couplings

*The rhythmic breathing activity is associated with characteristic pressure waves which are transmitted to the circulatory system, producing subtle but spatially-coherent patterns in peripheral blood flow rates. Accordingly, LDF signals of cutaneous perfusion exhibit an oscillatory mode linked to this physiological process. The present Chapter covers a wavelet phase coherence analysis focused on the detection of a functional coupling between breathing and specific morphological properties of peripheral LDF pulse waves, extracted by means of the novel multi-Gaussian PDA, presented in Chapter 3. The latter, in fact, might serve as a valuable tool for tracking the breathing function and its physiological coupling with the cardiovascular system, through the sole non-invasive analysis of peripheral pulse waves.*¹

¹A preliminary version of this chapter has been published as “Wavelet phase coherence between the microvascular pulse contour and the respiratory activity” in *World Congress on Medical Physics and Biomedical Engineering 2018: IFMBE Proceedings*, vol. 68, pp. 311-314, 2018.

5.1 Introduction

As introduced in Chapter 2, the time-frequency analysis of LDF signals recorded on the skin reveals the existence of several frequency intervals, associated to specific physiological processes of both central and local origin. Pulsatile components in blood perfusion related to the heartbeat and the respiratory activity arise from centrally generated pressure waves which propagate to the peripheral districts of the body circulation; conversely, the local control of the net vascular resistance to blood flow is related to characteristic low-frequency oscillatory components below 0.1 Hz, produced by the intrinsic and endothelium-dependent contraction and relaxation of the vascular smooth muscle cells, and the resultant modulation of the luminal radius. The complex functioning of the cardiovascular system can be seen to rely on a fine interaction and coordination of these oscillatory processes, in that it may represent a necessary precondition for a synergic adaptation of local blood supplies to time-varying environmental conditions and time- and spatially-varying metabolic needs. Therefore, assessing the coupling between these physiological mechanisms might yield information on the overall integrity and efficiency of the system.

In the field of biomedical signal processing, the frequency-resolved phase coherence and the bi-spectrum of two biological oscillations are often adopted in this regard, as a direct means to detect and characterize the degree of functional connectivity between the underlying physiological processes [98, 99]. A more sophisticated approach is represented by dynamical Bayesian inference [100, 101]. In this technique, the cardiovascular system is modeled as a network of coupled non-linear phase oscillators, whose time-evolving interactions are described in terms of coupling functions, that are sequentially inferred from information provided by consecutive time windows of the raw physiological data (e.g. R-R interval, breathing, tissue perfusion). This mathematical formalization enables the reconstruction of pairwise relations and connections of higher order existing within the network, providing specific information on both their strength and directionality (i.e. the *causality* of the interaction).

The above methodological framework, which is thoroughly reviewed in [102], has found application in the analysis of the ageing-related deterioration of cardio-respiratory couplings [103, 104], as the well-elucidated respiratory sinus arrhythmia, i.e. the characteristic variation of the heart rate during the breathing cycle, first reported by Hales in 1733 [105]. However, to the au-

thor's knowledge, only a number of recent studies have been focused on the physiological interaction between the cardiac and respiratory activities, and the peripheral tissue perfusion. For instance, Chalacheva et al. [106] have designed a Wiener cascade model (comprised of a dynamic linear stage and a second static nonlinear block) for characterizing the mechanisms governing the neurogenic vasoconstriction which follows spontaneous and induced sighs, suggesting a structural representation of the cardiovascular system which incorporates a coupling component between breathing and the peripheral vascular conductance. Furthermore, Ticcinelli et al. have recently investigated the coupling between the quasi-periodic myogenic component of cutaneous LDF signals and the physiological fluctuations in the R-R interval, in relation to ageing [107] and treated hypertension [108].

As discussed in Section 2.1, the shape of the pulse waves transmitted to the peripheral districts of the circulatory system conveys information on the physical properties of the vascular walls inside which they propagate. In this regard, pulse decomposition techniques represent a valuable approach for extracting this pulsatile component from photoplethysmographic or LDF signals recorded at the fingertips, and deriving a detailed and physiologically-motivated characterization of its morphological features. Nevertheless, no study has so far addressed the detection of oscillatory patterns in these properties, and their potential time-phase coordination with breathing. Accordingly, in the present work, the multi-Gaussian PDA previously introduced in Chapter 3 was applied in the context of a phase coherence analysis, with the aim to determine whether and which property of digital LDF pulse waves exhibits an oscillatory pattern linked to the central respiratory rhythm.

5.2 Materials and methods

A group of 21 non-smoker, healthy volunteers, aged from 20 to 30 years, was enrolled in the present preliminary study. Experimental data were collected in accordance with the guidelines of the Helsinki declaration. All the recruited participants agreed to be included in the study, signing an informed consent form after complete information on the purpose of the research and the related procedures was provided.

The measurement sessions involved the simultaneous acquisition of signals of cutaneous tissue perfusion and breathing activity: the former were obtained at the tip of the right forefinger using a Periflux 5000 LDF system (Perimed,

Sweden), whereas the latter was recorded with a BioHarness 3.0 wearable device for physiological monitoring (Zephyr Technology, US), and transmitted synchronously to a PC via Bluetooth. In detail, the BioHarness consists of an electronic module and a chest strap, which houses a pressure sensor pad able to detect the rhythmic expansion and relaxation of the thoracic cavity, linked to the respiratory activity. Hence, the data provided by this instrument are expressed in arbitrary units (AU). Both the LDF and breathing signals were digitized at a sampling frequency of 250 Hz. Measurements lasted five minutes and were performed in resting conditions, during spontaneous breathing (Fig. 5.1), in a temperature-controlled room ($T \approx 23^\circ\text{C}$). Additionally, a preliminary acclimatization interval of about 10 min was included at the start of the experiments, to allow the subjects' body temperature to be properly stabilized.

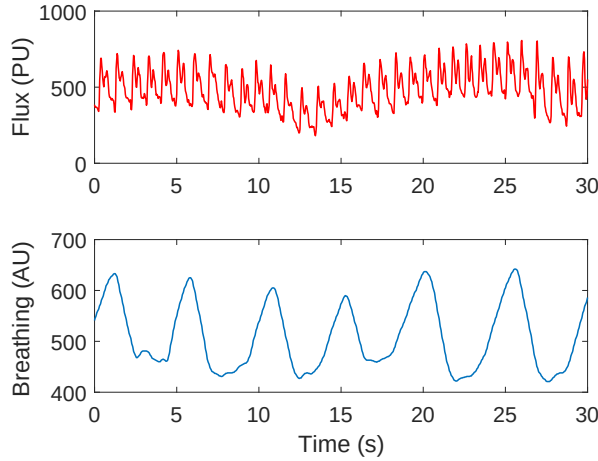


Figure 5.1: 30-s time window of an LDF signal of peripheral perfusion (top) and the corresponding breathing trace (bottom).

The LDF data of microvascular perfusion were processed with the novel multi-Gaussian PDA, whose implementation has been thoroughly covered in Chapter 3. The available temporal window allowed to analyze approximately 350-370 cardiac cycles for each subject, depending on their average heart rate. In this preliminary work, after the reconstruction of each separate pulse waveform, feature extraction solely focused on the following basic properties: the amplitude of the primary systolic component of the four-

Gaussian model, α_1 ; the area beneath it, A_s ; and, finally, the overall area under its diastolic phase, A_d . At this stage, a cubic spline-interpolation of the values associated with each cardiac cycle was applied in order to match the sampling time-grid of the respiratory signals. In this regard, the centroid of the diastolic phase of the waveform models was adopted in order to locate the original sample points of the A_d feature, whereas the α_1 and A_s properties were naturally associated with the mean μ_1 of the systolic Gaussian. For the sake of clarity, the extracted features are displayed again in Fig. 5.2.

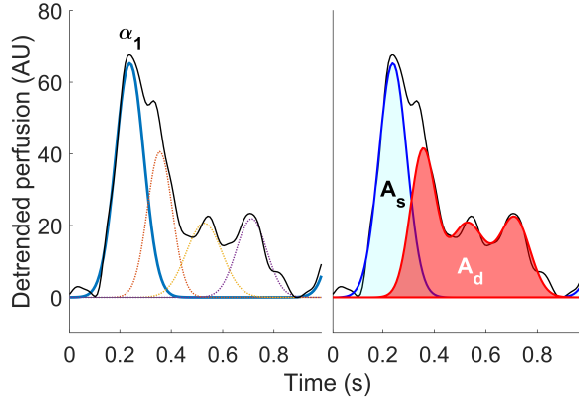


Figure 5.2: Multi-Gaussian modeling of the LDF pulse and model-derived shape features.

A general definition of phase coherence between two signals is provided by the following expression [109]:

$$C_\phi(f_k) = \frac{1}{N} \left| \sum_{n=1}^N e^{i(\phi_1(f_k, t_n) - \phi_2(f_k, t_n))} \right| \quad (5.1)$$

where the term $\phi_1(f_k, t_n) - \phi_2(f_k, t_n)$ indicates the phase difference between oscillatory components with characteristic frequency f_k at time t_n . Theoretically, if two generic oscillations remain phase-locked (i.e. they are temporally coherent) throughout the analyzed time window, then $C_\phi(f_k) = 1$; conversely, the absence of any tendency toward the preservation of a particular phase lag would be reflected by $C_\phi(f_k) = 0$. In the present study, the extraction of the above instantaneous phases was based on the continuous wavelet

transform of the original respiratory signals and the reconstructed time series of the interpolated waveform features; accordingly, the term *wavelet phase coherence* (WPC) will be henceforth adopted.

In detail, as standardly done when dealing with LDF signals of microvascular perfusion, this time-frequency analysis relied on the Morlet's mother wavelet, with central frequency $f_0 = 1$ Hz. 32 points were used to sample each octave of the frequency axis, so as to trade-off between resolution and computational times. Moreover, as described in Chapter 2, only the *cone of influence* of the resulting wavelet scalograms, where the relative distortion due to boundary effects was below 1%, was considered for further analysis. At each discrete time point, the average frequency in the nominal range of the respiratory activity, (0.145, 0.6) Hz, weighted with respect to the corresponding value of the wavelet power spectral density, was estimated so as to identify the instantaneous rate of their breathing-related mode.

The approach described by Tankanag et al. in [110] was followed to perform the subsequent WPC analysis. In detail, the coefficients corresponding to the respiratory *ridge* of the wavelet scalograms were used to derive the cosine and sine functions of the phase difference, $\Delta\phi$, between each couple of feature $x_i(t)$ and breathing $b(t)$ signals, as reported below:

$$\cos(\Delta\phi_{k,n}) = \frac{\Re(w_{b\ k,n}) \cdot \Re(w_{x_i\ k,n}) + \Im(w_{b\ k,n}) \cdot \Im(w_{x_i\ k,n})}{\sqrt{\Re(w_{b\ k,n})^2 + \Im(w_{b\ k,n})^2} \sqrt{\Re(w_{x_i\ k,n})^2 + \Im(w_{x_i\ k,n})^2}}, \quad (5.2)$$

$$\sin(\Delta\phi_{k,n}) = \frac{\Im(w_{b\ k,n}) \cdot \Re(w_{x_i\ k,n}) - \Re(w_{b\ k,n}) \cdot \Im(w_{x_i\ k,n})}{\sqrt{\Re(w_{b\ k,n})^2 + \Im(w_{b\ k,n})^2} \sqrt{\Re(w_{x_i\ k,n})^2 + \Im(w_{x_i\ k,n})^2}}, \quad (5.3)$$

where $\Re(w_{\star\ k,n})$ and $\Im(w_{\star\ k,n})$ respectively represent the real and imaginary parts of the wavelet coefficients at the k -th frequency sample and the n -th time instant. A global frequency-resolved WPC score was finally estimated as follows:

$$C_\phi(f_k) = \sqrt{\langle \cos(\Delta\phi_{k,n}) \rangle^2 + \langle \sin(\Delta\phi_{k,n}) \rangle^2} \quad (5.4)$$

where $\langle \cdot \rangle$ represents the average over the time domain of the signals, at each analyzed frequency f_k .

It is here relevant to emphasize how the correct interpretation of the resultant WPC values is significantly affected by the time scale of interest. WPC is, in fact, an inherently biased score, in that it tends to increase towards

the lower observable frequencies, due to the availability of fewer oscillation periods; furthermore, especially in the case of biological signals, WPC might assume high values due to autocorrelation phenomena, even when the dynamics underlying the couple of assessed time series is actually unrelated. Nevertheless, this potential source of unreliability can be accounted for by comparing the resulting $C_\phi(w_k)$ estimates against the phase coherence obtained between each signal and a corresponding *surrogate*, designed through phase randomization so as to retain all of the original information except for its time-phase properties [111]. Specifically, this strategy allows to selectively disrupt the properties associated with the null hypothesis to be tested, i.e. the instantaneous phase of each assessed $(b(t), x_i(t))$ couple is not reciprocally related. In detail, the surrogate signals were obtained by means of the iterative amplitude-adjusted Fourier transform (IAAFT) method [112]: a set of $N=100$ phase-randomized surrogates was generated from each $b(t)$ and $x_i(t)$ signal, where N was set so as to ensure the statistical repeatability of the test. Therefore, $N=100$ $C_{\phi,b,surr}(f_k)$ and $C_{\phi,x,surr}(f_k)$ surrogate curves were respectively obtained from each breathing signal and parameter of interest. Then, these sets were used to define the following significance threshold:

$$T_{C_{\phi,surr}}(f_k) = \overline{C_{\phi,surr}}(f_k) + 2 \cdot \sigma_{C_{\phi,surr}}(f_k) \quad (5.5)$$

where $\overline{C_{\phi,surr}}(f_k)$ indicates the mean WPC over the 100 surrogates, while $\sigma_{C_{\phi,surr}}(f_k)$ represents its standard deviation. Each couple $(b(t), x_i(t))$ was considered to be significantly phase-coherent at a specific frequency f_k , if $C_\phi(f_k) > T_{C_{\phi,surr}}(f_k)$ held for both the breathing and shape-feature signals.

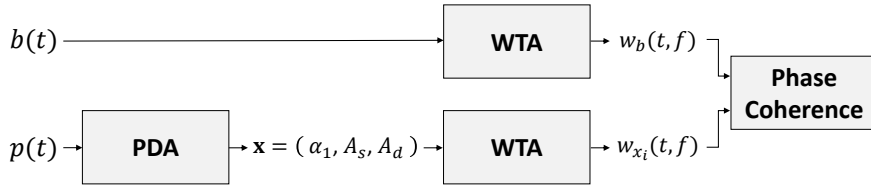


Figure 5.3: Wavelet phase coherence analysis: block diagram.

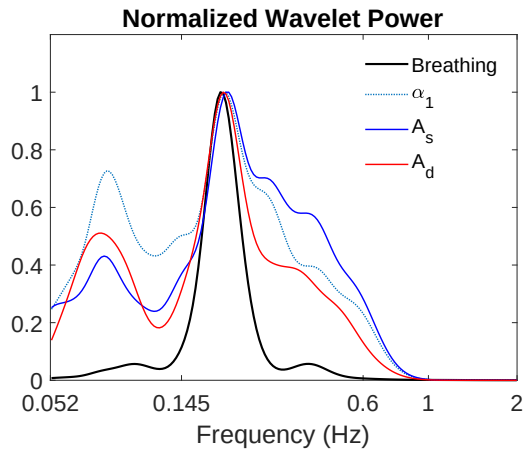
5.3 Experimental results

The time-averaged wavelet power spectra of the three shape feature signals, $x_i(t)$, were inspected in order to verify whether they exhibited a significant oscillatory component inside the frequency range of the breathing activity, i.e. $(0.145, 0.6)$ Hz. A considerable respiratory spectral content was indeed identified in most subjects (as in the example shown in Fig. 5.4a), thus confirming the hypothesis of a significant breathing-related modulation of the waveform features extracted by means of the multi-Gaussian PDA. This was quantified through the average power in the breathing interval, normalized with respect to the total power in the range $(0.02, 0.6)$ Hz, where 0.02 Hz corresponds to the lower observable frequency given the available signal length. In detail, the following group-wise median values and IQR were respectively found for the α_1 , A_s , and A_d features: 47.3 % (36.0, 49.9)%; 55.1 % (47.3, 59.3)%; 44.2 % (39.1, 52.4) %.

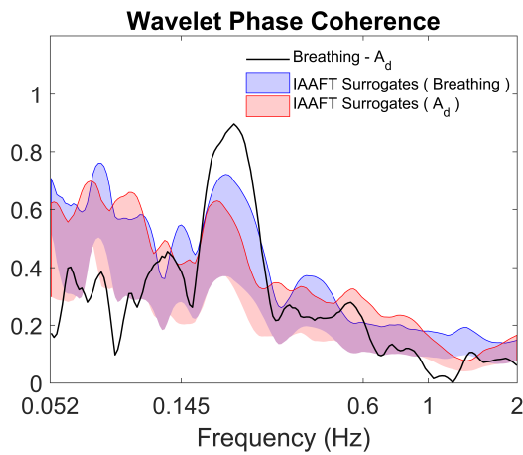
Regarding the characterization of their phase coherence with respect to the central breathing rhythm, the diastolic area of the LDF pulse, A_d , exhibited significant WPC values in 17 out of the 21 analyzed subjects (i.e. 81.0%). A sample WPC curve corresponding to one of these cases is shown in Fig. 5.4b. Overall, this subgroup of subjects was associated with a median value of the peak WPC of 0.78 (IQR: 0.63, 0.83). On the other hand, the features characterizing the systolic phase of the pulse waveforms, A_s and α_1 , did not show a consistent time-phase relationship with the respiratory activity except in a relatively small number of cases, reported in Table 5.1. The box plots of the significant WPC peaks, detected in the breathing interval, are displayed in Fig. 5.5.

Table 5.1: Wavelet phase coherence analysis: results.

FEATURE	COHERENCE	MEDIAN	IQR
A_d	17/21 subjects	0.78	(0.63, 0.83)
A_s	6/21 subjects	0.62	(0.56, 0.66)
α_1	8/21 subjects	0.65	(0.59, 0.68)



(a)



(b)

Figure 5.4: (a) Time-averaged normalized wavelet power spectra of the model-derived shape features, showing a peak in correspondence of the respiratory rhythm. (b) Sample WPC curve between breathing and A_d . The upper thick colored lines depict the significance thresholds, $T_{C_{\phi, \text{sur}}}(w_k)$, whereas the lower boundary of the shaded areas corresponds to the mean *surrogate* WPC.

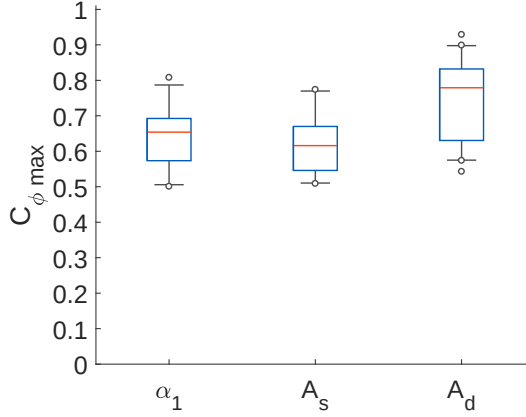


Figure 5.5: Peak WPC associated to the cases of significant coherence.

5.4 Conclusion

In summary, the study covered in the present Chapter investigated the presence of oscillations in basic shape features of LDF pulse waves, highlighting a significant phase coherence with the rhythm of the breathing activity. Interestingly, the diastolic phase of the waveforms, characterized by means of the area beneath its three-Gaussian model (A_d), exhibited a strong association with the breathing function in a considerable majority of the test subjects. This positively confirms the hypothesis of a significant functional coupling between this *central* physiological oscillation and peripheral perfusion. More specifically, this outcome might imply a particular effect of the respiratory activity on the secondary waves arising in the circulatory tree because of internal reflections and re-reflections, due to a mismatch in the effective vascular impedance encountered by the forward-travelling systolic pulse. Therefore, this result provides a preliminary indication of the suitability of the multi-Gaussian PDA, introduced in Chapter 3, for tracking the breathing function and detecting the potential deterioration of its physiological coupling with microvascular perfusion. In this regard, the feature A_d might represent a particularly useful target property.

Future work must deal with the acquisition and analysis of a wider set of signals and, furthermore, with a thorough physiological characterization of the adopted morphological features and their physical relationship with the respiratory dynamics.

Chapter 6

Analysis of vasomotor responses to a deep inspiration

*A rapid deep inspiration is known to trigger a characteristic transient vasoconstriction of arteriolar networks, which is reflected by a short-term decrease in cutaneous blood perfusion. Since this physiological reflex is mediated by the action of the sympathetic nervous system, the design of new measurement protocols and the development of robust signal processing techniques for the quantification of such neurogenic response would be of value in clinical scenarios, especially with a view to the non-invasive assessment of neurovascular deterioration in high-incidence chronic diseases, such as diabetes mellitus. The objective of the study covered in the present Chapter was to demonstrate the suitability of the multi-Gaussian PDA, introduced in Chapter 3, for the characterization of the physiological vasoconstrictive response to a standardized deep inspiratory gasp.*¹

¹A preliminary version of this chapter has been published as “Modeling of the microvascular pulse for tracking the vasoconstriction response to deep inspiratory gasp” in *World Congress on Medical Physics and Biomedical Engineering 2018: IFMBE Proceedings*, vol. 68, pp. 307-310, 2018.

6.1 Introduction

As discussed in the previous Chapter, the respiratory activity produces characteristic pressure waves which propagate through the circulatory system, giving rise to coherent oscillatory patterns in the peripheral perfusion, that are easily detectable with non-invasive optical techniques at the level of the cutaneous circulation [98].

