



Programa de Doctorado en Ciencias de la Salud

Programa de Doutoramento em Ciências da Saúde

Doctoral Program in Health Sciences

Contribución a un enfoque no convencional de la Función Vascular Periférica

- estudio del impacto fisiológico del masaje en la circulación del miembro inferior

Contribuição para uma abordagem não convencional da Função Vascular Periférica

- estudo do impacto fisiológico da massagem na circulação do membro inferior

Contribution to a non-conventional approach to Peripheral Vascular Function

- study of the physiological impact of massage on the circulation of the lower limb

Tesis Doctoral presentado por:

Tese Doutoral apresentada por:

Doctoral Thesis presented by:

Clemente José Gomes da Rocha

Alcalá de Henares, 2021

Contribución a un enfoque no convencional de la Función Vascular Periférica
- estudio del impacto fisiológico del masaje en la circulación del miembro inferior



En virtud del acuerdo de colaboración con la Universidade Lusófona
Em virtude do acordo de colaboração com a Universidade Lusófona
Under the collaboration agreement with the Universidade Lusófona

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Directores/Diretores/Directors:

Professor Doutor Luís Monteiro Rodrigues

Professora Doutora Júlia Buján Varela

Alcalá de Henares, 2021

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Contribución a un enfoque no convencional de la Función Vascular Periférica
- estudio del impacto fisiológico del masaje en la circulación del miembro inferior

Doña María Julia Buján Varela, Catedrática de Histología e Ingeniería Tisular en la
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CERTIFICA:

Que el presente trabajo titulado "CONTRIBUCIÓN A UN ENFOQUE NO CONVENCIONAL DE LA FUNCIÓN VASCULAR PERIFÉRICA- ESTUDIO DEL IMPACTO FISIOLÓGICO DEL MASAJE EN LA CIRCULACIÓN DEL MIEMBRO INFERIOR " realizado por Don Clemente José Gomes da Rocha en su Programa de Doctorado en Ciencias de la Salud de la Universidad de Alcalá (desarrollado en asociación con Universidad Lusófona de Humanidades e Tecnologías), reúne todos los requisitos científicos y formales para proceder a su lectura y defensa como Tesis Doctoral, para optar al Grado de Doctor por la Universidad de Alcalá.

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DECLARAÇÃO DE CONFORMIDADE

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Lisboa, 02 de junho de 2021



D. Pedro de la Villa Polo, Coordinador de la Comisión Académica del Programa de Doctorado en Ciencias de la Salud,

HAGO CONSTAR que la Tesis Doctoral titulada CONTRIBUCIÓN A UN ENFOQUE NO CONVENCIONAL DE LA ENFERMEDAD VASCULAR PERIFÉRICA: ESTUDIO DEL IMPACTO FISIOLÓGICO DEL MASAJE EN LA CIRCULACIÓN DEL MIEMBRO INFERIOR, presentada por D. Clemente José Gomes da Rocha, bajo la dirección del Dr. Luis Monteiro Rodrigues y la Dra. Julia Buján Varela, reúne los requisitos científicos de originalidad y rigor metodológicos, así como los aspectos éticos en la investigación, para ser defendida ante un tribunal. Esta Comisión ha tenido también en cuenta la evaluación positiva anual del doctorando, habiendo obtenido las correspondientes competencias establecidas en el Programa.

Para que así conste a los efectos del depósito de la tesis, se firma en Alcalá de Henares a 29 de julio de 2021.

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”Tuas forças naturais, as que estão dentro de ti, serão as que curarão suas doenças.”

Hipócrates

Resumen

El masaje es una técnica de manipulación conocida desde hace mucho tiempo, utilizada para múltiples propósitos principalmente asociados con la salud y el bienestar, a saber, en la rehabilitación y el tratamiento y alivio de los síntomas asociados con múltiples contextos osteo-mio-articulares y vasculares. Aceptado como beneficioso en muchas condiciones de salud y acogido por el paciente, recomendado para todos los grupos de edad, desde la infancia hasta las personas mayores, la demostración de su impacto fisiológico y los mecanismos involucrados es aún muy limitada, quizás incluso controvertida o no concluyente. Algunos estudios han sugerido una contribución positiva del masaje para modular el dolor, aliviar la fatiga y control da la ansiedad y el estrés, independientemente de las patologías subyacentes. Sin embargo, el uso de diferentes técnicas y protocolos de masaje, múltiples y variadas técnicas de grabación, y la casi imposibilidad de comparar resultados, son algunas de las dificultades que justifican estas complejidades.