Furthermore, breathing is associated with vasomotor responses mediated by the sympathetic and para-sympathetic nervous systems. In 1936, Bolton et al. [113] were the first to show how a deep inspiratory gasp (DIG) could evoke a transient vasoconstriction in the cutaneous circulation of fingers and toes which, however, was inhibited after the impairment of peripheral sympathetic pathways. Hence, the present manoeuvre has raised interest with respect to the design of novel measurement protocols for the clinical testing of autonomic function and baroreflex sensitivity. This gasp-induced reflex is known to be effected through the vasoconstriction of arterio-venous shunts, which abound in the cutaneous circulation of toes and fingertips particularly and are densely innervated, playing a key role in the physiological regulation of body temperature. The primary factor initiating the vasoconstrictive reflex has been identified with either the expansion of the thoracic cavity or with the stretch of intra-thoracic veins [114]; however, the specific physiological mechanisms have not been unequivocally elucidated yet.

In a preliminary study [115], involving 17 healthy male volunteers, Allen et al. have applied LDF and photoplethysmography with the aim of evaluating the repeatability of the quantitative changes in peripheral perfusion, photoplethysmographic pulse, and skin temperature which followed a finely standardized DIG manoeuvre. The median reduction in the LDF perfusion, contextually evaluated on the pulp of the index finger with respect to the peak value at baseline, was 93% (95% CI: 53.2%, 98.4%). This parameter exhibited a negligible degree of intra-subject variability both during the same measurement session (1.4%) and between two separate sessions, performed within four weeks (0.5%). Nonetheless, concerns have been raised upon the limited stationarity of the basal cutaneous perfusion and, therefore, on the general reliability of the adopted reference. Accordingly, in a subsequent work, the same author has regretted the unavailability of robust techniques, able to perform an automatic tracking and characterization of peripheral waveform features [116], in spite of the considerable attenuation induced by the DIG. In detail, the above statement concerned the challenge

of obtaining a reliable detection of the *foot*, or dominant peak, of the pulse waves with the aim of characterizing the DIG response through the estimate of the pulse arrival time (PAT), a physiological variable inversely linked to vascular tone. However, as formalized by the Moens-Korteweg model [72], the PAT estimate is subject to the influence of several covariates (i.e. blood viscosity, thickness of the vascular walls, luminal radius) and, secondly, still relies on the availability of a reference ECG signal.

Against this background, the objective of the present study was to demonstrate the suitability of the pulse decomposition algorithm (PDA), introduced in Chapter 3, for quantifying the transient physiological vasoconstriction induced by a rapid deep inspiration. This may support the advancement of this non-invasive physiological test for the clinical assessment of peripheral neuropathies.

6.2 Materials and methods

The present study involved the synchronous measurement of cutaneous perfusion, ECG and respiratory activity in 13 healthy volunteers (age: 26 ± 3 years; M/F: 10/3), evaluated at the Laboratory of Biomedical Engineering of the University of Florence. The research activity was carried out according to the guidelines of the Declaration of Helsinki: the enrolled participants received a detailed explanation of the research protocol, and filled an informed consent before the measurement sessions. In detail, microvascular perfusion was measured on the pulp of the right forefinger with a LDF Periflux 5000 system (Perimed, Sweden), whereas the breathing and ECG signals were recorded with a BioHarness 3.0 wearable telemetry device for physiological monitoring (Zephyr Technologies, US), and transmitted to a PC via Bluetooth protocol. All these physiological signals were digitized at a sampling rate of 250 Hz.

The measurement sessions were conducted in controlled thermal conditions ($T \approx 23^\circ\text{C}$), with the subjects sitting comfortably and leaning their ipsilateral forearm on a table, so as to minimise the probability of causing detrimental movement artefacts in the recorded data, especially in the LDF signals. The selected protocol included a 60-s baseline, after which a visual cue on the GUI of the data acquisition software signalled to the subjects to perform a rapid, deep inspiration; afterwards, they were instructed to hold their breath for 5 s until the activation of a second dedicated cue, before

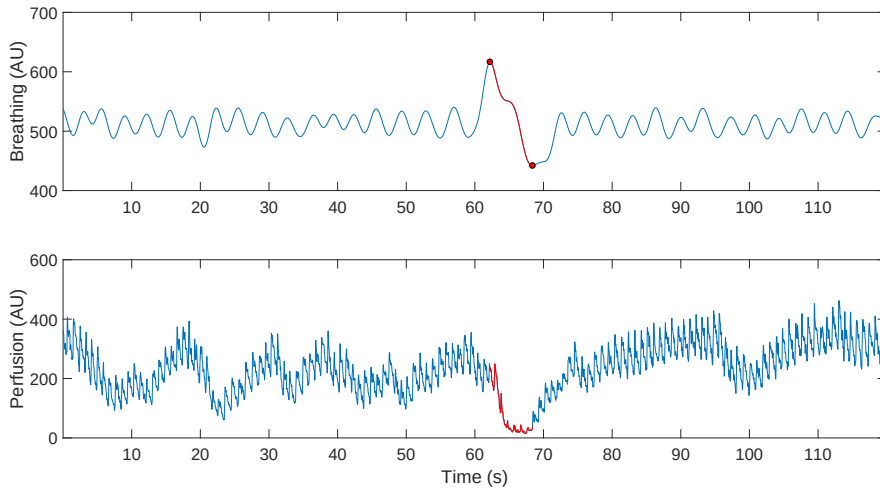


Figure 6.1: Sample signal of breathing activity, with the phase of the DIG manoeuvre properly highlighted in red (top). The corresponding LDF signal is shown below, where the reflex vasoconstriction is clearly visible.

exhaling and returning to breathing regularly for other 60s. As shown in Fig. 6.1, the points corresponding to the maximum and minimum expansion of the thoracic cavity, detected by the pressure transducer pad of the BioHarness' chest strap, were standardly adopted so as to obtain a precise windowing of the DIG manoeuvre, and thus be able to perform a comparative assessment of the algorithm with respect to the performance exhibited in standard basal conditions.

The LDF signals of cutaneous tissue perfusion were processed with the novel multi-Gaussian PDA, introduced in Chapter 3. In the present study, the amplitude α_1 of the primary systolic component of the pulse waveforms was adopted for assessing the physiological vasoconstrictive response to the DIG manoeuvre. In order to derive a global evaluation of the amplitude trend throughout the protocol, in spite of the varying basal perfusion levels of the different subjects, the above parameter was normalized with respect to the average, $\bar{\alpha}_1$, obtained during the baseline phase which preceded the DIG. Furthermore, thanks to the availability of the reference ECG, the analysis of the gasp-induced reflex also included the computation of the PAT, in analogy with the work by Allen et al. mentioned in the introductory section; in

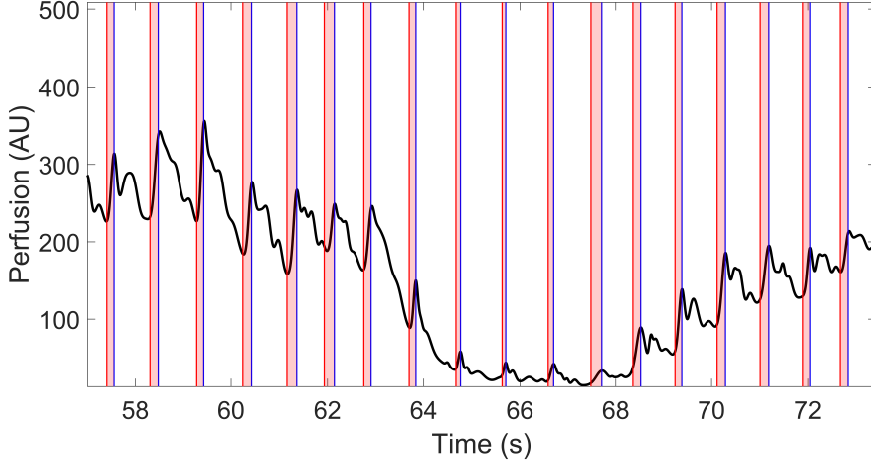


Figure 6.2: Computation of the PAT (highlighted in red), based on the R-peaks detected by the wearable telemetry device (vertical red lines) and the primary systolic components of the LDF pulse waves (vertical blue lines).

detail, the latter variable was obtained from the delay in between the time reference of the primary peak of the pulse wave models (i.e. μ_1), and the corresponding R peak of the ECG signal, as displayed in Fig. 6.2.

Three main aspects were probed for validating the accuracy of the PDA in detecting and reproducing the pulsatile component of the LDF signals during the transient vasoconstriction, namely: the rate of correctly reconstructed pulse waveforms relative to the actual number of cardiac cycles detected in the ECG provided by the BioHarness; the goodness of fit (GOF) achieved by the adopted four-Gaussian model, evaluated by means of the pulse-wise average coefficient of determination R^2 , and a normalized GOF parameter, GF_{norm} (whose expression is reported in Eq. 3.5); and, finally, the ability of the PDA to track the subjects' heart rate using the mean of the primary systolic components of the multi-Gaussian model, μ_1 , as performed for the estimation of the beat-to-beat PAT. The heart rate derived from the pulse decomposition analysis of the LDF signals (HR_{PDA}) was compared with the one obtained from the time interval separating the R-peaks detected in the ECG trace (HR_{ECG}): in this regard, for each analyzed subject, the root-mean-square error, RMSE, between the HR_{PDA} and the corresponding reference HR_{ECG} was computed separately for the baseline and the phase

of the DIG. Furthermore, the performance of the PDA was juxtaposed to the one achieved by performing a wavelet transform time-frequency analysis (WTA) of the LDF signals [47], as reported in Section 2.5. In detail, the instantaneous frequency, characterizing the cardiac modulation of the cutaneous blood perfusion, was estimated by reconstructing the ridge of the wavelet spectrogram inside the frequency range (0.6, 2) Hz [49].

6.3 Experimental results and discussion

A preliminary Shapiro-Wilk test revealed a significant deviation from normality in all data distributions ($p < 0.001$); accordingly, the non-parametric Wilcoxon signed-rank test for dependent samples was applied to perform the statistical comparisons, contextually required in the present study.

The multi-Gaussian PDA showed considerable accuracy in the automatic detection of the separate LDF pulse waves, with a median subject-wise PDR at baseline of 98.5% (IQR: 97.3%, 99.4%); interestingly, in spite of the attenuation of the LDF pulse waves, arising from the sympathetic reflex vasoconstriction, this performance did not significantly deteriorate during the nominal phase of the DIG (100%, IQR: 100%, 100%, $p = 0.286$), as shown in the box plot of Fig. 6.3.

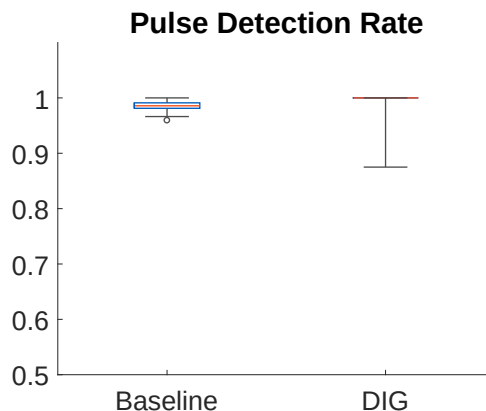


Figure 6.3: Rate of correctly reconstructed pulse waveforms (PDR): performance exhibited in standard basal conditions and during the reflex vasoconstriction, induced by the DIG.

The analysis of the GOF attained by the PDA during the transient vasoconstriction yielded promising results (expressed again as median and IQR). Indeed, as shown in Fig. 6.4a, the multi-Gaussian model of the LDF pulse waves did not exhibit a significant worsening of the GF_{norm} (0.90, IQR: 0.81, 0.94) relative to baseline (0.92, IQR: 0.87, 0.94; $p = 0.249$). Furthermore, no statistically significant deviation was demonstrated between the distributions of the mean R^2 (Fig. 6.4b), obtained from the phase of the DIG (0.973, IQR: 0.953, 0.980) and the baseline (0.973, IQR: 0.958, 0.978; $p = 0.552$) of each subject.

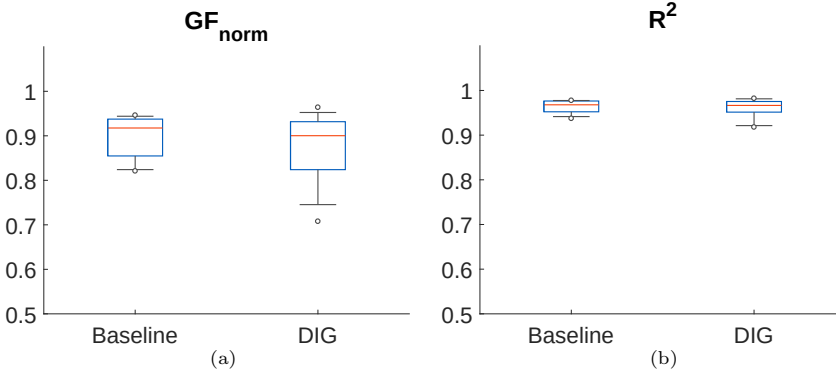


Figure 6.4: GOF of the four-Gaussian PDA: baseline vs. DIG.

Consistent results were found regarding the comparison of the HR_{PDA} estimates, for the indirect validation of the PDA robustness (Fig. 6.5a). The ability to track with continuity the reference HR_{ECG} of the wearable sensor (and thus the single pulse waves) was confirmed by the low RMSE obtained during the DIG (1.9 bpm, IQR: 1.4, 3.2 bpm). As expected in light of the results of the GOF analysis, these RMSE levels were not associated with a significant increase relative to the basal performance (1.8 bpm, IQR: 1.4, 2.7 bpm; $p = 0.861$).

Furthermore, in general, the pulse decomposition analysis of the LDF perfusion signals outperformed the time-frequency approach in the estimation of the instantaneous heart rate, as shown in Fig. 6.5b. Specifically, the multi-Gaussian PDA showed a significantly lower RMSE over the entire time domain with respect to the WTA (1.6 bpm, IQR: 1.4, 2.6 bpm; vs. 3.2 bpm, IQR: 2.4, 5.1 bpm; $p < 0.001$); in detail, the latter was associated with an increase in the median error score by 46.8%.

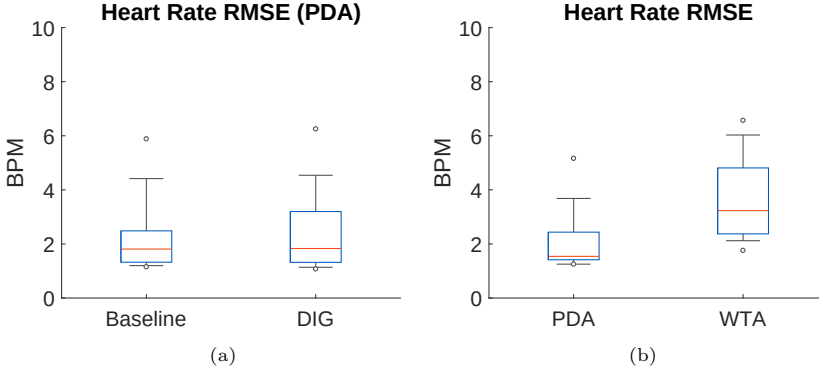


Figure 6.5: Heart rate validation. (a) PDA-based heart rate tracking: baseline vs. DIG; (b) PDA vs. wavelet transform analysis.

The subject-wise median trend of the baseline-normalized HR_{PDA} is shown in Fig. 6.6a, where a substantial agreement with respect to the corresponding quartiles of the reference HR_{ECG} data can be appreciated over the entire measurement protocol. Consistently with previous findings [116, 117], the DIG induced an increase in the heart rate of the analyzed subjects, which appears to peak in correspondence with the maximum expansion of their thoracic cavity, with a relative median rise of 13.3% (IQR: 7.1, 18.9%) above the average at baseline.

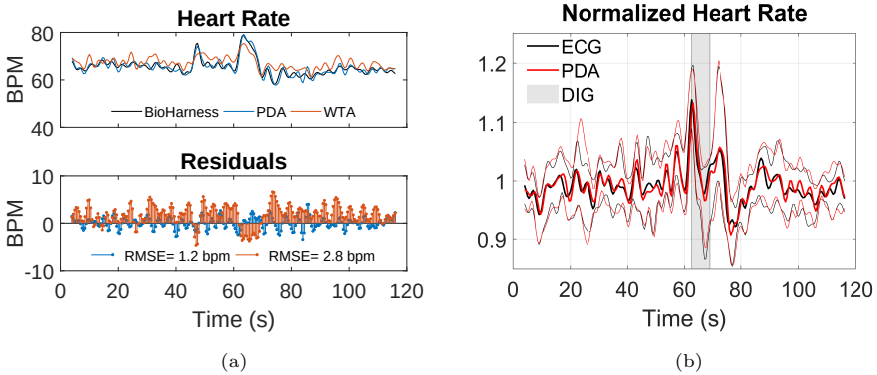


Figure 6.6: (a) Sample residual analysis of the heart rate obtained from the multi-Gaussian decomposition and the WTA of the LDF signals; (b) median trend of the baseline-normalized HR_{ECG} and HR_{PDA} signals.

Regarding the quantitative characterization of the reflex vasoconstriction induced by the DIG, as expected, the manoeuvre determined a reduction of the amplitude α_1 of the LDF pulse waves, whose normalized median trend is displayed in Fig. 6.7a. Overall, the maximum vasoconstrictive effect appears to occur approximately 3.0s after the end of the sudden inspiration, where a median decrease of 41.4% (IQR: 26.5, 68.2%) is observed. Fig. 6.7b shows the probability density functions of the α_1 parameter associated with the baseline, DIG and post-DIG phases of the measurement protocol. It is interesting to note how the latter period exhibits a trend towards supra-basal values; in fact, the analysis of the time dynamics of this reactive hyperaemia might yield valuable information on the microvascular responsiveness to a transient ischaemia.

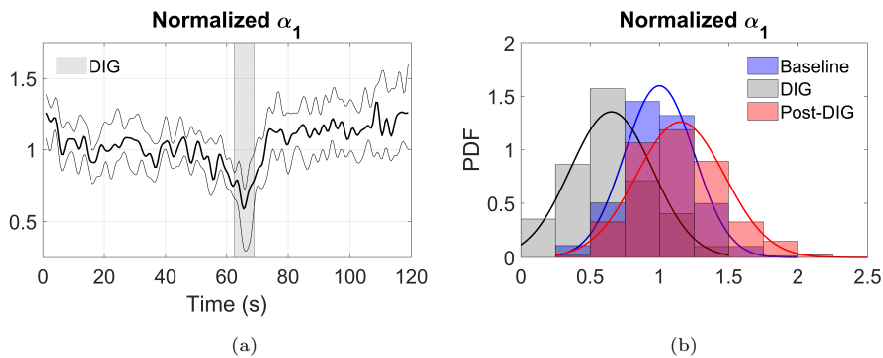


Figure 6.7: (a) Normalized trend of the systolic pulse amplitude, α_1 ; (b) probability density functions of the α_1 parameter derived from the baseline, DIG and post-DIG phases of the protocol.

Fig. 6.8 shows the effect of the DIG manoeuvre on the arrival time of the LDF pulse waves. Data obtained from the group of 13 control subjects would seem to indicate a reduction in this physiological variable, which is theoretically congruent with the underlying vasoconstriction and the resultant attenuation of the pulse amplitude. However, the overall magnitude of the changes detected in the PAT is milder and not statistically significant with respect to baseline. Furthermore, the observed trend differs from the biphasic response (i.e. first increasing and then decreasing below basal values) previously reported by Allen et al. in [116]. In this latter study, the PAT was derived from the instantaneous phase shift at the dominant *car-*

diac frequency of the ECG and digital PPG signals, extracted by means of a wavelet time-frequency analysis. Therefore, it would be relevant to compare these different approaches, extending the evaluation of the PAT response on a wider set of LDF or PPG signals.

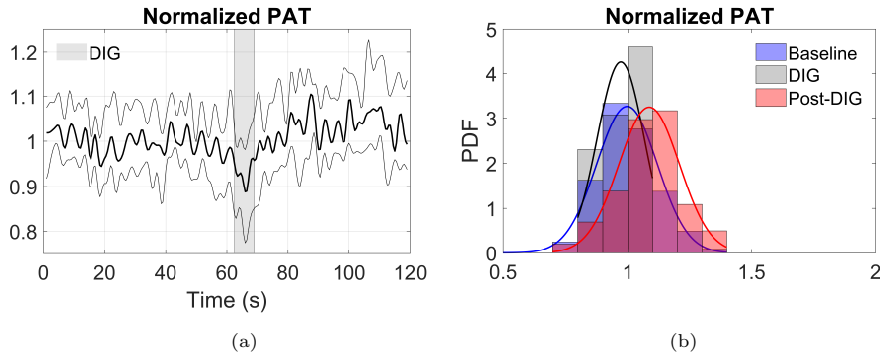


Figure 6.8: (a) Trend of the baseline-normalized PAT; (b) probability density functions of the PAT derived from the baseline, DIG and post-DIG phases of the protocol.

6.4 Conclusion

Collectively, the findings of the present investigation further support the reliability of the multi-Gaussian PDA with respect to the automatic tracking of LDF pulse waveforms in standard conditions of basal tissue perfusion, thus reinforcing the promising results already presented in Chapter 3.

In addition, it was thoroughly highlighted how the attained performance does not suffer a significant deterioration in correspondence with the transient vasoconstriction determined by the sympathetic reflex. Hence, the proposed technique appears to be suitable for characterizing the integrity of the neurovascular pathways underlying this vasomotor response, on the basis of a meticulous evaluation of the relative decrease in the amplitude of peripheral pulse waves, detected on the skin.

Nevertheless, it is relevant to emphasize how the robustness exhibited by the multi-Gaussian PDA might in general be exploited so as to obtain a comprehensive description of the morphological changes suffered by the LDF waveforms; in this regard, the present work could be extended with the analysis

of the *Stiffness* and *Reflection* indices, introduced in Section 3.3. Anyway, further work will be needed for expanding the current dataset and improving the statistical significance of the preliminary findings. Afterwards, a direct development of the present research activity could involve the comparative assessment of this vasomotor response in a pathological group, comprised of subjects suffering from established peripheral neuropathy (e.g. diabetic patients).

Chapter 7

Validation of physiological features of LDF pulse waves

Alterations in the physical properties of proximal and distal vascular walls are reflected by quantifiable modifications of the contour of peripheral pulse waves. In this regard, pulse decomposition, which allows for an accurate characterization of their morphological features, represents a promising technique for the non-invasive extraction of clinically relevant information from laser Doppler flowmetry signals of microvascular perfusion. However, no study regarding the physiological validation of the properties of LDF pulse waveforms is available in the literature. Therefore, in the present Chapter, we report a small-scale validation study aimed at assessing the correlation between established parameters of central and distal arterial compliance, based on a four-element Windkessel model of the circulatory system, and the Stiffness and Reflection indices of the LDF pulse, introduced in Chapter 3.

7.1 Introduction

Peripheral pulse waveforms originating from the cardiac activity are shaped according to the mechanical characteristics of the vascular walls inside which they propagate. Pulse decomposition techniques (Chapter 3) have emerged as a powerful approach for their systematic characterization, as they ensure a detailed reconstruction of the cardiac pulsatility thanks to a linear combination of basis functions (namely, log-normal, Rayleigh, or Gaussian kernels). In fact, the use of robust data models of the pulse wave contour lays a prerequisite foundation for the accurate determination of morphological descriptors of clinical relevance.

However, although the research activities reported in the present thesis relied on the analysis of laser Doppler flowmetry (LDF) signals of cutaneous perfusion, to the author's knowledge no study on the quantitative analysis of LDF waveforms and the validation of their properties is available in the current literature. Hence, in this regard, the physiologically-motivated features of the digital photoplethysmographic pulse, proposed by Millasseau et al. [71, 73, 74, 82], were adopted as a preliminary reference. In detail, as already mentioned in Section 3.3, these authors have derived an index of macrovascular stiffness (the so-called *Stiffness Index*, SI) from the relative timing of the systolic and diastolic phases of photoplethysmographic pulse waves, recorded on the pulp of the index finger. Moreover, they have identified a second marker of distal wave reflection in the relative amplitude of the early diastolic peak (or inflection point) with respect to the antegrade systolic pulse: this *Reflection Index* (RI) appears to be specifically associated with the vascular tone of peripheral resistance blood vessels.

Still, it is important to recall that photoplethysmographic signals reflect local changes in relative blood volume, whereas LDF is sensitive to the local tissue perfusion (i.e. the product of the concentration and mean speed of flowing erythrocytes). Thus, the LDF variant of the above parameters might be prone to interpretive errors. Therefore, it is of utmost importance to obtain a first indication regarding the underlying meaning and the reliability of the LDF-derived SI and RI, in order to support the clinical relevance of LDF and pulse decomposition methods for the non-invasive assessment of the circulatory system. To this end, we designed a small-scale validation study for evaluating the correlation between these indices and estimates of proximal and distal vascular compliance, derived from a third-order Windkessel lumped model of the arterial tree.

7.2 Experimental setup and subjects

The present investigation relied on data collected from 28 healthy volunteers (age: 24 ± 2 years; M/F: 17/11), recruited at the School of Engineering of the University of Florence. Research activities were conducted in accordance with the Declaration of Helsinki of the World Medical Association: complete information on the measurement protocol and the aim of the study was provided to the subjects involved, which signed an informed consent form before the start of the evaluations. The measurement sessions took place in a thermally stable environment ($22\text{--}24^\circ\text{C}$), and were performed in steady resting conditions, with the subjects in sitting position. The present validation study required the parallel acquisition of pulse waveforms of radial arterial pressure and skin microvascular perfusion. The latter were recorded by fixing the probe of a Periflux 5000 laser Doppler flowmetry (LDF) system (Perimed, Sweden) to the pulp of the right index finger, so as to ensure a stable interface and avoid movement-related artefacts. As reported in the other Chapters, the LDF signals were recorded at a sample rate of 32 Hz and the minimum available time constant of the instrument's output low-pass filter (i.e. 0.03 s) was selected so as not to dampen the cardiac pulsatility of the cutaneous perfusion. On the other hand, radial artery waveforms were recorded non-invasively at 200 Hz, by means of a HD/Pulse Wave CR-2000 tonometry system (Hypertension Diagnostics, US). In detail, the tonometer was placed over the ipsilateral wrist, in correspondence of the radial bony prominence. A proprietary wrist stabilizer was used to support the subjects' forearm in order to facilitate the correct positioning of the sensor and to limit undesired movements during the measurements. Moreover, prior to the start of the recordings, a blood pressure cuff was placed on the contralateral arm for calibrating the measured pressure waveforms by means of the oscillometric method [118]. The tonometer was carefully manipulated until qualitatively ideal waveforms and a stable trend were visualized on the display of the instrument's monitor. Then, 30-s signals of microvascular and arterial pulse waveforms were simultaneously acquired. These were systematically inspected to ensure the coincidence of the acquired time windows, and the cardiac cycles included within them. The experimental setup is shown in Fig. 7.1. In order to derive indices of central and distal vascular compliance, the CR-2000 system implements a contour analysis of the diastolic phase of the pressure pulse waves. The theory behind this technique is introduced in the following Section.



Figure 7.1: Picture of the experimental setup, showing the CR-2000 CardioVascular Profiling system (left) and the Periflux LDF system (right), the positioning of their respective proprietary sensors, and the blood pressure cuff needed for calibrating the radial pressure waveforms.

7.3 Diastolic pulse contour analysis

The analysis of the diastolic portion of radial pressure pulse waves represents an established non-invasive approach for deriving estimates of central and peripheral vascular compliance. The technique, originally proposed by Cohn et al. [119], relies on a modified third-order Windkessel model of the circulatory system [120]. The four lumped components of its electrical representation, shown in Fig. 7.2, have a direct physiological meaning: the inductance, L , represents the inertance of the blood flowing through the arterial tree; R corresponds to the total vascular resistance to blood flow; whereas the two capacitances, C_1 and C_2 , model respectively the central and peripheral vascular compliance, i.e. the tendency of proximal and distal arteries to stretch in response to higher intra-luminal pressure levels.

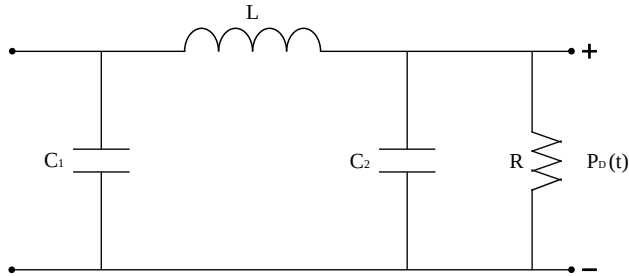


Figure 7.2: Electrical equivalent of the modified third-order Windkessel model.

It is here relevant to highlight that, according to this circuit-equivalent model of the cardiovascular system, current is analogous to blood flow, whereas voltage corresponds to blood pressure. Thus, the diastolic phase of the radial pulse waves can be modeled through the following expression [119]:

$$P_D(t) = p_1 \cdot e^{-p_2 \cdot t} + p_3 \cdot e^{-p_4 \cdot t} \cdot \cos(p_5 \cdot t + p_6). \quad (7.1)$$

In the above equation, the first addend accounts for the exponential decay of the diastolic portion of the pulse, whereas the second (a decaying sinusoid function) reproduces the dicrotic *incisura* of the waveform and the damped oscillations that follow it (Fig.7.3).

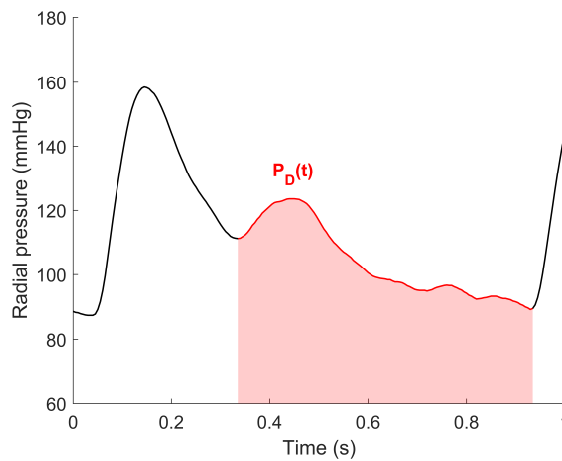


Figure 7.3: Sample radial pressure pulse.

Based on this set of parameters, the compliances C_1 and C_2 are computed as reported below [121]:

$$C_1 = \frac{2 \cdot p_4 \cdot [(p_2 + p_4)^2 + p_5^2]}{R \cdot p_2 \cdot (p_2 + 2 \cdot p_4)(p_4^2 + p_5^2)} \quad (7.2)$$

$$C_2 = \frac{1}{R \cdot (p_2 + 2 \cdot p_4)}. \quad (7.3)$$

According to these equations, C_1 and C_2 are inversely related to the total peripheral resistance, R ; this parameter must be derived from the ratio of mean arterial pressure and mean blood flow. While the former is directly measured, the latter is estimated on the basis of the following empirical model of cardiac output (CO) [122], i.e. the volume of blood pumped by the left ventricle per unit of time:

$$\text{CO} = \frac{\text{HR}}{1000} \cdot [-6.6 + 0.25 \cdot (T_e - 35) - 0.62 \cdot \text{HR} + 40.4 \cdot \text{BSA} - 0.51 \cdot A], \quad (7.4)$$

where HR is the mean heart rate (expressed in beats/min), T_e represents the mean ventricular ejection time (ms), whereas BSA and A correspond respectively to the body surface area (m^2) and the age (years) of the analyzed subject.

7.4 Pulse decomposition analysis

As can be observed in Eq. 7.1, in lumped element models, the variables of interest depend only on time; thus, any wave propagation or reflection phenomena are neglected. However, as originally hypothesized by Wetterer [123], and thoroughly discussed by Westerhof et al. in [124], the arterial tree is not a reflectionless system, and secondary retrograde components arise from the forward-travelling systolic pulse at points of significant impedance mismatch (e.g. at vascular bifurcations); moreover, blood pressure changes do not occur instantaneously, but in fact are propagated through the vascular system at varying finite speeds, depending on the local biomechanical characteristics of the vessels (i.e. luminal radius, wall thickness and Young's modulus), as formalized by the Moens-Korteweg equation [72]. Accordingly, arterial pulse waveforms can be regarded as the summation of these forward and counter-propagating components, whose relative superposition and amplitude is believed to carry implicit information on the vascular biomechanics.

This is the fundamental concept underlying the physiologically-motivated properties of the digital volume pulse (DVP), defined by Millasseau and co-workers, and the techniques of automatic pulse decomposition, such as the multi-Gaussian algorithm presented in Chapter 3. In particular, as already reported in Section 3.3, a feature of large artery stiffness (i.e. the *Stiffness Index*, SI), has been identified with the inverse delay time separating the systolic and diastolic phases of DVP waves [71]. As performed in this specific investigation, this property is generally weighted by the height of the analyzed subject, so as to take the variation in arterial path lengths into account (under the assumption of a linear relation). Moreover, the relative amplitude of the DVP diastolic peak (or, alternatively, the diastolic inflection point) with respect to the main systolic wave, has been proposed as a surrogate marker of peripheral reflection (thus termed *Reflection Index*, RI), linked to the tone of distal resistance vessels [74]. The derivation of these characteristics is recalled in Fig. 7.4:

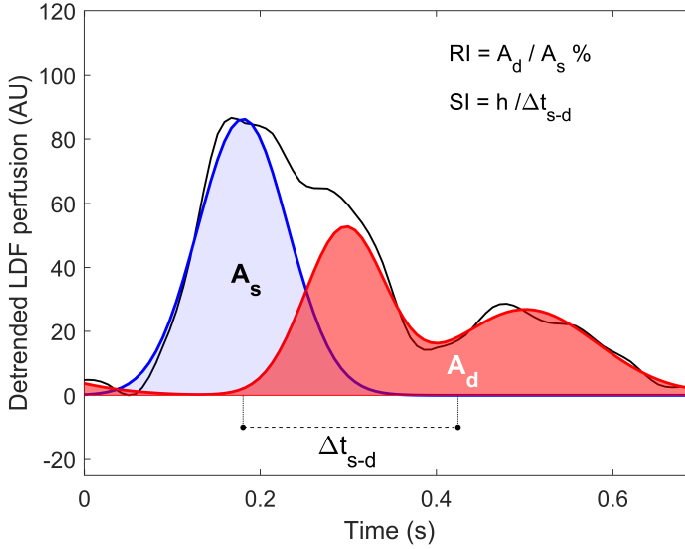


Figure 7.4: Pulse decomposition and extraction of the *Stiffness* and *Reflection* indices, where h represents the height of the subject. The diastolic time reference, used for estimating the former parameter, is identified with the centroid of the three diastolic Gaussian terms (see Section 3.3).

In order to support the physiological interpretation of these properties, the present validation study aimed at assessing the relationship between the *Stiffness* and *Reflection* indices and the parameters of central and distal vascular compliance (i.e. C_1 and C_2), that are derived from the Windkessel-based modeling of arterial pressure pulse waves during diastole. To this end, pulse-wise averaged SI and RI estimates were obtained by applying the novel multi-Gaussian decomposition algorithm to the LDF recordings of cutaneous perfusion. The results of the present regression analysis are discussed in the following Section.

7.5 Experimental results and discussion

Notwithstanding the particular care taken in the positioning of the tonometer's probe, data pertaining to 5 out of the 28 recruited volunteers had to be excluded from the correlation analysis, since the recordings of radial artery pressure exhibited an unstable trend or pulse waveforms which were evidently not suitable for the analysis of the diastolic contour and, thus, the generation of reliable reference values of central and distal arterial compliance. A sample case is displayed in Fig. 7.5.

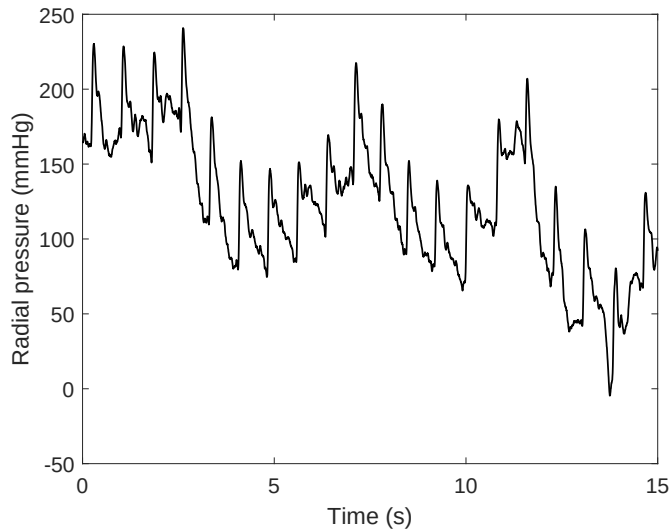


Figure 7.5: Low-quality recording of radial artery pressure.

The general characteristics of the subjects eventually included in the validation study are listed below, in Table 7.1.

Table 7.1: Test subjects: general characteristics.

Characteristics		Mean	σ
Age	(years)	24	3
Height	(cm)	175	8
Weight	(kg)	73.3	12.6

N: 23; M/F: 16/7.

Interestingly, a statistically significant and qualitatively strong correlation was detected between the SI and the compliance parameter C_1 , derived from the Windkessel model of the circulatory system (Fig. 7.6). In detail, a Spearman's correlation coefficient $\rho = -0.567$ was observed ($p < 0.001$). As expected, the analysis highlighted the presence of an inverse relationship between these variables, in agreement with their opposite physiological meaning: the SI is in fact conceived as a surrogate marker of large artery *stiffness*, whereas C_1 is a parameter of central vascular *compliance*.

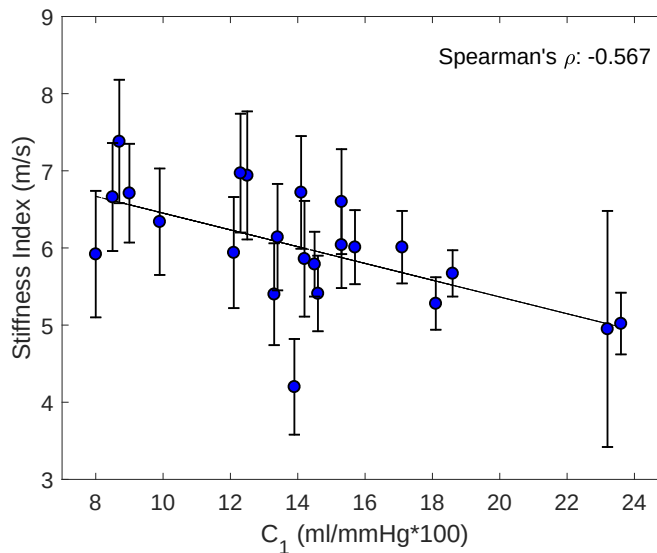


Figure 7.6: Scatter plot showing the correlation between the large artery *Stiffness Index* (SI) and the Windkessel-based central compliance, C_1 .

However, as can be observed in Fig. 7.7, no appreciable association could be demonstrated between the RI and the distal compliance, C_2 , which exhibited a non-significant Spearman's ρ of -0.163 ($p = 0.589$). Therefore, this specific outcome casts some doubts on the actual reliability of the RI as a marker of peripheral vascular tone.

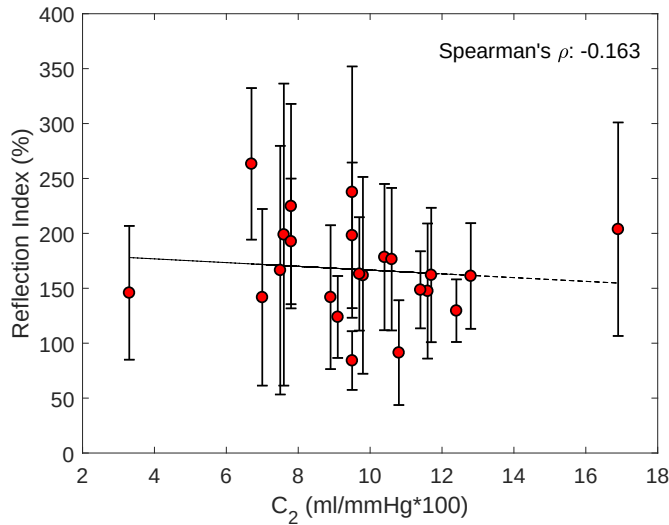


Figure 7.7: Scatter plot showing the correlation between the peripheral *Reflection Index* (RI) and the Windkessel-based distal compliance, C_2 .

Nevertheless, a couple of relevant methodological observations must be made. First, the LDF and pressure pulse waves used for estimating the above parameters were acquired at different anatomical locations (namely, the radial bony prominence and the pulp of the index finger); thus, the observed disagreement could have been partly due to an underlying heterogeneity in the local properties of the distal vascular tree. Secondly, it is relevant to recall how the multi-Gaussian decomposition of peripheral pulse waves and the Windkessel-based analysis of diastolic pressure contours rely on conflicting assumptions regarding the origin of the secondary waves that follow the systolic pulse: in fact, as discussed in Section 7.4, the former method regards measured pulse waveforms as the superposition of an antegrade systolic component and multiple retrograde wavelets arising from phenomena of internal reflection, whereas the latter ascribes the presence of secondary diastolic os-

cillations to the combined effect of arterial compliance and blood inertance (which, in turn, is affected by blood density and cross-sectional vessel area), neglecting any wave propagation mechanism.

For the sake of completeness, Table 7.2 summarizes the descriptive statistics of the variables of interest, together with the macro-hemodynamic parameters involved in the derivation of the Windkessel compliance estimates:

Table 7.2: Vascular and haemodynamic parameters: descriptive statistics.

Characteristics		Mean	σ
SI	(m/s)	6.0	0.8
RI	(%)	167.1	42.7
C_1	(ml/mmHg*100)	14.2	4.1
C_2	(ml/mmHg*100)	9.7	2.7
HR	(bpm)	69.7	8.9
MAP	(mmHg)	91.8	8.8
CO	(l/min)	5.5	0.5

HR: heart rate; MAP: mean arterial pressure; CO: cardiac output.

7.6 Conclusion

In conclusion, the experimental results presented in this Chapter corroborate the physiological interpretation of the SI as a non-invasive feature of proximal artery stiffness. Therefore, this small-scale validation study encourages the adoption of LDF and pulse decomposition techniques, for the non-invasive assessment of vessel wall alterations at the macrovascular level of the circulatory system. On the other hand, no significant relation could be identified between the index of peripheral reflection, RI, estimated from the relative area beneath diastolic LDF contours, and the index of distal compliance, C_2 , of the modified four-element Windkessel model of the arterial system. Although this outcome might be biased from methodological issues (namely, the distance between the LDF and tonometry sensors), further investigation is required before any reliable conclusions on possible alterations in the peripheral vessel tone can be drawn on the basis of this surrogate marker.

Chapter 8

Assessment of vascular abnormalities in type 1 diabetes

The burden of type 1 diabetes (T1D) is growing worldwide, stressing the requirement to limit the threat of its long-term complications. The development of methods for the early diagnosis and the non-invasive monitoring of T1D patients is thus widely recognized as one of the greatest priorities of modern clinical research. In this regard, the validation of bioengineering techniques for the assessment of vascular function, based on optical modalities such as laser Doppler flowmetry (LDF), is deemed of utmost importance. The objective of the study covered in the present Chapter was to identify alterations in LDF signals of microvascular perfusion at the foot of T1D patients and, moreover, to investigate their potential association with the long-term quality of metabolic control. To this end, the characterization of the LDF signals relied on the range of methods, introduced in the previous Chapters, namely the multi-Gaussian decomposition of peripheral pulse waves, the wavelet transform analysis of the myogenic vasomotion and, finally, the modeling of the cutaneous hyperaemic response to local thermal stimuli.