Nuestro principal objetivo fue estudiar el impacto fisiológico del masaje en el miembro inferior humano y los mecanismos de adaptación implicados, en poblaciones sanas, previamente seleccionadas y caracterizadas. Para registrar eventos de microcirculación y con el fin de garantizar la total comodidad y seguridad de los participantes, utilizamos técnicas ópticas no invasivas, ampliamente probadas, confiables y reconocidas, como la flujometría láser Doppler (FLD), una de las más reconocidas “gold-estándares” y fotoplefetismografía (FPG), que se ha utilizado como un medio complementario de cuantificación de la microcirculación. Adicionalmente, y con el fin de ampliar la capacidad de analizar directamente estas señales biológicas, necesariamente complejas por su naturaleza oscilatoria y no estacionaria, recurrimos a otras estrategias matemáticas para el procesamiento de señales. Usamos la transformada de Ondeleta (TO), que ha sido adaptada y utilizada para la descomposición de estas señales biológicas con el fin de mejorar expandir las capacidades de cuantificación, contribuyendo a la estandarización de procedimientos, desde el nivel de definición del dibujo. Experimental y, más Recientemente, el análisis de entropía como una forma de analizar la complejidad de las señales biológicas.

El presente proyecto contó con la colaboración de 98 participantes (32.6 ± 15.5 años) de ambos sexos (47 hombres y 51 mujeres). En los artículos aquí descritos utilizamos un total de cuatro grupos, el primero de los cuales fue el masaje con 32 participantes (19.8 ± 1.5 años), con 16 hombres y 16 mujeres, y en el segundo grupo, el RVA (Reflejo Venoarteriolar), el grupo estuvo formado por 12 participantes ($26,0 \pm 5,0$ años), con 5 hombres y 7 mujeres. Los dos últimos grupos, con personas mayores, fueron masaje (52.6 ± 6.2 años), con 11 hombres y 11 mujeres, y en el último grupo, sometido al procedimiento RVA (50.3 ± 5.6 años), con 5 hombres y 5 mujeres. Se confirmó que todos los participantes seleccionados eran normotensos y sin signos de deterioro vascular, con un índice tobillo-brazo (ITB) > 1.0 . El proyecto fue evaluado previamente por el Comité de Ética institucional (CE 03/2013.12) y todos los procedimientos siguieron los principios de buena práctica clínica establecidos por la Declaración de Helsinki y modificaciones posteriores. El protocolo de masaje se evaluó en dos de sus variantes en cuanto a la dirección de aplicación: proximal o ascendente y descendente o distal. En todos los casos, el procedimiento se aplicó a uno de los miembros inferiores (pierna y pie) previamente aleatorizado, sirviendo el otro miembro como control. El procedimiento de masaje experimental involucró tres fases de registro continuo, en supinación, con la Fase I correspondiente al registro de reposo (basal), durante 10 minutos, Fase II, de provocación con la aplicación de masaje de piernas y pies durante 5 minutos. Minutos y Fase III, recuperación durante 10 minutos. La perfusión sanguínea se evaluó simultáneamente por FPG (PLUX Biosystems, Portugal) y por FLD (PF 5010, Perimed, Suecia), con las sondas aplicadas en ambos pies, en la superficie plantar de los dedos 1 y 2, respectivamente. La presión arterial, medida en el brazo, también se registró en las tres fases del protocolo. La aplicación de las dos variantes del protocolo, cuyo orden de aplicación fue previamente aleatorizado, fue intermediada por un período de lavado de treinta minutos.

Para el “refinamiento analítico” de las señales FLD, usamos otra herramienta de refinamiento analítico, análisis de textura (AT) e se analizó uno subgrupo de masaje con 29 voluntarios (19.9 ± 1.6 años, 13 hombres y 16 mujeres), como se describió anteriormente y usando la TO, las señales FLD se descompusieron en sus componentes oscilatorios, para evaluar los posibles mecanismos involucrados. Se consideraron los siguientes rangos de frecuencia: de 0.05 a 2 Hz: - cardíaco de 0.6 Hz a 2 Hz; respiratorio

de 0.145 Hz a 0.6 Hz; miogénico de 0.052 Hz a 0.145 Hz; simpático de 0.021 Hz a 0.052 Hz; endotelial dependiente de óxido nítrico (endoDON) de 0.0095 Hz a 0.021 Hz; y endotelial independiente del óxido nítrico (endoION) de 0.005 a 0.0095 Hz.