8.1 Introduction

The prevalence of type 1 diabetes (T1D) is steadily rising worldwide [125]. In this regard, the development of methods for the early diagnosis and non-invasive monitoring of T1D patients is widely recognized as one of the greatest priorities of the clinical research in this field. T1D is characterized by absolute insulin deficiency, abrupt onset of symptoms, proneness to ketosis and dependency on exogenous insulin to sustain life. It is the result of pancreatic beta cells destruction and its development requires a genetic predisposition to the disease and putative environmental triggers, that activate pathogenetic mechanisms leading to a progressive loss of pancreatic beta cells. Its detrimental impact on the patients' quality of life stresses the urgency of developing novel clinical screening tests for the early detection of long-term chronic complications. It is nowadays established that such complications are closely correlated with an imbalance in glycemic control. In detail, a central pathophysiological pathway has been identified in the tissue oxidative stress induced by supernormal glucose concentrations: these determine the build-up of advanced glycation end-products (AGE) via non-enzymatic processes which, in turn, enhance the formation of stable cross-links between inelastic collagen fibrils [126, 127]. This is known to induce tissue mechanical stiffening and, thus, to cause a decrease in joint range of motion (ROM), involving particularly the distal joints of the limbs [128, 129], even in young T1D patients [127]. Furthermore, due to this mechanism, diabetes plays a pivotal role in the development of macro- and microvascular complications [130–132], with alterations in the control mechanisms and the biomechanics of the blood circulation (e.g. angiogenesis dysregulation, thickening of the basement membrane, impairment of physiological vasodilatory pathways).

In this regard, the cutaneous microcirculation is deemed as a valuable translational model for evaluating the general status of the microvascular districts of the circulatory system [41, 133]. Therefore, it offers a convenient window to non-invasive diagnostic approaches aimed at assessing diabetes-related vascular disorders. In recent years, optical modalities such as laser speckle contrast imaging and laser Doppler flowmetry (LDF) have been widely applied [134], in combination with a range of pharmacological and physical stimuli, in order to investigate neurovascular and endothelial functions. In particular, as introduced in Chapter 2, the rapid local warming of cutaneous tissue above $\approx 42^\circ\text{C}$ is known to evoke a transient (3–5 min) vasodilatory

reflex, mediated by the action of local afferent sensory nerves [9]; thus, the present test could be applied to monitor the integrity of peripheral nerve fibers in otherwise asymptomatic T1D patients. Moreover, the normal microcirculation is characterized by non-stationary oscillations around 0.1 Hz, produced by the spontaneous adaptation of the vascular smooth muscle cell tone, in response to cyclic variations in transmural pressure [135]. However, there is evidence indicating a decrease in the 0.1-Hz modulation of microvascular perfusion at the foot of neuropathic T1D subjects [136]. Therefore, the time-frequency analysis of this *myogenic* vasomotion mechanism might hold the potential for detecting and preventing early microvascular complications. The aim of the study presented in this Chapter is to verify the deterioration of physiological properties of the foot microcirculation in T1D patients, and to investigate their possible relationship with glycemic control, limited joint mobility and muscle weakness.

8.2 Study participants

The present research study was carried out in accordance with the guidelines of the Declaration of Helsinki: detailed information on the involved procedures and their purpose was provided to the subjects, which gave their written informed consent prior to the measurement sessions. Participant recruitment was non-randomized. 47 patients diagnosed with T1D were enrolled and evaluated in the period from December 2016 to March 2018, at the Diabetes Unit of the San Jacopo General Hospital (Pistoia, Italy). The control group comprised, on the other hand, 63 healthy individuals without any diagnosed pathology. All the enrolled participants were non-smokers. Data regarding age, gender, and BMI were collected; furthermore, T1D duration, T1D-related complications, and longitudinal records of glycated haemoglobin (HbA1c) concentrations were retrieved from each patient's health record. In clinical practice, HbA1c is routinely evaluated as a parameter of long-term glycemic control or, more specifically, as an index of average plasma glucose concentrations. HbA1c was measured every three months at baseline, by means of high-performance liquid chromatography. The measurement sessions involved the evaluation of right ankle ROM, hand grip strength, and microvascular perfusion at the foot, as detailed in the following paragraphs. The general characteristics of the study participants are summarized in Table 8.1.

Table 8.1: Study participants: general characteristics.

Characteristics		Control (n=63)	T1D (n=47)	p
Sex	(M/F)	41/22	23/24	0.090 ^{α}
Age	(years)	35.9 $\pm$ 3.2	40.5 $\pm$ 2.5	0.130 ^{β}
Heart Rate	(bpm)	78.0 $\pm$ 12.6	75.9 $\pm$ 13.0	0.278 ^{β}
T1D duration	(years)	-	21.6 $\pm$ 2.1	-
HbA1c ^{δ}	(%)	-	8.0 $\pm$ 0.2	-

α : Pearson's χ^2 test; β : Mann-Whitney U-test; γ : Student's t-test;

δ : HbA1c levels recorded on the day closest to the measurement session.

8.3 Hand grip and ankle mobility evaluation

Hand strength was assessed in each subject by means of a Jamar Hydraulic Hand Dynamometer (Lafayette Instrument Company, USA), a validated device, considered as the current gold standard for quantitative measurements of hand maximum isometric muscle strength. During the evaluation, subjects were seated comfortably on a chair without armrests. The ipsilateral shoulder was adducted and neutrally rotated, with the elbow flexed at 90°, and the forearm and wrist in neutral position. Standard verbal encouragement using the same tone of voice (“squeeze the handle/button as hard as possible”) was used during the measurement sessions. Three consecutive grip strength values were obtained at 15-s intervals and the average result was recorded.

Ankle joint mobility (AJM) was assessed in plantar and dorsiflexion by the same observer, by means of an inclinometer (Fabrication Enterprises, USA), in accordance with the protocol described by Francia et al. in [137]: patients lied supine, with the subtalar joint in neutral position, and the ipsilateral knee extended and placed over a rigid 5-cm high support. The maximum range of dorsal and plantar flexion was determined by marking the fifth metatarsal bone with a demographic pen and then positioning the inclinometer along the diaphysis of the bone, with one extremity over the distal condylus. Both angular motions were recorded as the mean of three consecutive measurements.

8.4 Microvascular perfusion analysis

Signals of cutaneous perfusion were acquired from the plantar side of the right hallux using a Periflux 5000 Laser Doppler Flowmetry (LDF) system (Perimed, Sweden). In order to retain the heartbeat-related pulsatile components of the signals, the time constant of the instrument's Perfusion Monitoring Unit was set at the minimum value of 0.03 s. The LDF measurements were carried out under controlled thermal conditions (22–24 °C), after a 10-min acclimatization time had passed. Participants were carefully instructed to relax and to avoid abrupt movements during the measurement, so as to minimize the risk of motion-related artefacts. The general protocol included a baseline phase, and a 5-min interval of local thermal stimulation, where the LDF probe was heated up at a stable temperature of 43 °C. However, this test could be performed in 20 controls (i.e. $\approx 32\%$) and 25 T1D patients ($\approx 53\%$) only. As previously discussed, the available time-frequency domain where boundary distortions are negligible should last at least 5 fundamental cycles [49], in order for a statistically robust assessment of the myogenic microvascular flowmotion to be achieved. Accordingly, the wavelet transform analysis (WTA) of the myogenic flowmotion required the availability of a minimum 3.5-min baseline, free of significant artefacts; this prerequisite was met in 53 controls (i.e. 84.1%) and 37 T1D patients (78.7%).

8.4.1 Myogenic vasomotion analysis

Cutaneous blood perfusion is continuously regulated by the concurrent action of neurogenic, myogenic and endothelial control mechanisms. These non-autonomous, non-stationary oscillatory processes are typically investigated by means of WTA [47], in that it offers the maximum feasible joint time-frequency resolution (specifically, when Gaussian-enveloped basis functions are used). The myogenic activity is generally associated with the frequency range (0.052, 0.145) Hz: as described in Chapter 2, its characteristic power, $P_{w,myo\%}$, was obtained by integration of the wavelet spectrogram (Fig. 8.1) within the above range, and by averaging with respect to time. Furthermore, in order to cope with the inter-subject variability in the local microvessel density and, thus, in the absolute levels of LDF perfusion, properly normalized estimates were computed. To this end, the time-averaged power of the cardiac pulse waves propagating to the capillary beds was adopted as normalization factor; this was obtained from the standard range (0.6, 2) Hz.

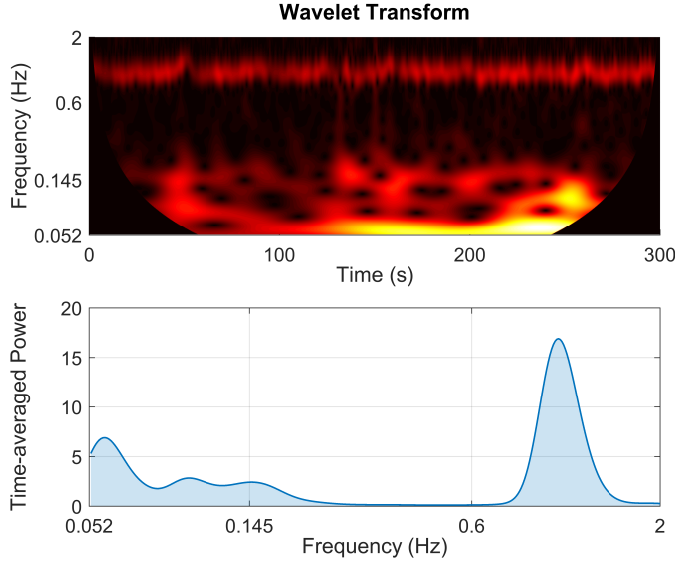


Figure 8.1: Wavelet spectrogram (top), where the black areas indicate the domain where the transform was not defined, to avoid the influence of boundary effects; time-averaged power (bottom), where the grid highlights the myogenic frequencies, and the cardiac range used for normalization.

8.4.2 Multi-Gaussian modeling of peripheral pulse wave

Arterial wall stiffening affects the velocity of the pulse waves, which propagate from the aortic valve to the peripheral districts of the circulatory system. Nevertheless, as introduced in Chapter 3, alterations of the vascular biomechanical properties also exert a sensible effect on their morphology: this can be conveniently monitored with single-probe optical techniques, such as LDF, and characterized quantitatively by means of decomposition methods. In the present investigation, the novel four-Gaussian pulse decomposition algorithm (PDA), presented in Chapter 3, was applied to the baseline of the LDF signals. From each identified model of pulse waveform, the large artery *Stiffness Index* (SI) and the *Reflection Index* (RI), linked to peripheral vessel tone, were estimated [74]. Furthermore, the relative amplitude of the systolic component, α_1 , was recorded. These parameters, whose definition is recalled in Fig. 8.2, were averaged for each subject.

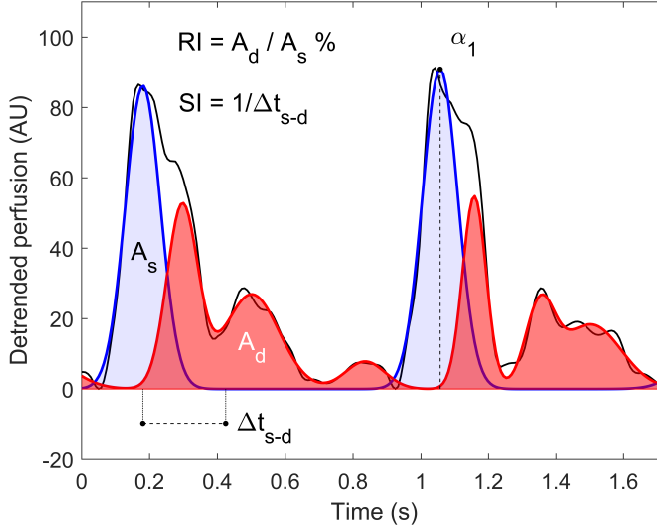


Figure 8.2: LDF pulse analysis and feature extraction, where RI: *Reflection Index*; SI: *Stiffness Index*; α_1 : relative amplitude of the systolic component. Systolic and diastolic phases, as detected by the PDA algorithm, are highlighted in *blue* and *red*, respectively.

8.4.3 Local thermal hyperaemia: modeling and analysis

The Temperature Unit of the LDF system was used to provide a precise thermal stimulus at the measurement site. As mentioned earlier, the rapid vasodilatory reflex, mediated by afferent sensory fibers, was evoked by heating up the LDF thermostatic probe at a stabilized temperature of 43 °C. The hyperaemic response was represented by the following bi-exponential data model (Fig. 8.3):

$$F(t) = \begin{cases} k + A_e \left(e^{\frac{-(t-t_d)}{\tau_1}} - e^{\frac{-(t-t_d)}{\tau_2}} \right) & t \geq t_{43}^{\circ\text{C}} \\ k & t < t_{43}^{\circ\text{C}} \end{cases} \quad (8.1)$$

where t denotes time, k is the mean basal perfusion, and t_d accounts for the delay in the vasodilatory reflex after the start of the heating stimulus at $t_{43}^{\circ\text{C}}$. The adopted model was fitted to the LDF perfusion data using a *trust-region* algorithm for constrained non-linear optimization. Afterwards, the

identified values of the five parameters were used to locate the maximum peak of the thermal hyperaemia; this value was then normalized to the average perfusion at baseline, $\bar{f}_{bsl}$:

$$\Delta f_{peak\%} = \frac{f_{peak} - \bar{f}_{bsl}}{\bar{f}_{bsl}} \quad [\%], \quad (8.2)$$

where f stands for *blood flow*; furthermore, the $F(t)$ data model allowed for a robust characterization of the hyperaemic response in terms of relative increase in signal power (P):

$$\Delta P_{43^\circ\text{C}} = 10 \log_{10} \left(\frac{P_{43^\circ\text{C}}}{P_{bsl}} \right) \quad [\text{dB}]. \quad (8.3)$$

Finally, in addition to these model-based parameters, the present analysis involved the estimation of the percentage increase in the mean raw perfusion, $\Delta \bar{f}_{43^\circ\text{C}\%}$:

$$\Delta \bar{f}_{43^\circ\text{C}\%} = \frac{\bar{f}_{43^\circ\text{C}} - \bar{f}_{bsl}}{\bar{f}_{bsl}} \quad [\%]. \quad (8.4)$$

To this end, a fixed 60-s transition window was ignored after the end of the baseline, as shown in Fig. 8.3.

8.5 Results and discussion

Control and T1D groups were compared in terms of AJM, grip strength, and microvascular parameters. Data non-normality was assessed with the Shapiro-Wilk test: significantly non-normal variables were then analyzed using the non-parametric Mann-Whitney U test; otherwise, the Student's t-test for independent samples was adopted. The results of this statistical comparison are summarized in Table 8.2, where data are reported as median and interquartile range (IQR). In detail, no significant difference in AJM nor hand grip could be determined between the available groups of T1D and control subjects. Moreover, no significant relation could be demonstrated between the latter and the LDF parameters. On the other hand, T1D patients were associated with a lower absolute LDF perfusion at baseline, $\bar{f}$ (C: 82 (117) AU; T1D: 42 (102) AU; $p=0.036$), which was consistently reflected by a lower α_1 amplitude of the systolic pulse waves (C: 59 (94) AU; T1D: 41 (65) AU; $p=0.004$). Instead, the present analysis did not highlight any statistical difference in the RI and SI features of the LDF pulse, nor in the

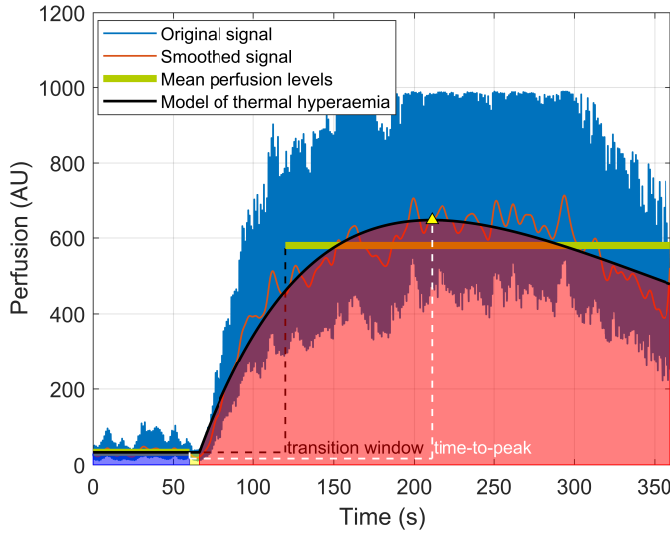


Figure 8.3: Modeling of the local hyperaemia response to thermal stimulation. The delay between the start of the heating stimulus and the onset of the vasodilatory reflex is highlights in *yellow*, whereas baseline is represented in *blue*.

normalized myogenic power at baseline, $P_{w,myo\%}$. As already pointed out in Section 8.4, local thermal hyperaemia could be evaluated only on a subset of the enrolled subjects (thus limiting the resultant statistical sensitivity). Nevertheless, a significantly higher relative hyperaemic response emerged in the T1D group, in terms of power ($\Delta P_{43^{\circ}\text{C}}$), mean ($\Delta \bar{f}_{43^{\circ}\text{C}\%}$) and peak ($\Delta f_{peak\%}$) perfusion values (Table 8.2). Since rapid local warming of skin above $\approx 42^{\circ}\text{C}$ determines the full relaxation of microvascular smooth muscle cells and, thus, the achievement of maximal levels of blood perfusion, these results suggest that the lower parameters of basal flow imply the presence of a larger vasodilatory reserve and, possibly, a higher vascular resistance at rest. The longer time needed to achieve full vasodilation would also explain the higher (though not significantly, $p = 0.107$) response delay t_d , observed in T1D patients. On the other hand, the remaining four parameters of the $F(t)$ model did not exhibit any significant difference between the groups, possibly because of their large coefficients of variation.

Table 8.2: Comparison of the microvascular parameters by diabetes status.

Characteristics	N_C	N_{T1D}	Control	T1D	p
			Median (IQR)	Median (IQR)	
$\bar{f}$ [AU]	63	47	82 (117)	42 (102)	0.036 ^{†*}
$P_{w,myo}\%$ [%]	53	37	27.1 (47.9)	34.9 (62.5)	NS [†]
RI [%]	63	47	82.7 (35.5)	98.0 (45.3)	NS [†]
SI [1/s]	63	47	3.6 (0.7)	3.5 (0.6)	NS [†]
α_1 [AU]	63	47	59 (94)	41 (65)	0.004 ^{†*}
$\Delta f_{peak}\%$ [%]	17	24	2.6 (6.5)	10.7 (19.7)	0.016 ^{†*}
Δt_{peak} [s]	17	24	135.0 (56.5)	156.4 (51.1)	NS [‡]
$\Delta P_{43}^{\circ C}$ [dB]	20	25	12.2 (11.3)	19.8 (15.6)	0.027 ^{‡*}
$\Delta \bar{f}_{43}^{\circ C}\%$ [%]	20	25	321 (608)	942 (1719)	0.038 ^{†*}
A_e [AU]	20	25	33159 (53676)	43379 (46000)	NS [†]
t_d [s]	20	25	4.1 (14.8)	7.3 (13.4)	NS [†]
τ_1 [s]	20	25	144.1 (270.3)	174.4 (74.5)	NS [†]
τ_2 [s]	20	25	121.0 (91.7)	142.3 (65.1)	NS [†]

†: Mann-Whitney U-test; ‡: Student's t-test;

*: statistical significance;

NS: not significant.

The results of the correlation analysis between the LDF variables and the average annual HbA1c are shown in Table 8.3. While most of the investigated variables were not associated with meaningful Spearman's ρ values, it is interesting to observe a significant, though moderate, positive correlation in the arterial *Stiffness Index*, SI ($\rho = 0.322$, $p = 0.026$), shown in Fig. 8.4. This parameter is generally estimated by taking the inverse of the delay between the primary systolic waves and a corresponding diastolic peak reference. In the present work, this secondary point was specifically identified with the diastolic centroid of the pulse model (Fig. 8.2, *red* area). The SI has been proposed as a marker of large artery stiffness, showing a positive correlation with arterial PWV ($r = 0.67$, $p < 0.001$ [73]) and age ($r = 0.63$, $p < 0.001$ [74]). However, to avoid any confusion, it is here emphasized that the partial correlation obtained for this index was comparable and still significant after removing the effect of age ($\rho_{age} = 0.335$, $p = 0.026$).

Table 8.3: Correlation between the LDF parameters and the average HbA1c.

Characteristics		N	Spearman's ρ	p
$\bar{f}$	[AU]	45	0.046	NS
$P_{w,myo} \%$	[%]	35	0.054	NS
RI	[%]	45	-0.130	NS
SI	[1/s]	45	0.332	0.026 *
α_1	[AU]	45	-0.015	NS
$\Delta f_{peak} \%$	[%]	24	-0.257	NS
Δt_{peak}	[s]	24	-0.095	NS
$\Delta P_{43} ^\circ C$	[dB]	25	0.001	NS
$\Delta \bar{f}_{43} ^\circ C \%$	[%]	25	-0.273	NS
A_e	[AU]	25	0.285	NS
t_d	[s]	25	0.139	NS
τ_1	[s]	25	-0.449	0.028 *
τ_2	[s]	25	0.008	NS

★: statistical significance;

NS: not significant.

A protracted impairment in glycemic control is deemed to further tissue mechanical stiffening, through the build-up of AGE, and the enhanced cross-linking of inelastic collagen fibrils. Indeed, this outcome is consistent with the presence of a relation between arterial stiffening and a long-term imbalance in plasma glucose concentrations. This, however, did not correspond to a significant reduction in ankle ROM. Therefore, the multi-Gaussian decomposition and the morphological characterization of peripheral LDF pulse waves might represent a valuable approach for the early detection of diabetes-related vascular complications. Nevertheless, this result must be confirmed on a richer dataset, validating the SI estimates against measurements of carotid-femoral PWV, derived with standard clinical procedures. A significant, though moderate, association with HbA1c emerged also for the τ_1 parameter ($\rho = -0.449$, $p = 0.028$), which characterizes the temporal dynamics of the hyperaemic response upon heating. However, the large inter-subject variability and the lower sample size available for this analysis preclude us from raising any solid conclusion on its physiological meaning.

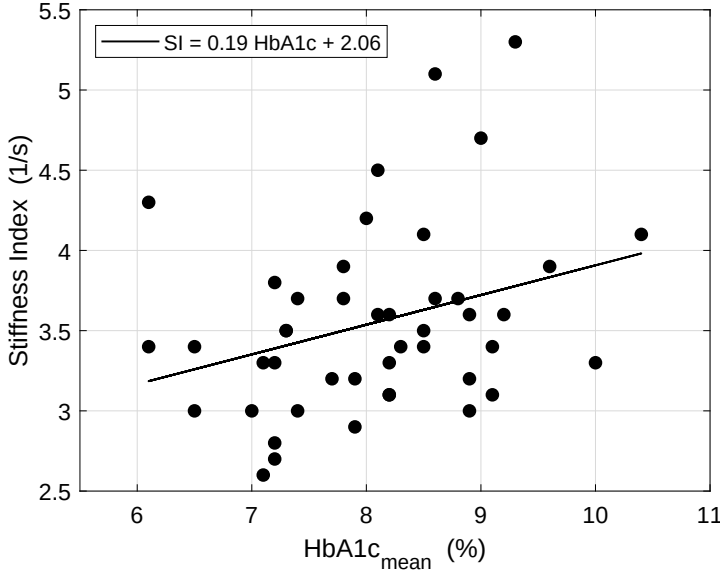


Figure 8.4: Scatter plot showing the relation between annual average HbA1c concentrations and the pulse-derived *Stiffness Index* (SI), with superimposed regression line.

8.6 Conclusion

In the present study, the potential alteration in the properties of microvascular LDF signals, acquired at the foot of T1D patients, was assessed by means of PDA and time-frequency methods applied in basal conditions and, moreover, through tests of thermal hyperaemia, analyzed on the basis of a bi-exponential data model. Furthermore, in this research activity, the existence of possible relations between the microvascular parameters and the quality of annual glycemic control, in terms of average HbA1c, hand grip strength, and ankle ROM, was investigated. Our findings indicate that large artery stiffness, evaluated through the PDA-based *Stiffness Index*, SI, holds a relation with the long-term loss of glycemic control over the past year.

Chapter 9

Related works

This Chapter introduces two research activities related to the main subject of the present thesis. Namely, the first section deals with a fully automated method devoted to the quantification of capillary flow rates from dark field microscopy videos of the sublingual microcirculation. The developed technique exploits image segmentation and a multi-object tracking algorithm for reconstructing the path of the flowing erythrocytes, and derive their mean axial speed. The method has been validated against reliable velocity values obtained from the manual analysis of space-time diagrams of the capillary flow, exhibiting a high level of agreement for speeds up to 300 $\mu\text{m/s}$.

*In the second section, we present a novel method for the characterization of the spatial distribution of cutaneous perfusion, based on the analysis of photoplethysmography images captured with a high-speed camera. Briefly, this technique involves the generation of spatial maps of the pulse propagation delay, inside the superficial microvascular beds, and the application of textural analysis for the quantitative assessment of the resulting pattern.*¹

¹A preliminary version of the first section of the present chapter has been published as “Particle tracking for the assessment of microcirculatory perfusion” in *Physiological Measurement*, vol. 62, pp. 74-78, 2017.

A preliminary version of the second section has been published as “Evaluation of spatial distribution of skin blood flow using optical imaging” in *CMBEBIH 2017: IFMBE Proceedings*, vol. 38, pp. 358-373, 2017.

9.1 Automatic assessment of erythrocyte flow in dark field microscopy

Circulatory shock is a widespread clinical condition that affects about 30% of the patients admitted to European intensive care units (ICU) [138]. The most prevalent form is septic shock, in which tissue oxygenation becomes compromised due to the disruption of the physiological (myogenic, metabolic and neurohumoral) mechanisms that regulate the microvascular blood flow in order to meet the local metabolic requirements of the organism [139]. In this pathological condition, microvascular perfusion can still be deteriorated even after the normalization of standard macrohemodynamic parameters: a dysfunctional microcirculation, in fact, may not be reflected by the state of the systemic circulation as a consequence of arterio-venous shunting. In the light of the above, since the microcirculation of non-vital districts is the first to reflect the characteristic derangements of circulatory shock, monitoring the peripheral perfusion of ICU patients may provide valuable biomarkers for the early detection of a potential impairment of the oxygenation of vital organs. Furthermore, the direct assessment of the microvascular perfusion may allow the identification of suitable end-points for guiding hemodynamic resuscitation, able to ensure an adequate correction of local blood flow deficits, without compromising the oxygen transport efficiency due to excessive hemodilution [140].

Recent years have witnessed the development of subsequent generations of compact, mobile, non-invasive microscopy devices [39, 141, 142] that enable the direct visualization of the sublingual microcirculation at the bedside of ICU patients, through dark field illumination (Section 1.2.3). Clinical studies conducted with these hand-held microscopes have found that microcirculatory alterations are best correlated with the occurrence and severity of multi-organ failure [143] and, furthermore, that a persistent deterioration in the microvascular perfusion after 24 hours following the onset of shock represents an accurate predictor of ICU mortality [144] (even in the absence of abnormal values of conventional hemodynamic and oxygen-derived parameters). However, a subjective assessment of microvascular alterations, based on the visual inspection of the recorded videos, is not feasible because of the complexity of the underlying patterns; this, moreover, makes the evaluation particularly prone to intra- and inter-observer reproducibility issues. In this regard, the definition of a consensual scoring system by

the microcirculation research community, including several quantitative and semi-quantitative parameters of capillary density and perfusion, have laid the basis for the establishment of a standardized procedure that favours the achievement of improved reproducibility and reliability levels [145,146]. Such framework still entails time-consuming operator-dependent tasks. Nevertheless it has provided the active investigators with an effective reference for the development of suitable image processing algorithms, able to translate these microvascular parameters into the critical care practice, thanks to a partial automation of the protocol [147].

Two commercial software packages, dedicated to the computer-assisted analysis of dark field microscopy images of the microcirculation, are nowadays available for research purposes, namely: Automated Vascular Analysis (AVA, MicroVision Medical, he Netherlands) and CytoCam Tools (CCT, Braedius, the Netherlands). Both feature vessel detection tools and modules for the semi-automated evaluation of the microcirculation, including the assessment of capillary flow rates, in accordance with the established consensus. However, a comprehensive quantitative analysis of erythrocyte flow velocities necessitates an accurate segmentation of the microvascular structures, which would be extremely demanding if performed manually by a single operator. In AVA, the automatic detection and reconstruction of the visualized microvascular networks is based on the segmentation algorithm described by Steger in [148], whereas CCT adopts a similar approach, developed by Frangi et al. [149]. In general, these image processing techniques require the video frames to be preliminarily stabilized, so as to correct for potential displacements of the field of view during the recording; specifically, the present image registration is performed through a normalized 2-D cross-correlation of the consecutive frames. Afterwards, these are time-averaged in order to smooth the gray level discontinuities caused by the plasma gaps between the flowing erythrocytes and, thus, enhance the visualization of the vascular structures. After the above preprocessing steps, both segmentation methods employ a tensor representation of the resultant time-averaged image which, thanks to an eigenvector analysis of local Hessian matrices, allows the delineation of basic linear structures (i.e. characterized by gray levels that mainly change in one direction only). In these widely used software packages, the measurement of erythrocyte flow velocities relies on the semi-automated analysis of particular *space-time diagrams* (STDs), generated from the individual capillaries, identified at the preceding segmentation stage [150].

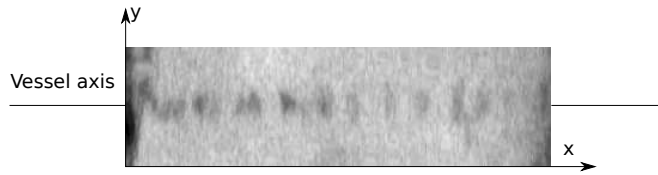


Figure 9.1: Sample frame of a capillary, where the luminal axis has been identified and properly straightened in order to generate the STD.

In detail, blood microvessels are initially straightened through an adequate transformation of the original image (Fig. 9.1). Then, the diagram is generated by arranging the gray level intensities of the centerline pixels, corresponding to the subsequent video frames, into adjacent columns (or rows, depending on the software package), as shown in Fig. 9.2. Thus, one image coordinate indicates the position along the vessel axis, whereas the second characterizes the temporal evolution of the gray level intensities: in other words, depending on the chosen orientation, columns (rows) represent the spatial distribution of the gray levels along the vessel axis at a specific time point, while rows (columns) show how the intensity varies with time at a specific location.

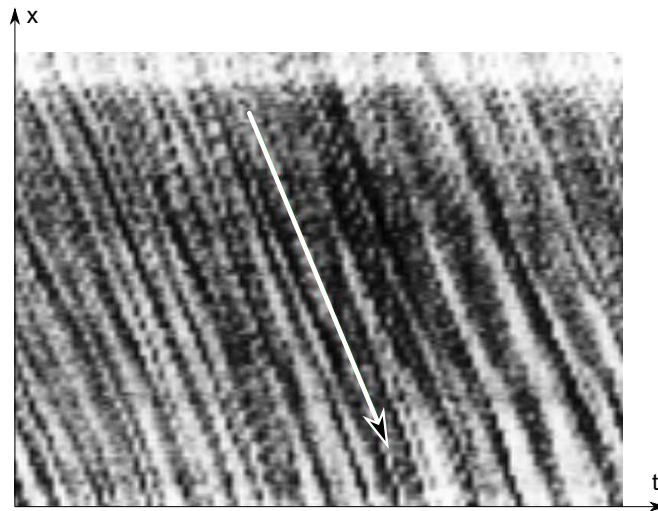


Figure 9.2: Sample STD of a capillary, where the arrow indicates the approximate average slope of the line pattern, corresponding to the mean velocity.

In STDs, erythrocyte flow creates characteristic linear, dark, oblique structures, which alternate with the lighter pattern produced by plasma gaps. It is straightforward to see that the higher the velocity of an erythrocyte is, the larger will be the distance travelled in the time interval between two consecutive frames, and hence the local slope of the associated dark track, with respect to the temporal axis. In fact, in STD analysis, the estimate of the mean erythrocyte velocity is usually obtained from the average slope of a number of lines, manually traced by an operator so as to reproduce the underlying line pattern of the diagram. In addition, AVA supports an automated assessment of the pattern orientation, based on the Hough Transform [147]. It is important to note how the proper detection of linear structures requires the presence of an adequate contrast along the longitudinal direction of the vessel: in other words, the analyzed video must offer a proper resolution of the flowing erythrocytes, with acceptable contrast and focus. Therefore, the measurement conditions and the specifics of the microscopy systems must ensure the visualization of the granular nature of the erythrocyte flow through the imaged capillary bed. Regardless from image quality, ambiguities in the interpretation of the STDs may nevertheless arise: if the luminal radius of the microvessel of interest is large enough, erythrocytes might in fact occlude or move past each other, leading to uncertainty in the physical correspondence of the objects observed through consecutive frames, especially at high cell densities. Thus, STDs may display lines which cannot be unequivocally related to separate flowing cells. Furthermore, STDs are often difficult to interpret, due to the uneven spacing of the erythrocytes, and the fluctuations in flow speed both over time and along the vessel length. Therefore, the operator is usually required to perform a visual best-fit of several lines over different regions of the diagram, implementing a manual sampling of the erythrocyte speed in space and time. However, this approach is time-consuming, inherently subjective, and does not thoroughly consider the spatio-temporal variance of the estimates. The manual evaluation of the erythrocyte flow velocity is thus one of the major limitations holding back a widespread clinical application of these portable microscopes to the study of the mucosal microcirculation. Therefore, much research is ongoing with the aim to provide an operator-independent assessment of microcirculatory parameters, but no validated method is available so far [151]. Among the possible strategies, the most recent methods proposed in the available literature include *dynamic time warping* [152], *optical flow* estimation [153], and

block matching algorithms [154]. A preliminary work [155] demonstrated that erythrocyte segmentation and video tracking, applied to microscopy videos captured with CytoCam, could provide accurate velocity estimates up to $200\text{ }\mu\text{m/s}$. Adopting the same approach, in the present study the original technique has been evolved toward a fully automated method for the robust quantification of capillary flow speeds. Briefly, the proposed algorithm performs an automatic segmentation of the erythrocytes inside every frame of the microscopy videos, so as to derive an estimate of their instantaneous position along the microvessel axis, and then reconstructs their individual path by means of a Kalman filter-based multiple-object tracking technique, returning a cell-wise averaged estimate of the capillary flow rate.

9.1.1 Preliminary study

A preliminary study was conducted in order to identify the velocity range which can be generally assessed from videos recorded with state-of-the-art portable dark field microscopes. To this end, the present analysis relied on 920 STD-based estimates of erythrocyte velocity, obtained from videos of the sublingual microcirculation, captured with the CytoCam microscopy system [39] in porcine models of circulatory shock. The velocity distribution observed in this dataset is shown in Fig. 9.3.

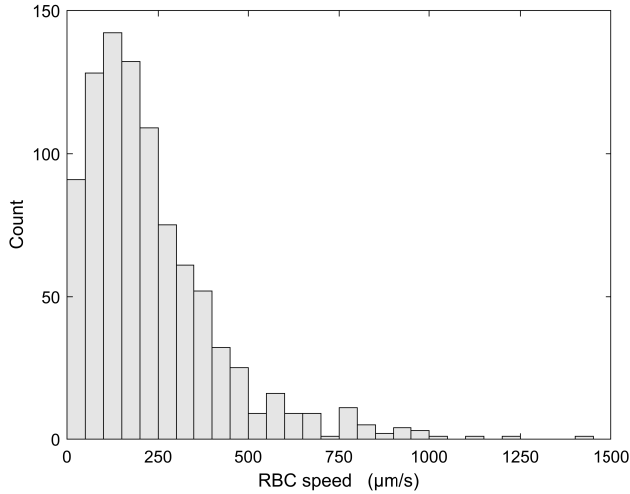


Figure 9.3: Velocity distribution of the reference dataset.

A considerable number of samples appears to be comprised between 100 and 300 $\mu\text{m/s}$, whereas relatively few values are found above 500 $\mu\text{m/s}$: in detail, statistical analysis revealed a median capillary flow speed of 187 $\mu\text{m/s}$, and an interquartile range given by $307 \mu\text{m/s} - 114 \mu\text{m/s} = 193 \mu\text{m/s}$. It must be emphasized that, in STD-based velocity estimation, higher values tend to be increasingly inaccurate, due to the greater sensitivity to slight adjustments of the lines traced by the operators: in fact, even pixel-size displacements of the reference points can produce significant differences in the resultant velocity value. Evidently, a reliable evaluation of erythrocyte velocities above 500 $\mu\text{m/s}$ is extremely challenging and feasible only when the operator manages to perform a proper adjustment of the capillary detections, so as to obtain an overall length which allows to sample the motion of fast-flowing erythrocytes for an adequate period of time.

9.1.2 Dataset

The agreement between the proposed automatic method and the reference STD-based manual assessment of capillary perfusion was evaluated on a set of videos of the sublingual mucosal microcirculation, that had been acquired during the clinical experimentation of the CytoCam system [39] on patients undergoing on-pump coronary bypass surgery [156]. In detail, the microcirculation videos were captured perioperatively by a trained operator, at a resolution of 0.7 $\mu\text{m/pixel}$, and a frame rate of 25 Hz. In order to ensure a thorough and reliable validation, a set of quality requirements was imposed on the microvessels, so as to ensure the estimation of accurate reference velocity values, by means of the STD approach. Accordingly, only capillary detections exhibiting adequate focus and contrast levels were taken into consideration, so as to allow the visual perception of the *granular* nature of the erythrocyte flow and, thus, the generation of STDs presenting a clear line pattern that could be reliably interpreted. Vessel segmentation was performed with CCT: a dataset of 50 capillaries, complying with the above quality requirements, was collected. Afterwards, the *Capillary Network Analysis* module of the CCT software was used to generate the corresponding STDs; their subjective manual evaluation was standardized as follows: from each image, three clearly perceptible traces associated to the flow of different erythrocytes were identified, and the corresponding speeds were averaged so as to produce the reference value used for validating the results returned by the erythrocyte tracking algorithm. The latter was applied on videos of the

capillary flow, provided for each detection by the same module. These videos have a standardized format, with a fixed height of 100 pixels and a variable width, according to the length of the detected microvessel. Since the capillary segmentation algorithm used by CCT may occasionally return false detections in the background or, alternatively, spurious results caused by portions of separate vessels that are improperly chained together, a manual adjustment of these instances was performed so as to guarantee an univocal correspondence between detections and underlying capillaries.

9.1.3 Erythrocyte segmentation

Erythrocyte segmentation is carried out independently on each video frame. In dark field microscopy, erythrocytes appear as dark blobs flowing on a lighter background. However, salt-and-pepper noise generally corrupts the original images, which require preliminary enhancement in order to improve the accuracy of the successive automatic detection. The preprocessing stage of the proposed algorithm is comprised of four main operations: after inverting the original image, a median filter, with a 7×7 kernel size, is applied for noise reduction; next, a top-hat filter, based on a disk-shaped structuring element with a 16-pixel radius, is used for reducing potential background inhomogeneities; afterwards, small artefacts are suppressed by means of a morphological opening operation, based on a disk structuring element with a 4-pixel radius [157]. The size of the above structuring elements was adequately tuned in order to avoid the undesirable elimination of the target erythrocytes. In detail, human normocytic erythrocytes have an average diameter of about $7.5 \mu\text{m}$ [2]. Therefore, in the microscopy videos captured with CytoCam, their expected average size is $7.5/p_s = 10.7$ pixel (where p_s is the available spatial resolution). Since the top-hat filtering and morphological opening operations tend to remove bright details that are respectively larger and smaller than the employed structuring element, the selected configuration adequately suits the requirement. An example of the intermediate and final results of the preprocessing module is shown in Fig. 9.4.

As can be observed in this sequence, flowing erythrocytes tend to be aligned along the centerline of the image, which corresponds to the microvessel axis; consequently, potential residual *blobs*, close to the upper and bottom edges of the video frame, can be regarded as spurious detections produced by the segmentation process. Hence, a space-dependent contrast enhancement, which increases the image contrast near the horizontal axis of the video frames

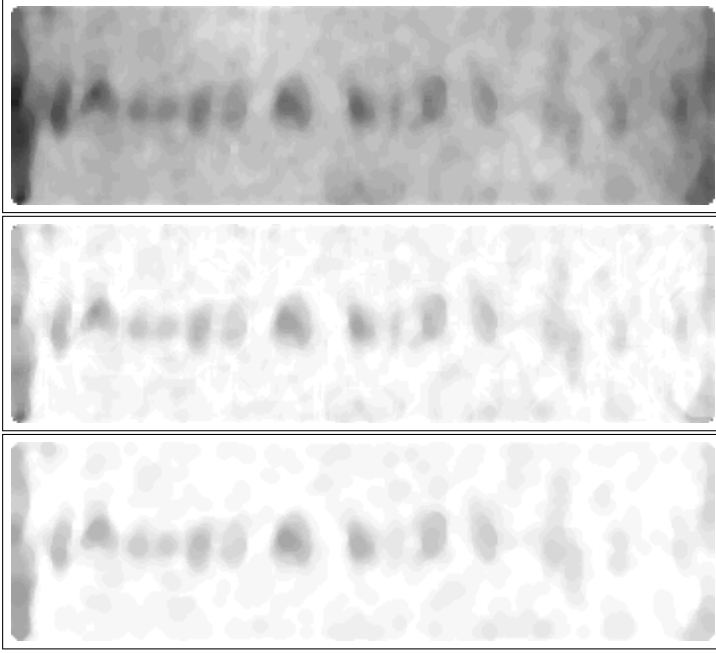


Figure 9.4: Output of the preprocessing stage (excluding the gray level inversion): output of the median filter (top); (inverted) output of the top-hat filter (middle); results of the morphological opening (bottom).

and decreases it towards the top and bottom edges, is implemented at this stage, so as to limit those false detections. In detail, contrast enhancement is performed by weighting the pixel intensities with a Gaussian mask $M(x, y)$, whose expression is reported below:

$$M(x, y) = \exp \left[-\frac{1}{2} \left(\frac{y - y_a}{\sigma_y} \right)^2 \right], \quad (9.1)$$

where $y_a = 50$ is the vertical coordinate of the vessel axis and $\sigma_y = 20$. Finally, connected bright regions are isolated and segmented from the background by means of the Otsu's method for image thresholding [158]. The images obtained from the space-dependent contrast enhancement, the connected-component analysis, and the Otsu thresholding are shown in Fig. 9.5: as thoroughly explained in the following paragraph, the centroids of the dark *blobs* are fed to the tracking algorithm as noisy estimates of the instantaneous position of the flowing erythrocytes.

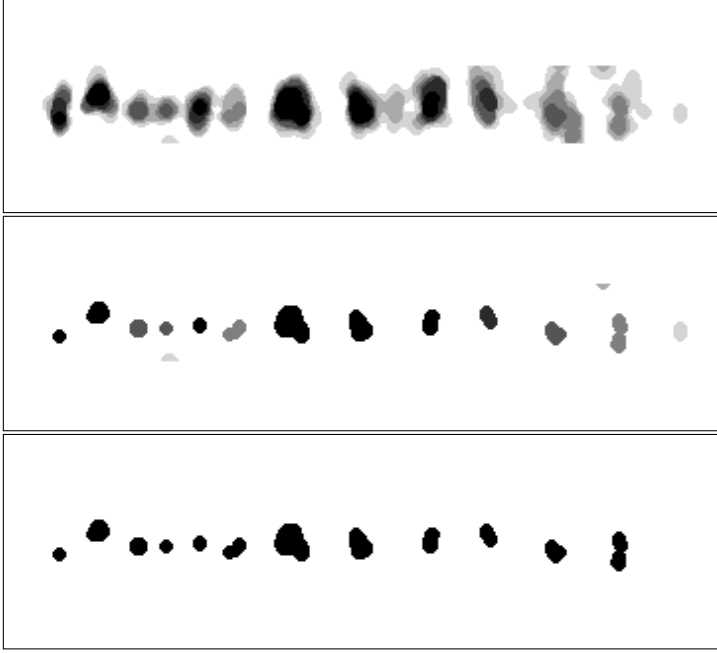


Figure 9.5: Steps of the segmentation stage (inverted gray scale): contrast enhancement (top); connected-component extraction (middle); Otsu thresholding (bottom).