Para validar a TO en señales FPG, otra herramienta de refinamiento analítico, utilizamos datos de un grupo de 12 voluntarios (26.0 ± 5.0 años, 5 hombres y 7 mujeres), sometidos a dos protocolos, un masaje como se describe anteriormente y también a un protocolo RVA, en el que los participantes realizó un procedimiento en decúbito supino de 10 minutos con piernas extendidas, Fase I (línea de base); 10 minutos con un miembro pendiente a 50 cm del nivel del corazón, Fase II (prueba); y 10 minutos después de volver a la posición inicial, Fase III (recuperación). El miembro de prueba fue aleatorizado y el contralateral permaneció inmóvil y sirvió como control. En esta validación de la complejidad de la señal FPG, también analizamos la textura de las escalogramas de TO, utilizando entropía multiescala (EME), un procedimiento estándar, como comparación, y la utilidad del AT en el análisis de la complejidad en las señales en señal FPG se confirmó, aunque se encontró una menor sensibilidad de esta técnica en las zonas espectrales de frecuencias más bajas en relación con la entropía multiescala.

En este estudio se utilizaron dos grupos de individuos sanos, pero mayores, sujetos a la aplicación de los mismos protocolos, masaje y RVA, para evaluar y comparar las respuestas identificadas con los grupos de jóvenes.

Nuestro proyecto se estructuró en cuatro hitos, siendo el primero la definición del procedimiento experimental, lo que permitió observar de inmediato que el impacto del masaje en la perfusión se observaba en ambas extremidades, a pesar de ser aplicado solo a una de las extremidades. El segundo hito, en el que pretendemos profundizar en el análisis de la perfusión, también verificamos que el análisis de correlación de las señales brutas de LDF y FPG confirmó correlaciones significativas, especialmente en las fases I y III, con correlaciones débiles a moderadas aún existentes en el análisis de correlación de las componentes de ambas señales obtenidas con la TO, coincidiendo la dirección de respuesta de las señales y sus componentes en general, aunque con diferentes intensidades de registro. Con este hito, aún confirmamos la utilidad del análisis de texturas en el análisis de complejidad en señales FPG, aunque hay menos sensibilidad de esta técnica en las zonas espectrales de frecuencias más bajas en relación

a la entropía multiescala. En el tercer hito, confirmamos la adecuación de las tecnologías de medición no invasivas elegidas y encontramos que el espectro promedio de los perfiles de señal de FLD y FPG tienen diferentes amplitudes, independientemente de los estímulos que los hacen variar, habiendo observado que el FLD parece ser más sensible a los componentes de baja frecuencia, mientras que el FPG parece tener una mayor capacidad de discriminación. Este estudio confirmó la ventaja del uso simultáneo de las dos tecnologías para evaluar de manera complementaria eventos que implican alterar la perfusión micro circulatoria de una manera más integral, ya que las tecnologías ópticas utilizadas interactúan de manera diferente con los tejidos. En el último hito, confirmamos que, en las condiciones experimentales actuales, el masaje implica más que un cambio en la microcirculación local que llamamos Respuesta de Adaptación Hemodinámica Inmediata (o PAHR *Prompt Adaptive Hemodynamic Response*) que impacta toda la hemodinámica cardiocirculatoria sistémica.

Esta respuesta fue abordada de forma complementaria en individuos igualmente sanos, pero mayores, constituyendo el último manuscrito de esta colección, recientemente enviado para publicación. Aquí demostramos que, aunque los tipos de respuestas obtenidas son similares a las obtenidas en participantes jóvenes, esta PAHR es dependiente de la edad y por tanto menos evidente, aunque presente, lo que refuerza el interés por utilizar este potencial indicador en un entorno clínico.

Con base en los resultados obtenidos en estas publicaciones, pudimos fundamentar las siguientes conclusiones principales:

- que el procedimiento experimental que diseñamos demostró ser riguroso, fácilmente reproducible y totalmente adaptado a los propósitos del estudio, garantizando al mismo tiempo la total comodidad y bienestar de los participantes.
- que las tecnologías elegidas para evaluar la microcirculación, a saber, FLD y FPG, resultaron adecuadas para el estudio, ya que los resultados permitieron describir con precisión cuantitativa la microcirculación periférica in vivo, en condiciones dinámicas como las que intervienen en el masaje, habiéndose verificado que el uso de ambas tecnologías proporciona un análisis más sostenido de los fenómenos en cuestión.
- que la maniobra de masaje cuando se aplica a la extremidad inferior de individuos jóvenes y sanos, permite incrementar el flujo micro circulatorio en la extremidad inferior

masajeada, pero también en la extremidad contralateral, este efecto, independientemente de la dirección en la que se aplique el masaje, influyendo en la hemodinámica en general, afectando la frecuencia cardíaca y la presión arterial.

- que esta respuesta hemodinámica al masaje que identificamos en este estudio (PAHR), aunque similar, es claramente dependiente de la edad.