9.1.4 Kalman filtering

In the context of classic experimental fluid dynamics, the application of Kalman filtering techniques to the problem of video tracking can be traced back to 20 years ago [159, 160]. Recently, this approach has been translated to the biomedical field, in order to address the challenge of estimating erythrocyte speed [161, 162]. However, to the author’s knowledge, this method has never been implemented *in vivo* nor extended to clinical scenarios. The Kalman filter deals with the estimation of the state of a linear, stochastic, discrete-time process, governed by the following general difference equation [163]:

$$\vec{s}_t = A\vec{s}_{t-1} + B\vec{u}_{t-1} + \vec{n}_{p,t-1}, \quad (9.2)$$

where $\vec{s}_t$ represents the state vector of the object being tracked at time t , $\vec{u}_t$ is the (optional) control input vector, $\vec{n}_{p,t-1}$ is the process noise, whereas

A and B are the matrices that define the process, by relating the previous state and the current input to the current process state. Furthermore, the general equation describing the measurements performed on the process is given by:

$$\vec{z}_t = C\vec{s}_t + \vec{n}_{m,t}, \quad (9.3)$$

where $\vec{z}_t$ is the measurements vector, $\vec{n}_{m,t}$ represents the localization noise, and C is the measurement matrix. In the Kalman filter theory, the random processes $\vec{n}_{p,t-1}$ and $\vec{n}_{m,t}$ are hypothesized to be white, reciprocally independent and Gaussian; thus, their covariance matrices are diagonal. Moreover, although the process and measurement matrices could, in general, be time-varying, in the present context they are assumed to be constant.

A linear motion model with constant velocity was considered; accordingly, the specific expression of the state equation of the i -th object is the following:

$$\begin{bmatrix} x_{i,t} \\ v_{x\ i,t} \\ y_{i,t} \\ v_{y\ i,t} \end{bmatrix} = \begin{bmatrix} 1 & 1 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 0 \end{bmatrix} \begin{bmatrix} x_{i,t-1} \\ v_{x\ i,t-1} \\ y_{i,t-1} \\ v_{y\ i,t-1} \end{bmatrix} + \begin{bmatrix} \delta_p x_{i,t} \\ \delta_p v_{x\ i,t} \\ \delta_p y_{i,t} \\ \delta_p v_{y\ i,t} \end{bmatrix}, \quad (9.4)$$

where the state of the system at an arbitrary time t is given by the object's position and speed $(x_{i,t}, v_{x\ i,t}, y_{i,t}, v_{y\ i,t})$. As can be deduced from the elements of the A matrix, the motion model assumes the vertical position of the erythrocytes to be constant and the vertical speed to be null; therefore, any observed variability in the vertical direction is attributed only to the process noise. Furthermore, since image segmentation and blob analysis provide an estimate of the tracked object's position, in this specific implementation of the Kalman filter, the measurement equation becomes:

$$\begin{bmatrix} \bar{x}_{i,t} \\ \bar{y}_{i,t} \end{bmatrix} = \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 \end{bmatrix} \begin{bmatrix} x_{i,t} \\ v_{x\ i,t} \\ y_{i,t} \\ v_{y\ i,t} \end{bmatrix} + \begin{bmatrix} \delta_m x_{i,t} \\ \delta_m y_{i,t} \end{bmatrix}, \quad (9.5)$$

where $\delta_m x_{i,t}$ and $\delta_m y_{i,t}$ are the horizontal and vertical components of the measurement error, respectively.

From a mathematical standpoint, the Kalman filter would require the *a priori* knowledge of the process and measurement covariance matrices. In the proposed algorithm, the erythrocyte flow was hypothesized to closely follow uniform motion along the axial direction of the microvessels (i.e. the

x image coordinate); therefore, the $\delta_p x_{i,t}$ and $\delta_p v_{x\ i,t}$ variances were assigned a very small value: 0.0001 pixel^2 . On the other hand, having assumed that $v_{y\ i} = 0$, a higher level of process disturbance was introduced into the model for the y coordinate ($\delta_p y_i = 100 \text{ pixel}^2$), in order to take the variability in the vertical position of the erythrocytes properly into account, and improve the tracking robustness. Moreover, the variance of the localization errors, $\delta_m x_{i,t}$ and $\delta_m y_{i,t}$, was tailored according to the size of the erythrocyte detections, which were expected to have a radius lower than $4 \mu\text{m}$. Accordingly, $\delta_m x_{i,t}$ and $\delta_m y_{i,t}$ were set to $(2 \mu\text{m}/p_s)^2 = 8.2 \text{ pixel}^2$.

9.1.5 Particle tracking

Video frame enhancement and segmentation provide the Kalman Filter with a set of noisy observations $\tilde{z}_{i,t}$ of the motion process of each erythrocyte. However, since for every frame the segmentation algorithm leads to several detections of varying shape and size, a crucial difficulty underlying the correct reconstruction of the motion of multiple objects, through video processing, is encountered: the *correspondence problem*, i.e. the problem of ensuring the correct matching between each physical moving object and the successive locations associated to it over consecutive frames, in a scenario with no self-evident correspondence criterion. To this end, the inherent *a priori* information in the motion model of the Kalman filter was exploited; more specifically, the previous position of each erythrocyte being tracked is projected forward in time, according to the hypothesis of uniform motion, and the Euclidean distances between each *a priori* estimate and the new measured positions are computed. This array of distances between predicted and measured positions provides a means for deriving a cost function for each of the possible combinations: in detail, the optimal association between active tracks and new detections is identified through the minimization of the overall assignment cost (i.e. the global distance), based on the assignment algorithm described by Munkres [164]. The present approach maximizes the probability of maintaining the biunivocal correspondence between tracks and detections, provided that the sampling time interval between consecutive frames is narrow enough to make the displacement of the flowing erythrocytes significantly shorter than their average reciprocal distances. It must be emphasized that the adopted assignment algorithm does not necessarily imply the assignment of a new position to each of the currently active tracks, thus allowing the presence of unassigned tracks.

These unassigned instances are not immediately erased; instead, they are marked as “invisible” and kept active, in order to cope with possible mis-detections, and preserve the tracking robustness even in presence of substantial background noise: a visibility feature, computed as the ratio of the number of locations assigned to each track and the number of frames, is exploited at a successive selection step.

Conversely, new detections, not linked to any of the existing tracked objects, are used as seed points for initializing the state of new tracks: in detail, the initial condition for the erythrocytes’ position, $(x_{i,t}, y_{i,t})$, is obtained from the centroid of each unassigned detection, whereas the velocity components are preliminarily assumed to be null. Therefore, the initial state of the tracked particle is given by:

$$\vec{s}_{i,t} = [x_{i,t}, 0, y_{i,t}, 0]'. \quad (9.6)$$

Ideally, this mechanism is designed to handle the situations where new erythrocytes enter the capillary, coming into view for the first time, in order to avoid incorrect associations to active tracks related to different moving cells. In practice, this strategy has also proved to be useful for resuming the tracking of erythrocytes which are lost by their original tracks.

According to the Kalman filter theory, the *a priori* error covariance P_0^- of the estimated state, $\vec{s}_t$, must be set upon track initialization:

$$P_0^- = \begin{bmatrix} \delta_p x_{i,0} & 0 & 0 & 0 \\ 0 & \delta_p v_{x\ i,0} & 0 & 0 \\ 0 & 0 & \delta_p y_{i,0} & 0 \\ 0 & 0 & 0 & \delta_p v_{y\ i,0} \end{bmatrix}. \quad (9.7)$$

The selection of the elements of P_0^- follows the same rationale adopted for the process noise, except for the variance of the axial velocity component $\delta_p v_{x\ i,0}$: in specific, this is set to a very high value, so as to allow the filter to rapidly adapt the corresponding state component to the detected displacements, and avoid being trapped in the initial condition:

$$\delta_p x_{i,0} = \delta_p y_{i,0} = (4\ \mu\text{m}/p_s)^2 = 32.7\ \text{pixel}^2 \quad (9.8)$$

$$\delta_p v_{x\ i,0} = 10\ 000\ \text{pixel}^2 \quad (9.9)$$

$$\delta_p v_{y\ i,0} = 0\ \text{pixel}^2. \quad (9.10)$$

The motion model provides an *a priori* estimate of the state $\vec{s}_{i,t}^-$ of each active track, inside the current video frame under processing. All estimates are associated with an error covariance given by:

$$P_t^- = AP_{t-1}A^T + Q, \quad (9.11)$$

where P_{t-1} represents the *a posteriori* estimate of the error covariance at the previous frame, and Q is the covariance of the process noise. In the case of the tracks assigned with new detections by the Munkres algorithm, the *a priori* estimates are integrated with the information carried by the corresponding measurements: the *a posteriori* state $\vec{s}_{i,t}$ is in fact computed as the linear combination of the model prediction $\vec{s}_{i,t}^-$ and the weighted residual between $\vec{s}_{i,t}^-$ and the actual measurement $\vec{z}_{i,t}$:

$$\vec{s}_{i,t} = \vec{s}_{i,t}^- + K_t (\vec{z}_{i,t} - \vec{s}_{i,t}^-). \quad (9.12)$$

In the above expression, the weight K_t represents the Kalman filter gain at time t which, in the present tracking problem, is given by:

$$K_t = \frac{P_t^-}{P_t^- + R}, \quad (9.13)$$

where R is the covariance of the measurement noise. The covariance of the overall estimate error is likewise updated as:

$$P_t = (I - K_t)P_t^-, \quad (9.14)$$

where I denotes the identity matrix. On the other hand, the state of the tracks which are left unassigned, for which no measurement is thus available, is updated only via the predictions returned by the adopted uniform motion model:

$$\vec{s}_{i,t} = \vec{s}_{i,t}^- \quad (9.15)$$

$$P_t = P_t^-. \quad (9.16)$$

Once the set of detection/tracking operations described above has been completed on the current frame, unreliable tracks, identified as those having age less than 5 frames and visibility lower than 60%, are suppressed and discarded.

Moreover, a further selection strategy is implemented in order to properly deal with the erythrocytes which have flowed out from the analyzed capillary. In fact, the corresponding “lost” tracks need to be promptly disabled so as not to match them with different moving erythrocytes, thus preventing the consequent corruption of the reconstructed trajectory. In the proposed algorithm, a track is considered to be lost if marked as “invisible” for 3 consecutive frames. Lost tracks, differently from spurious tracks, may correspond to a correct tracking process and, thus, are included in the final estimation of the average flow velocity. Therefore, although removed from the tracking process, the corresponding paths are properly saved.

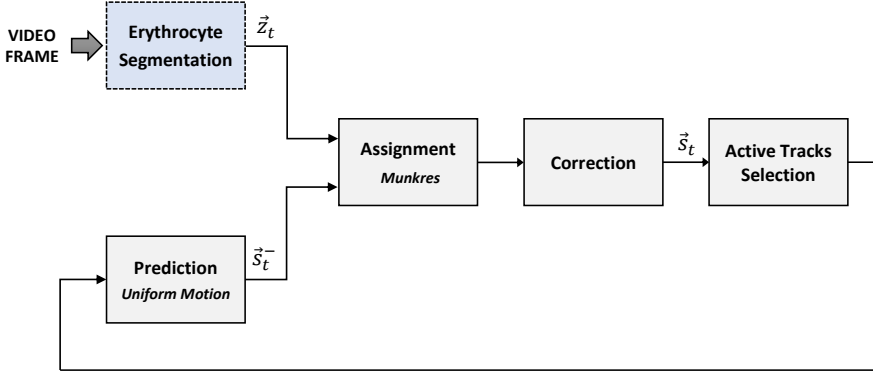
Once the iteration over the video frames is completed, a post-processing module limits the detrimental impact of noisy/invalid trajectories, produced by potential static artefacts and matching errors (i.e. different erythrocytes associated to the same track). In detail, this module retains only those tracks that completely fulfil the following list of requirements:

- ratio of the maximum excursion along y to the maximum excursion along x lower than 0.5: cells not moving along the vessel axis are rejected;
- number of changes in the sign of $v_{x\ i,t}$ lower than 3: a large number of changes in the flow direction is likely related to matching errors;
- flow direction consistent with the majority of the flowing erythrocytes: at high flow rates, an “aliasing” effect may be observed, with different cells matched to the same track throughout the video frames;
- $|\bar{v}_{x\ i,t} - v_{x\ mean}| < 1.65 \cdot v_{x\ \sigma}$, where $\bar{v}_{x\ i,t}$ is the average velocity of the i^{th} track, whereas $v_{x\ mean}$ and $v_{x\ \sigma}$ are respectively the mean and standard deviation of the overall velocity distribution: estimates must be within the 90% confidence interval of the sample mean velocity.

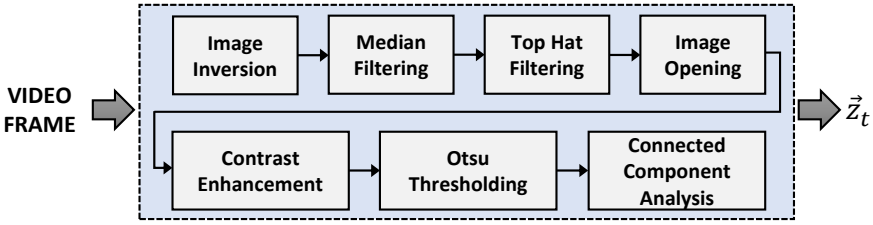
Finally, the capillary flow speed v_c (expressed in $\mu\text{m/s}$), is computed as the average of the mean axial velocity, $\bar{v}_{x\ i,t}$, of the reliable instances satisfying the above inclusion criteria, weighted by their respective length, so as to assign a more important contribution to long-lasting, stable tracks:

$$v_c = f \cdot p_s \cdot \frac{\sum_{i=1}^{N_t} N_i \cdot \bar{v}_{x\ i,t}}{\sum_{i=1}^{N_t} N_i} \quad (9.17)$$

where $f = 25$ Hz is the video frame rate, p_s is the pixel size, N_t is the number of reliable tracks, whereas N_i represents the tracking time of the i^{th} track.



(a)



(b)

Figure 9.6: Automated assessment of erythrocyte velocities: (a) multiple object tracking: block diagram; (b) erythrocyte segmentation: block diagram.

9.1.6 Results and discussion

A preliminary evaluation was carried out in order to compare the velocity distribution of the selected capillaries with the reference dataset of 920 microvascular velocity samples (Section 9.1.1). The histogram obtained from this test subset, by means of the STD technique (Fig. 9.7), confirms that the frame rate offered by the CytoCam system generally allows the evaluation of capillary flow speeds lower than $400 \mu\text{m/s}$, with a considerable percentage of cases below $300 \mu\text{m/s}$. Therefore, the present analysis confirms that the selected dataset fits the velocity distribution that can be expected in the general population.

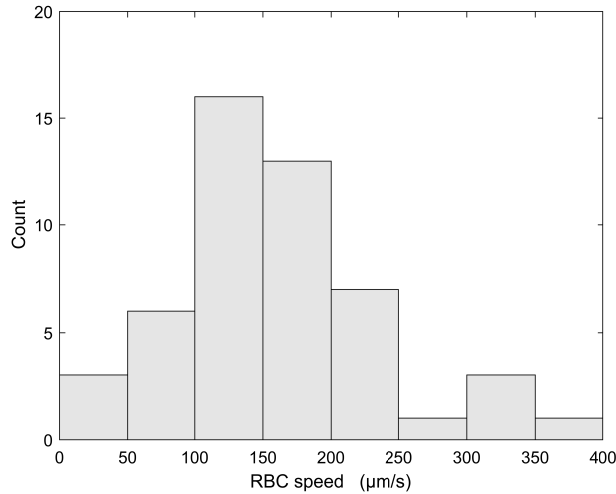


Figure 9.7: Velocity distribution of the test dataset.

In a first phase, the agreement between the automatic technique and the STD approach was evaluated qualitatively, through a visual assessment of the tracking procedure. To this end, the proposed algorithm generates STD-like images, which display the reconstructed path of each tracked object throughout the video sequence, as shown in Fig. 9.8. These diagrams report both accepted and rejected tracks, thus providing a feedback on the possible issues that might affect the tracking process, the possible limitations of the Kalman filter configuration and, moreover, a direct means to verify the inclusion of spurious tracks in the computation of the final velocity estimate. In this regard, the visual inspection of these images may highlight quasi-horizontal, irregular lines, corresponding to false detections produced by local quasi-static noise; furthermore, as previously mentioned, they may occasionally reveal spurious tracks which exhibit an opposite orientation with respect to the actual erythrocyte flow.

Overall, the qualitative assessment of the diagrams highlighted the suitability of the multi-object tracking algorithm, and supported the effectiveness of the inclusion/rejection strategy for the derivation of reliable estimates of capillary flow velocity.

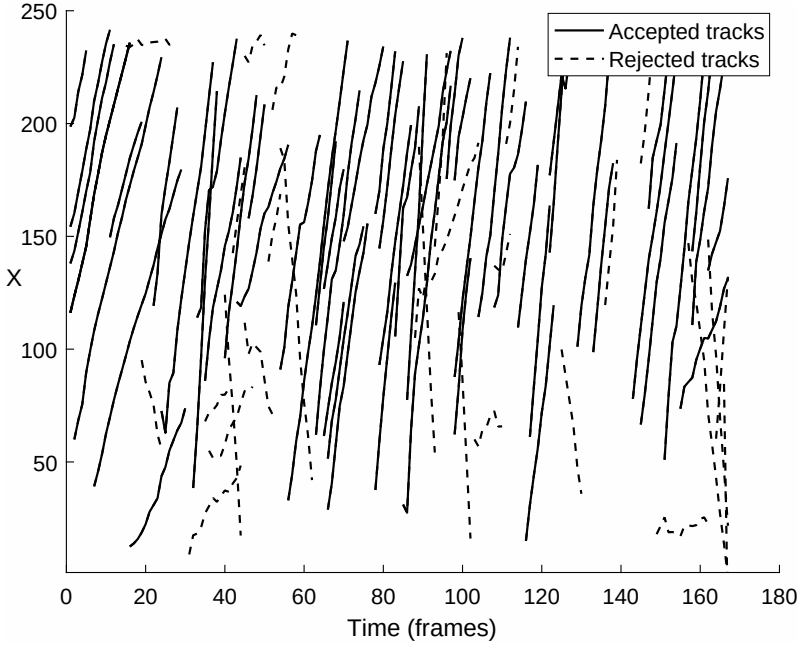


Figure 9.8: Reconstructed paths of the detected tracks, simulating a *space-time diagram*.

The quantitative validation of the performance of the video tracking algorithm consisted in comparing its velocity estimates against the corresponding results provided by the established manual STD technique, by means of correlation and Bland-Altman analyses.

The correlation plot between the manually and automatically derived estimates is shown in Fig. 9.9. The resulting sample Pearson's correlation coefficient is $r = 0.951$, which corresponds to a coefficient of determination R^2 of 0.905. This outcome expresses a strong, positive, and statistically significant ($p < 0.001$) linear correlation between the compared velocity variables, indicating that 90.5% of the variance in the automatically-derived speeds is predictable from the reference STD-based estimates. Furthermore, the linear least-squares fit of the velocity data returned a regression line of equation $y = mx + p = 0.953x - 5.066$: because of the almost unitary slope ($m = 0.953$), the correlation data already suggest a high degree of agreement.

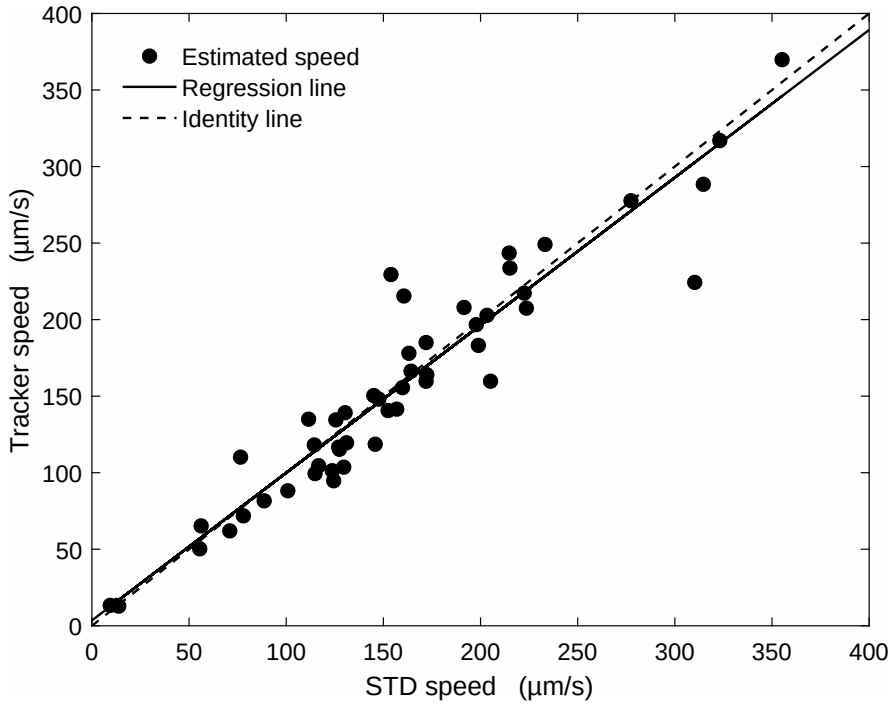


Figure 9.9: Correlation plot between the STD- and tracking-based velocity estimates.

Fig. 9.10 shows the Bland-Altman plot [165]. Only a slight bias was detected between the compared techniques, identified by a mean velocity difference d of $-2.2 \mu\text{m/s}$ (confidence interval: $-4.6 < d < 0.9 \mu\text{m/s}$; significance level: $\alpha = 0.05$). Regarding the evaluation of the 95% lower and upper limits of agreement, the analysis respectively returned: $l_{low} = -49.0 \mu\text{m/s}$ (confidence interval: $-60.8 < l_{low} < -37.2 \mu\text{m/s}$; $\alpha = 0.05$) and $l_{up} = 44.6 \mu\text{m/s}$ ($32.9 < l_{up} < 56.4 \mu\text{m/s}$; $\alpha = 0.05$).

However, the values shown here are affected by the contribution of the three outliers (annotated in Fig. 9.10), which present a large disagreement with respect to the reference velocity returned by the STD analysis. The maximum theoretical speed that allows a reliable tracking depends on the acquisition rate of the microscopy device, the size of the flowing erythrocytes, and their relative distance, Δx .

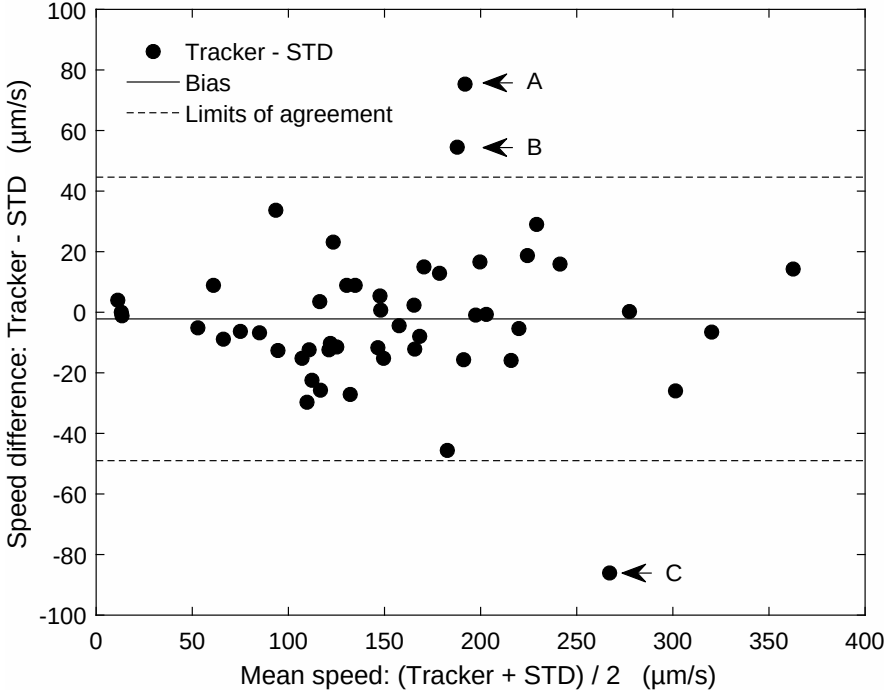


Figure 9.10: Bland-Altman plot of the STD- and tracking-based velocity estimates. Three outliers, labelled with A, B, and C, were detected.

The basic strategy, i.e. associating new detections with the closest predicted locations of the moving objects, yields a correct match if:

$$\Delta x > 2 \cdot v_x / f. \quad (9.18)$$

In the worst case scenario, where erythrocytes having a diameter equal to the lowest extreme of the normocytic range, $D_{min} = 6 \mu\text{m}$, are adjacent to each other, the maximum speed that can be reliably estimated is $v_x = (D_{min}/2) \cdot f = 3 \cdot 25 \mu\text{m/s} = 75 \mu\text{m/s}$: this is substantially below the velocity range of interest. Nevertheless, erythrocytes are generally spaced unequally from each other, providing some sort of “contextual information”. Thus, in practice, the global matching algorithm is able to achieve a stable tracking of the separate moving targets even at higher speeds; specifically, the numerical results observed in the present validation study indicate that a correct matching is obtained for speeds up to $300 \mu\text{m/s}$.

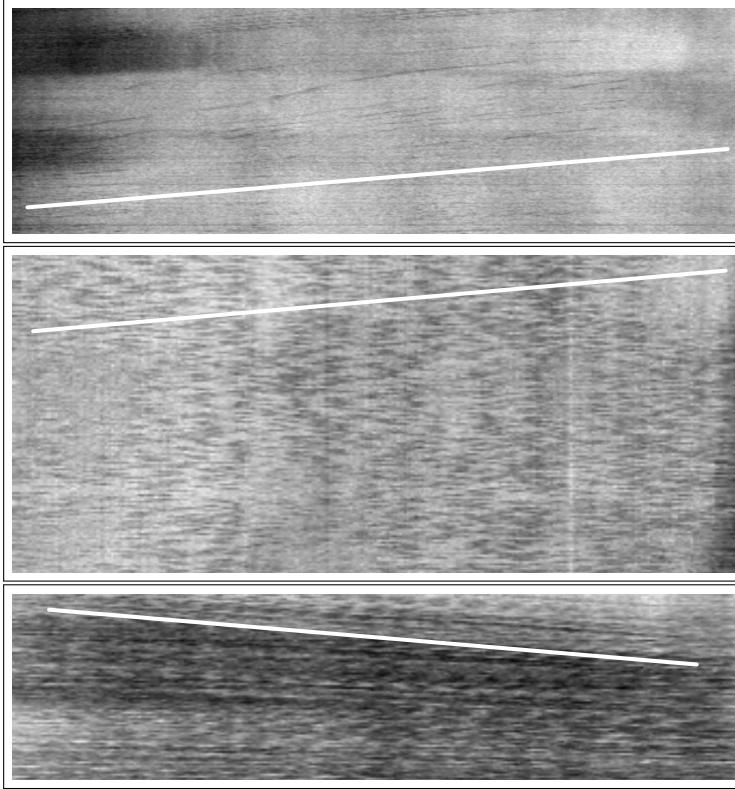


Figure 9.11: Noisy STDs corresponding to the velocity outliers identified during validation (top: A; middle: B; bottom: C). The slope of the white lines represents the estimate returned by the Kalman filter-based tracking.

At higher speed ranges aliasing effects progressively intensify, and increasing mismatches between tracks and physical objects are observed, leading to average speed estimates that are significantly lower than the actual flow velocity. However, the strategy adopted for rejecting velocity outliers and, in general, for handling spurious unreliable tracks, limits the impact of these artefacts on the final estimates.

As previously highlighted in Fig. 9.10, a rather large disagreement between the video tracking and the STD techniques was identified in 3 out of the 50 capillaries, included in the test dataset. A retrospective analysis of the corresponding STDs revealed two distinct situations (Fig. 9.11).

The top box in Fig. 9.11 shows the STD associated with the velocity sample A: it can be clearly observed how the erythrocyte velocity undergoes appreciable changes in different regions of the diagram. Thus, computing a unique velocity estimate is almost meaningless, as the average speed cannot reflect the spatio-temporal heterogeneity of the microvascular flow pattern. In these cases, a more thorough description of the velocity distribution could possibly provide valuable clinical data. On the other hand, samples B and C (Fig. 9.11, middle and bottom boxes) correspond to very noisy diagrams which, however, still reflect the presence of a uniform motion pattern. Therefore, in this case, the average speed returned by the tracker (represented by the white line) may still constitute a reasonable estimate of the erythrocyte flow velocity. However, especially for sample B, assessing the actual orientation of the STD pattern is extremely challenging, in that it is too blurred for allowing a reliable delineation of the traces produced by the erythrocyte flow.

Therefore, excluding the above outliers from further consideration is legitimate, according to the present discussion. This evidently leads to a sensible improvement of the overall accuracy: indeed, after removing these instances, the resultant correlation coefficient is $r = 0.978$, whereas the regression line becomes $y = mx + p = 1.01x - 4.68$, indicating an almost perfect agreement with the manually estimated values.

9.1.7 Conclusion

The proposed automatic method is able to provide a quantitative assessment of erythrocyte flow speeds, from videos of the sublingual microcirculation captured *in vivo* with the CytoCam IDF microscopy system.

With respect to our previous work, described in [155], the current technique features improved image enhancement and segmentation algorithms which successfully limit the number of mis-detections and, furthermore, a refined motion model underlying the prediction stage of the Kalman filter, which yield a reliable video tracking over an extended range of flow velocities. Furthermore, the post-processing module effectively reduces the number of spurious tracks which are included in the final speed computation, thus improving the overall reliability of the automatic estimates.

In detail, the outcome of the correlation and Bland-Altman analyses indicate that the Kalman filter-based video-tracking technique is able to assess erythrocyte flow velocities up to 300 $\mu\text{m/s}$.

The method, however, is characterized by some degree of instability at higher ranges, with problems predominantly arising from the erroneous matching of erythrocytes and detections. The major cause of this limitation is certainly represented by the current video frame rate of the system, i.e. 25 Hz. Thus, the robustness of the tracking process may directly benefit from the improved specifications, offered by the upcoming generations of the device. In fact, the acquisition rate of the CytoCam portable microscope is expected to be increased to 60 Hz: this amelioration should theoretically allow to measure erythrocyte velocities up to about 600 $\mu\text{m/s}$.

9.2 Contactless assessment of skin perfusion with camera-based PPG

Historically, cardiovascular research has been mainly focused on the heart and the major blood vessels. However, in several major diseases, the assessment and normalization of central hemodynamic parameters have been shown to be inadequate, and that a more in-depth knowledge of the microcirculatory flow is required for guaranteeing a positive clinical outcome [166]. Therefore, in the last decades, there has been an increasing interest regarding the physiology and the pathological deterioration of this long-neglected level of the vascular system. Thanks to its accessibility, skin represents a convenient target for investigating the microcirculation, in that allows the *in vivo* utilization of non-invasive techniques [167]. However, average cutaneous perfusion levels have revealed to be unsuitable for detecting pathological conditions [168], without the application of adequate thermal or biochemical stimuli [169,170]. Thus, several analysis frameworks have been proposed in order to support the interpretation and improve the significance of microvascular perfusion data, e.g. the time-frequency analysis of spontaneous fluctuations, and the time-domain characterization of stimulus-response mechanisms by means of data modeling techniques.

The latter generally requires an external input for triggering the control pathways involved in the local regulation of blood perfusion. In this regard, experimental protocols often rely on the local application of heating or cooling stimuli, within safe temperature ranges. In specific, local skin heating above $\approx 42^\circ\text{C}$ elicits the activation of neurological and biochemical feedback mechanisms, which increase the local perfusion rates in order to enhance heat dissipation and thus compensate for the temperature rise. In detail, the neurogenic pathway manifests itself as an early hyperaemic peak whereas the biochemical mechanism, involving the endothelial release of nitric oxide (NO), has a delayed activation and produces a sustained and confined vasodilation of the microvessel beds [9,45,171,172].

Laser Doppler flowmetry (LDF) represents an established technique for the non-invasive acquisition of microvascular perfusion signals (Section 1.2.1). However, despite offering adequate temporal resolution, LDF does not enable the spatial characterization of the microvascular flow. In order to overcome this inherent limitation, laser speckle contrast imaging (LSCI) has been recently adopted in microvascular research (Section 1.2.2). Briefly, the LSCI

technique is based on the use of an infrared laser beam which is diffusely backscattered by the erythrocytes flowing in the superficial microvessels, yielding a dynamic interference speckle pattern, whose local temporal statistics are related to the tissue blood flux. Nevertheless, state-of-the-art LSCI systems offer a reduced temporal resolution with respect to LDF [173].

A relatively new, low-cost, alternative method is imaging photoplethysmography (IPPG), which relies on standard high-speed cameras and incoherent light sources for assessing cutaneous blood fluctuations [174]. As LSCI, IPPG is fully contactless and, thus, may be suitable for the long-term bedside monitoring of critically ill patients (e.g. in intensive care scenarios); on the other hand, it has the disadvantage of being sensitive only to variations in the relative blood concentration inside the effective measurement volume. Nevertheless, due to the pulsatile nature of the microvascular flow, these two measurands are correlated, as blood concentration changes correspond to the net difference between inflowing and outflowing blood volumes. In general, since it combines high temporal and spatial resolution, IPPG offers a means to perform a comprehensive non-invasive assessment of the microvascular flow dynamics and spatial distribution. In specific, the present technique has been successfully applied to detect regional asynchronicities in facial blood perfusion [175] and, moreover, to estimate the interval between QRS complexes and peripheral pulse waves [176].

Following each heartbeat, in fact, a pressure pulse travels from the aortic root towards the peripheral capillary beds. This pulse wave, then, propagates over the skin surface following the bifurcations of the cutaneous microvascular network, producing characteristic spatio-temporal changes in tissue perfusion. In practice, pulse wave propagation may be described by detecting, during each cardiac cycle, the instant corresponding to the maximum blood concentration, i.e. the peak of the pressure pulse. However, in IPPG, the identification of the pulse peak at pixel level is associated with very poor SNR levels. Improved robustness may nevertheless be achieved by estimating the time delay between the local cardiac-related blood pulsatility and a reference heartbeat signal, derived from the average intensity of the recorded IPPG frames. This approach enables the generation of particular images representing the spatial distribution of the propagation delay. The geometric organization of the skin microcirculation has been often represented as a fractal-like tree [177, 178]; hence, it is reasonable to expect that the above distribution would retain the spatial properties of the microvascular

geometry. Indeed, researchers have already considered fractal properties for characterizing the temporal dynamics of blood flow [179,180]. Accordingly, in the present study, fractal analysis has been adopted in order to characterize IPPG-derived images of the pulse propagation delay and, specifically, to investigate how thermal stimulation interacts with the spatio-temporal distribution of cutaneous perfusion, by assessing the textural changes of the delay maps in response to vasodilatory and vasoconstrictive thermal stimuli.

9.2.1 Experimental setup

The experimental setup used in the present research activity combines a polarized green light source and a high performance camera, similarly to the one described by Kamshilin et al. in [174]. More specifically, the light source, used for illuminating the target skin area, consisted of a wideband (warm white light) MC-WHLP1 LED (Thorlabs Inc., US), equipped with a linear polarizer LPVIB100 (Thorlabs Inc., US) and a green emission filter MF525-39 (Thorlabs Inc., US) with central wavelength $\lambda_c = 525\text{ nm}$ and bandwidth $\Delta\lambda = 39\text{ nm}$. An ORCA-Flash 4.0 V2 Digital CMOS camera (model C11440-22CU, Hamamatsu, JP) was used to capture 256×256 pixel images at 400 Hz from 3 cm-wide surfaces, with a 16-bit gray-level resolution. The camera was equipped with a 8.0 mm EFL, F/1.4, 2/3" image format lens (model MVL8M23, Thorlabs Inc., US), and a linear polarizer, identical to the one mounted on the light source. In detail, the above setup would theoretically allow to derive pixel-level estimates of the pulse propagation delay with a temporal resolution of 2.5 ms.

Measurements were carried out on the dorsal aspect of the hand and lasted 20 s, i.e. 8000 video frames. Particular care was devoted to avoid any relative displacements of the field of view during the recording time, in order to prevent the generation of undesired movement artefacts. Accordingly, the hand was positioned on a stable surface, so as to minimize fatigue and possible vibrations. Three different experimental conditions were tested, namely: basal perfusion, heat-induced hyperaemia, and cold-induced vasoconstriction. The baseline measurement was acquired at a controlled room temperature (22 °C), after proper acclimatization of the subjects. On the other hand, heat stimulation was performed by directing a hot air flow towards the dorsal side of the hand. The air source temperature was regulated at about 50 °C, so as to elicit the maximum physiological hyperaemic response. Conversely, cold stimulation was obtained by immersing the hand

in cold water (with ice cubes keeping the temperature at 0°C) immediately before the start of the measurement. Additionally, during the recording, an ice pack was placed between the table surface and the hand of the subjects in order to maintain the stimulation.

9.2.2 Extraction of the reference heartbeat signal

In IPPG images, extracting the heartbeat-related blood pulsatility at pixel level is a very challenging task, because of the limited SNR of the resulting data. Fig. 9.12 shows a sample signal, $s(x, y, t)$, obtained by averaging the gray level intensity over a 3×3 pixel region (centred on (x, y)): in fact, the cardiac component cannot be distinguished from the superimposed noise.

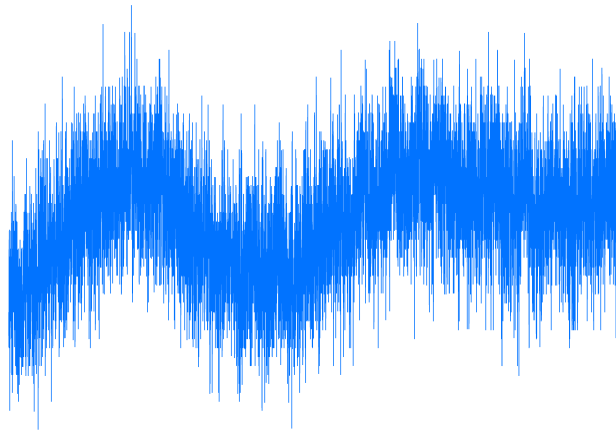


Figure 9.12: Sample PPG signal derived from a 3×3 pixel region.

Therefore, a 200 pixel-wide region of interest (ROI) was selected for this purpose. As expected, averaging the image intensity over a larger ROI yielded a cleaner heartbeat reference, $s_R(t)$, as shown in Fig. 9.13; this generally exhibits characteristic pulse waveforms, overlying low-frequency fluctuations caused by temporal variations in the illumination system and by the physiological mechanisms modulating the local perfusion rates. It is relevant to observe that the obtained signal, being proportional to the intensity of the backscattered green light, is inverted with respect to the relative blood volume inside the superficial microvascular beds. However, the analysis was only aimed at estimating a spatial map of the pulse propagation delays.

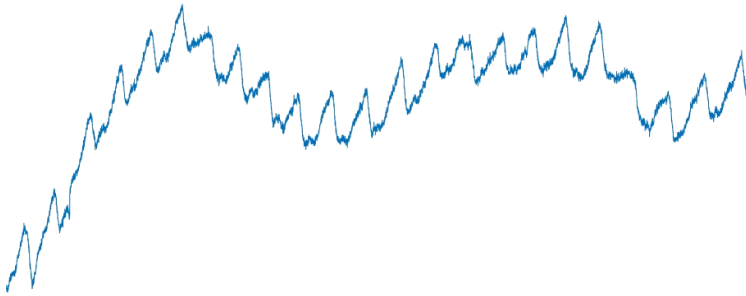


Figure 9.13: Sample PPG signal estimated from the reference 200×200 pixel ROI.

A few preprocessing operations were applied so as to extract the heartbeat frequency range from the original reference signal, $s_R(t)$: in detail, a low-pass digital Butterworth IIR filter of order 12, and cut-off frequency $f_c = 2.4$ Hz, was applied for attenuating the *unphysiological* high-frequency harmonics, whereas a high-pass Butterworth filter (order: 3; $f_c = 0.4$ Hz) was used for removing the baseline drift. An example of the resulting filtered reference is displayed below, in Fig. 9.14.

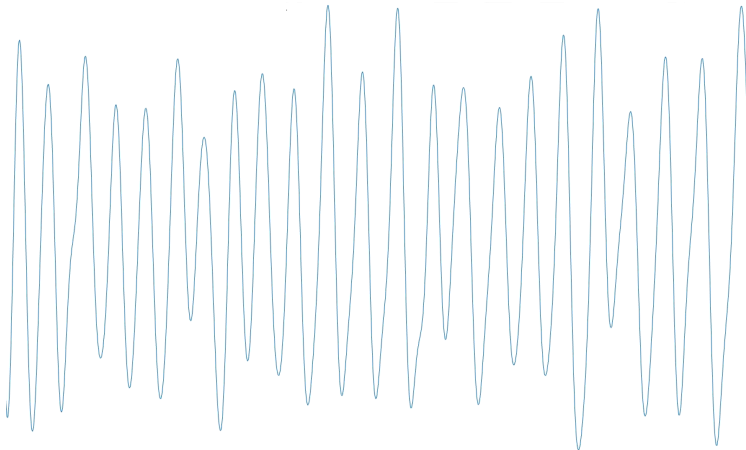


Figure 9.14: Filtered reference signal.

9.2.3 Estimation of pulse propagation delays

Due to the quasi-periodic nature of the reference signal, pulse propagation times can be mapped through the analysis of the cross-correlation between $s_R(t)$ and the signals $s(x, y, t)$, obtained locally from 3×3 pixel ROIs. These underwent a preliminary filtering process, similarly to the one used for the heartbeat reference; however, a more conservative low-pass filtering was applied in this case (order: 6, cut-off frequency: $f_c = 3.2$ Hz).

In detail, the maximum point of the cross-correlation function corresponds theoretically to the optimal match between the local and the reference waveforms, and can be assumed as a global estimate of their relative time shift. However, because of the quasi-periodicity of the heartbeat, the above function exhibits several, almost equally high peaks. Additionally, the low SNR characterizing the local $s(x, y, t)$ signals can lead to highly variable cross-correlation values. Therefore, a straightforward peak detection could produce unreliable estimates. Thus, this operation was confined within a search interval corresponding to half of the expected cardiac cycle duration, i.e. ≈ 700 ms: in detail, the maximum of the cross-correlation identifying the relative delay, $\Delta t(x, y)$, was hypothesized to lie within the range $(-175, +175)$ ms, i.e. $(-70, +70)$ frames. The resultant estimates were then rescaled to the range $(0, 255)$ and represented as a gray level image (Fig. 9.15). A possible extension to the present approach may consist in the detection of the QRS complex in synchronous ECG signals, in order to identify the ventricular systole and, thus, derive accurate spatial maps of the pulse transit time. In this study, instead, the above propagation delay was computed with respect to a local arbitrary PPG signal (i.e. the average gray level intensity over the reference ROI); accordingly, the resulting delay estimates can assume both positive and negative values (i.e. $s(x, y, t)$ respectively following or preceding the reference pulse wave signal, $s_R(t)$).

9.2.4 Delay image analysis

Two methods were applied to assess the spatial heterogeneity of the propagation delay image $\Delta t(x, y)$. The first is based on textural parameters derived from gray-level co-occurrence matrices (GLCM) [181]. GLCM represent the second-order distribution of the gray level intensity, $P_\delta(i, j)$: in specific, $P_\delta(i, j)$ is the probability that two pixels, separated by an offset δ , have respectively gray levels i and j .

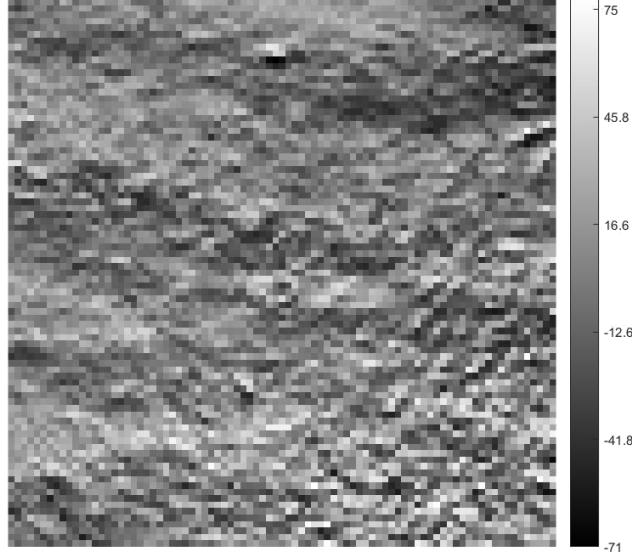


Figure 9.15: Spatial distribution of the pulse propagation delays, represented as a gray scale image.

In general, GLCM that are particularly concentrated along the principal diagonal correspond to coarse textures, whereas scattered elements are related with fine-grained patterns. The probability distribution directly depends on δ : since small values usually carry most of the textural information, in the present analysis a fixed $\delta = (1, 0)$ pixel offset was selected. Several textural parameters were adopted in order to characterize the GLCM: *contrast* (c), *homogeneity* (h), *energy* (e), and *correlation* (r). Their expressions are reported below:

$$c = \sum_{i,j} |i - j|^2 P(i, j), \quad (9.19)$$

$$h = \sum_{i,j} \frac{P(i, j)}{1 + |i - j|}, \quad (9.20)$$

$$e = \sum_{i,j} P(i, j)^2, \quad (9.21)$$

$$r = \sum_{i,j} P(i, j) \frac{(i - \mu_i)(j - \mu_j)}{\sigma_i \sigma_j}, \quad (9.22)$$

where μ_i , μ_j , σ_i , σ_j represent the mean and standard deviation of the marginal probability matrices, respectively obtained by summing $P(i, j)$ along rows and columns.

The second approach is based on fractal geometry, and specifically involved the estimation of the fractal dimension, D_f , of the propagation delay maps. Due to its ease of implementation, the *box counting* algorithm [182] is the most widely applied technique in fractal image analysis. The box counting algorithm is based on the computation of the number N_ϵ of ϵ -wide boxes required to “cover” the delay image intensity (which can be regarded as a 3-D landscape, $(x, y, \Delta t(x, y))$). In detail, the domain of the intensity surface is split into n_s squares of size ϵ ; next, for each square s , the number of boxes needed for covering the corresponding portion of landscape is estimated as:

$$N_{s,\epsilon} = \text{ceiling} \left(\frac{\max_s(\Delta t(x, y)) - \min_s(\Delta t(x, y))}{\epsilon} \right). \quad (9.23)$$

Then, the total box number N_ϵ is directly computed by summing the $N_{s,\epsilon}$ values obtained from each of the n_s square sub-domains:

$$N_\epsilon = \sum_{s=1}^{n_s} N_{s,\epsilon}. \quad (9.24)$$

This procedure is iterated over a grid of ϵ values. The fractal dimension D_f is finally obtained from the slope of the regression line between N_ϵ and $\log \epsilon$, inside the range where the curve $N_\epsilon(\log \epsilon)$ exhibits an approximate linear trend (i.e. in correspondence with the spatial scales where the image has underlying fractal properties). In the present analysis, ϵ values ranging from 3 to 15 pixel were specifically evaluated in this regard.

The fractal dimension of the pulse delay maps was assessed also by means of the *blanket* method [183]. This technique is a variation of the box counting approach, where the $\Delta t(x, y)$ image is covered with a blanket composed of triangles of base ϵ . More specifically, each square sub-domain s (as defined above) is divided up into two equal triangles along its diagonal; next, the global area A_ϵ of the resulting surface is computed. In the blanket method, the fractal dimension D_f is then obtained from the slope m_r of the regression line between A_ϵ and $\log(\epsilon)$, based on the relation $D_f = 2 - m_r$.

Furthermore, the *lacunarity* of the pulse delay maps was evaluated, in order to characterize their spatial heterogeneity. Several expressions of this metric have been proposed; they nevertheless share the concept of measuring data

variability with respect to their mean. The adopted definition is based on the variance in the number of surface points included in each of the ϵ -wide boxes used to estimate the fractal dimension [184]. More specifically, considering the number of points $n_\epsilon(i)$ inside each box i , the lacunarity index was defined as:

$$L_\epsilon = \sigma_\epsilon^2 / \mu_\epsilon^2, \quad (9.25)$$

where μ_ϵ and σ_ϵ respectively represent the mean and standard deviation of $n_\epsilon(i)$.

9.2.5 Results

The visual assessment of the pulse delay images highlighted that the hot and cold thermal stimuli produce, as expected, different spatial patterns. As shown in Fig. 9.16, skin cooling at 0°C yields a more complex, heterogeneous pulse propagation pattern with respect to the basal reference. On the contrary, heating above $\approx 42^\circ\text{C}$ appears to be associated with a more homogeneous perfusion, with milder variations between neighbouring regions.

The observed effect was quantified by means of GLCM-derived and fractal textural metrics, as detailed in the previous paragraph. In specific, a decrease in the GLCM contrast (Eq. 9.19) was progressively detected for the cooling, baseline, and heating experimental conditions. Accordingly, an opposite trend emerged in the GLCM homogeneity (Eq. 9.20), energy (Eq. 9.21), and correlation parameters, which undergo a consistent increase with temperature (Table 9.1). Similar results were obtained with the fractal analysis (Table 9.2). Specifically, the fractal dimension, related to the complexity of the pulse delay pattern, undergoes a gradual reduction with increasing temperatures. A significant drop in fractal dimension is observed particularly for the heating stimulation. This trend is consistently confirmed by the box counting and blanket methods whereas, on the other hand, the effect of cooling is only evident in the fractal dimension estimated by means of the latter technique. The tested thermal conditions also determined sensible changes of the lacunarity index, which is meant to characterize the local heterogeneity of the pulse propagation time; however, the observed outcome conflicts with the hypothesized trend, i.e. lower lacunarity (homogeneity) in the heating condition, and vice versa. Nevertheless, the interpretation of this metric is more difficult, as it depends not only on the actual irregularity of the pattern but also on the estimated fractal dimension.

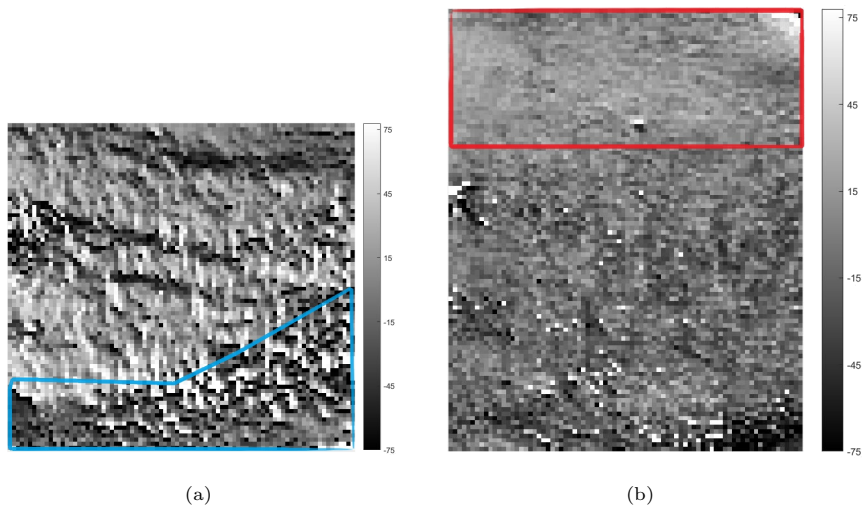


Figure 9.16: Spatial distribution of the pulse propagation times: skin cooling (left); skin heating (right). The field of view corresponds to the one displayed in Fig. 9.15

Table 9.1: GLCM-derived textural parameters.

	Cooling	Baseline	Heating
Contrast	2.45 ± 1.47	0.69 ± 0.44	0.24 ± 0.15
Homogeneity	0.77 ± 0.04	0.83 ± 0.04	0.90 ± 0.02
Energy	0.09 ± 0.04	0.14 ± 0.05	0.30 ± 0.09
Correlation	0.65 ± 0.10	0.76 ± 0.07	0.72 ± 0.80

Each parameter is reported as the *mean* $\pm$ σ of three evaluations.

Table 9.2: Fractal parameters.

	Cooling	Baseline	Heating
Box counting dimension	2.79 ± 0.17	2.63 ± 0.15	2.49 ± 0.17
Blanket dimension	2.98 ± 0.30	2.98 ± 0.23	2.83 ± 0.31
Lacunarity	0.77 ± 0.04	0.83 ± 0.04	0.90 ± 0.02

Each parameter is reported as the *mean* $\pm$ σ of three evaluations.

9.2.6 Conclusion

The experimental results indicate that thermal stimulation induces alterations in the spatial distribution of cutaneous blood flow. Specifically, cooling is associated with a more inhomogeneous pattern with respect to basal perfusion conditions. In this case, the increased heterogeneity in the pulse propagation times might be indicative of a varying degree of vasoconstriction inside the imaged microvascular beds. Furthermore, the effect might have been amplified by the lower functional microvessel density (i.e. the increased distance between the perfused capillaries). Conversely, skin heating above $\approx 42^{\circ}\text{C}$ increased the spatial homogeneity of the pulse propagation, suggesting that the capillary beds within the analyzed ROIs underwent an equivalent degree of vasodilation.

Up to date, there have been several investigations aimed at a thorough characterization of microcirculatory flow patterns by means of non-invasive optical techniques; these may provide a valuable alternative to traditional monitoring methods [175]. This specific pilot study showed that IPPG may provide information concerning the spatial distribution of the cutaneous perfusion, that cannot be quantified with established techniques, such as LDF or LSCI. The latter, in fact, despite providing a more accurate measure of tissue flow rates, has a limited resolution due to the inherent spatio-temporal integration of the raw speckle patterns [185]. The local heterogeneity of microvascular blood flow is of high interest in critical care; therefore, the proposed technique may constitute a valuable extension to the methods devoted to the quantitative analysis of PPG signals. Nevertheless, its potentiality needs to be further assessed with respect to pathological conditions, that are known to play a role in the deterioration of this fundamental level of the cardiovascular system (e.g. diabetes).

Chapter 10

Conclusion

10.1 Summary of contribution

The main core of the research activity covered in the present thesis was the development and the validation of a novel multi-Gaussian decomposition method for the robust detection and characterization of the peripheral LDF pulse wave, and the non-invasive extraction of clinically relevant features. As confirmed by the *goodness of fit* and the coefficient of determination observed in a dataset comprised of more than 20000 reference cardiac cycles (Chapter 3), the multi-Gaussian PDA exhibits an extremely good accuracy when applied to the reconstruction of the cardiac pulsatility of the cutaneous microvascular perfusion, in conditions of baseline blood flow. Moreover, the reconstruction of the LDF waveform, i.e. the pulse-to-pulse identification of the multi-Gaussian model, demonstrates high stability relative to the initial conditions adopted in the numerical optimization of its twelve parameters, hence supporting the reliability of the extracted descriptors. Furthermore, the study reported in Chapter 6 thoroughly highlighted how the marked attenuation of the cardiac pulsatility, due to the transient neurogenic vasoconstriction produced by a rapid inspiratory manoeuvre, does not lead to a significant decline in the achieved performance, corroborating the clinical relevance of the proposed PDA method for the non-invasive assessment of neuromotor mechanisms. In Chapter 5, the multi-Gaussian PDA was applied in combination with a wavelet phase coherence analysis in order to explore the presence of a potential synchronization of the respiratory activity and the LDF pulse waveform which, as extensively discussed in the present work, is

deemed to carry implicit information on the physical properties of the vascular walls. This study led to the detection of a significant coherence between the breathing rhythm and the diastolic phase of the cutaneous perfusion pulse and, thus, to the identification of a non-invasive marker of possible clinical relevance, which may specifically reflect the functional coupling of these physiological oscillatory processes. The ability of the proposed methodological framework to open a window on the alterations of the vascular system is supported by the results of the study outlined in Chapter 4, in which morphological features of the LDF pulse waveform, derived from its multi-Gaussian decomposition, were used to train a supervised support vector machine classifier, which exhibited promising performance in the detection of subjects of different age. The small-scale validation study described in Chapter 7 in fact confirms the interpretation of the LDF *Stiffness Index* as an indirect marker of proximal vascular stiffness, hence encouraging the adoption of pulse decomposition analysis for the non-invasive detection of alterations involving the macrovascular level of the circulatory system. On the other hand, the preliminary results of this study raise questions upon the actual physiological meaning of the *Reflection Index* of distal artery tone, stressing the need for further research. Chapter 8 focused on the non-invasive identification of diabetes-related vascular alterations, based on an extensive analysis of the LDF perfusion measured on the hallux, targeting the myogenic *vasomotion*, the endothelium-dependent reactive vasodilation to thermal stimulation, and the indirect markers of vascular stiffness, extracted by means of the multi-Gaussian PDA. Moreover, the present study explored the possible relation of the microvascular parameters of interest to the long-term metabolic balance. Consistently with the previous findings and with the known role of diabetes in the progressive stiffening of the vascular walls, a significantly strong correlation was detected between the large artery *Stiffness Index* and the loss of glycemic control over the past year. Finally, Chapter 9 dealt with the development of advanced methods for the quantitative analysis of the microcirculation, based on two separate imaging modalities recently introduced in microvascular research, namely: portable incident dark field (IDF) microscopy and imaging photoplethysmography (IPPG). In detail, the first section showed the feasibility of a fully automated approach to the *in vivo* assessment of capillary flow velocities (up to $\approx 300 \mu\text{m/s}$) from videos of the sublingual microcirculation, captured with the CytoCam IDF system. The second section, instead, introduced a new technique for the contactless char-

acterization of the spatial distribution of the microvascular blood flow, which combines IPPG and the textural analysis of local pulse propagation delay maps. Overall, the present thesis covered the implementation of an advanced multifaceted methodological framework, aimed to the extraction of clinically relevant information from the extensive quantitative analysis of signals and images of microvascular tissue perfusion, recorded non-invasively on the skin. The following section outlines the possible future developments.

10.2 Directions for future work

The pulse decomposition method introduced in the present thesis relies on a data model comprised of four Gaussian terms, which guarantee an accurate fit of pulse waveforms, measured non-invasively on the skin by means of LDF. Nevertheless, many types of basis function and different model orders can be tested. Therefore, future research activities might address the data-driven selection of the *best fitting* model, by tuning the nature and the number of components through the evaluation of established parameters such as the Akaike's Information Criterion [186], as recently performed by other investigators for the digital PPG pulse [70, 187]. In order to account for the spatial heterogeneity in the microvascular perfusion, highlighted in Chapter 2, and in the expectedly different propagation dynamics of the arterial pulse wave, the above analysis might involve separate measurement sites and the consideration of different modeling strategies. Furthermore, future studies might also deal with the adaptation and translation of the pulse decomposition framework, developed for LDF, to the analysis of IPPG maps of cutaneous perfusion, in order to explore the feasibility of a fully non-invasive, contactless optical technique for the early detection and monitoring of vascular conditions. Regarding IPPG, the experimental setup outlined in Chapter 9 could be integrated with ECG cardiac monitoring so as to derive *true* maps of the local pulse arrival times.

As emphasized in the present work, further research is needed to improve the clinical understanding of the features adopted for characterizing the LDF waveform; in this regard, their change in response to known alterations in the biomechanical properties of the arterial walls, or in relation to specific pathological states, need to be thoroughly analyzed. This is of utmost importance in order to confirm the suitability of LDF pulse decomposition for the non-invasive assessment of the circulatory system. Therefore, further work may

be devoted to the adequate expansion of the small-scale validation study, reported in Chapter 7, and to the improvement of the preliminary experimental setup, that showed limitations regarding the validation of the feature of peripheral arterial tone. Moreover, additional work may be devoted to the introduction of a feature selection stage in the pulse classification method, described in Chapter 4. This may clarify the significance of the single descriptors, and possibly guide the definition of new markers associated with an improved predictive accuracy with respect to specific classification tasks, targeting different pathological conditions. In particular, the above machine learning scheme, that exploits LDF, decomposition analysis and supervised classification for identifying abnormal patterns in the peripheral pulse waveform, may be applied in the diabetic population with the aim to design a clinical decision support system for the early detection and prevention of vascular complications. To this end, the study must involve a thorough clinical characterization of the test subjects. Accordingly, the control-case study reported in Chapter 8 could be expanded, involving a wider group of patients properly stratified with respect to the quality of glycemic control and the duration and severity of microvascular complications. In this regard, the study might consider continuous glucose monitoring in addition to the longitudinal record of the HbA1c concentrations. Furthermore, future activities may consider the application of pulse decomposition analysis for evaluating the neurogenic vasoconstriction induced by deep inspiratory tests, in order to probe the feasibility of this non-invasive approach, and its relevance with respect to the screening of peripheral neuropathies.

Finally, the wavelet phase coherence between the respiratory activity and the LDF pulse waveform, thoroughly characterized by multi-Gaussian decomposition, suggests the presence of a physiological coordination. Nevertheless, in future research, the coupling between these oscillatory processes might be further investigated by means of more sophisticated approaches, able to characterize time-evolving interactions in terms of strength and directionality, or causality. In this regard, the future methodological framework might include measures of functional connectivity, as the *generalized partial directed coherence*, applied in neuroscience to assess multivariate linear relationships [188], or refer to the theory behind the dynamics of non-linear coupled oscillators, recently reviewed by Rosenblum in [189], and consider the implementation of advanced techniques, such as *dynamical Bayesian inference* [100, 101].

Appendix A

Publications

This research activity has led to several publications in international journals and conference proceedings. These are summarized below.¹

International Journals

1. **M. Sorelli**, C. Ince, and L. Bocchi. “Particle tracking for the assessment of microcirculatory perfusion”, *Physiological Measurement*, vol. 38, iss. 2, pp. 358-373, 2017. [DOI: 10.1088/1361-6579/aa56ab]
2. **M. Sorelli**, Z. Stoyneva, I. Mizeva, and L. Bocchi. “Spatial heterogeneity in the time and frequency properties of skin perfusion”, *Physiological Measurement*, vol. 38, iss. 5, pp. 860-876, 2017. [DOI: 10.1088/1361-6579/aa5909]
3. **M. Sorelli**, A. Perrella, and L. Bocchi. “Detecting vascular age using the analysis of peripheral pulse”, *IEEE Transactions on Biomedical Engineering*, vol. 65, iss. 12, pp. 2742-2750, 2018. [DOI: 10.1109/TBME.2018.2814630]
4. A. Lima, T. van Rooij, B. Ergin, **M. Sorelli**, Y. Ince, P. A. C. Specht, E. G. Mik, L. Bocchi, K. Kooiman, N. de Jong, and C. Ince. “Dynamic contrast-enhanced ultrasound identifies microcirculatory alterations in sepsis-induced acute kidney injury”, *Critical Care Medicine*, vol. 46, iss. 8, pp. 1284-1292, 2018. [DOI: 10.1097/CCM.0000000000003209]

¹The author’s bibliometric indices are the following: *h*-index = 3, total number of citations = 14 (source: ResearchGate on Month January, 2019).

Submitted

1. **M. Sorelli**, P. Francia, L. Bocchi, A. De Bellis, R. Anichini. "Assessment of cutaneous microcirculation by laser Doppler flowmetry in type 1 diabetes", *Microvascular Research*, 2018. (Submitted on 22/10/2018)

International Conferences

1. **M. Sorelli**, L. Bocchi, C. Ince. "Monitoring the microcirculation at the bedside using hand-held imaging microscopes", in *Proc. of 37th International Conference of the IEEE Engineering in Medicine and Biology Society*, Milano (IT), 2015.
2. **M. Sorelli**, Z. Stoyneva, I. Mizeva, L. Bocchi. "Location-dependent microvascular response to thermal stimulation", in *Proc. of International Conference on Biological Oscillations*, Lancaster (UK), 2016.
3. **M. Sorelli**, A. Perrella, L. Bocchi. "Cardiac pulse waves modeling and analysis in laser Doppler perfusion signals of the skin microcirculation", in *Proc. of International Conference on Medical and Biological Engineering (CMBEBIH)*, Sarajevo (BiH), 2017.
4. L. Frassinetti, F. Giardini, A. Perrella, **M. Sorelli**, L. Sacconi, L. Bocchi. "Evaluation of spatial distribution of skin blood flow using optical imaging", in *Proc. of International Conference on Medical and Biological Engineering (CMBEBIH)*, Sarajevo (BiH), 2017.
5. **M. Sorelli**, A. Perrella, L. Bocchi. "Modeling of the microvascular pulse for tracking the vasoconstriction response to a deep inspiratory gasp", in *Proc. of World Congress on Medical Physics and Biomedical Engineering (IUPESM)*, Praga (CZ), 2018.
6. A. Perrella, **M. Sorelli**, F. Giardini, L. Frassinetti, P. Francia, L. Bocchi. "Wavelet phase coherence between the microvascular pulse contour and the respiratory activity", in *Proc. of World Congress on Medical Physics and Biomedical Engineering (IUPESM)*, Praga (CZ), 2018.
7. **M. Sorelli**, A. Perrella, P. Francia, A. De Bellis, R. Anichini, L. Bocchi. "Multi-Gaussian decomposition of the microvascular pulse detects alterations in type 1 diabetes", in *Proc. of World Congress on Medical Physics and Biomedical Engineering (IUPESM)*, Praga (CZ), 2018.
8. **M. Sorelli**, A. Ponzecchi, L. Bocchi. "Validation of physiological features of vascular stiffness extracted through the Pulse Decomposition Analysis of laser Doppler flowmetry pulse waves", in *Proc. of International Conference on Biological Oscillations*, Wien (AT), 2018.

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