Palabras llave: masaje; cardiovascular; hemodinámica; FLD; FPG; PAHR

Resumo

A massagem é uma técnica manipulativa há muito conhecida, utilizada com múltiplas finalidades associadas principalmente à saúde e ao bem-estar, designadamente na reabilitação e no tratamento e alívio de sintomas associados a múltiplos contextos osteo-mio-articulares e vasculares. Aceite como benéfica em muitas condições de saúde e bem acolhida pelo paciente, recomendada para todos os grupos etários, desde a infância aos indivíduos mais velhos, a demonstração do seu impacto fisiológico e mecanismos envolvidos é ainda muito limitada, porventura até controversa ou inconclusiva. Alguns estudos têm sugerido uma contribuição positiva da massagem na modulação da dor, alívio da fadiga e no controle da ansiedade e do stress, independentemente das patologias de base. Contudo, a utilização de diferentes técnicas e protocolos de massagem, múltiplas e variadas técnicas de registo, e a quase impossibilidade de compararmos resultados, são algumas das dificuldades que justificam estas complexidades.

O nosso trabalho teve por objetivo central, estudar o impacto fisiológico da massagem no membro inferior humano e os mecanismos de adaptação envolvidos, em populações saudáveis, previamente selecionadas e caracterizadas. Para registo de eventos da microcirculação e no sentido de garantir o total conforto e segurança dos participantes, recorreremos a técnicas óticas não invasivas, amplamente testadas, fiáveis e reconhecidas para este fim, como a fluxometria por laser Doppler (FLD) um dos mais reconhecidos “gold-standards” e a fotopletismografia (FPG), que vem sendo usada como meio complementar de quantificação da microcirculação. Adicionalmente, e com o objetivo de estender a capacidade de análise direta destes sinais biológicos, necessariamente complexos pela sua natureza oscilatória e não-estacionária, recorreremos a outras estratégias matemáticas para processamento de sinais. Utilizámos a transformada de Ondeleta (TO), que tem vindo a ser adaptada e utilizada para decomposição destes sinais biológicos com o intuito de melhorar expandir as capacidades de quantificação, passando por contribuir para a normalização de procedimentos, desde logo ao nível da definição do desenho experimental e, mais recentemente a análise de entropia como forma de analisar a complexidade de sinais biológicos.

O presente projeto contou com a colaboração de 98 participantes (32.6 ± 15.5 anos) de ambos os sexos (47 homens e 51 mulheres). Nos artigos aqui descritos, utilizamos um total de quatro grupos, sendo o primeiro de massagem com 32 participantes (19.8 ± 1.5 anos), com 16 homens e 16 mulheres, e no segundo grupo, do procedimento de RVA (Reflexo Veno-Arteriolar), foi de 12 participantes (26.0 ± 5.0 anos), com 5 homens e 7 mulheres. Os dois últimos grupos, com indivíduos mais velhos, foram um de massagem (52.6 ± 6.2 anos), com 11 homens e 11 mulheres, e no último grupo, submetido ao procedimento de RVA (50.3 ± 5.6 anos), com 5 homens e 5 mulheres. Todos os participantes selecionados foram confirmados como sendo normotensos e sem sinais de comprometimento vascular, com índice tornozelo-braço (ITB) > 1.0 . O projeto foi previamente avaliado pela Comissão de Ética institucional (CE 03/2013.12) e todos os procedimentos observaram os princípios da boa prática clínica estabelecidos pela Declaração de Helsínquia e subsequentes emendas. O protocolo de massagem foi avaliado em duas das suas variantes em termos de direção da aplicação: proximal ou ascendente e descendente ou distal. Em todos os casos o procedimento foi aplicado num dos membros inferiores (perna e pé) previamente aleatorizado, servindo o outro membro como controlo. O procedimento experimental de massagem, envolveu três fases de registo contínuo, em supinação, com a Fase I a corresponder ao registo em repouso (*baseline*), durante 10 minutos, a Fase II, de provocação com aplicação de massagem da perna e pé durante 5 minutos e a Fase III, de recuperação durante por 10 minutos. A perfusão sanguínea foi avaliada simultaneamente por FPG (PLUX Biosystems, Portugal) e por FLD (PF 5010, Perimed, Suécia), com as sondas aplicadas em ambos os pés, na superfície plantar dos dedos 1 e 2, respetivamente. A pressão arterial, medida no braço foi igualmente registada nas três fases do protocolo. A aplicação das duas variantes do protocolo, cuja ordem de aplicação foi previamente aleatorizada foi intermediada por um período de *washout* de trinta minutos.

Para “refinamento analítico” dos sinais de FLD, foi também analisado um subgrupo de massagem com 29 voluntários (19.9 ± 1.6 anos, 13 homens e 16 mulheres), conforme descrito acima e com recurso à TO, foram decompostos os sinais de FLD nas suas componentes oscilatórias, para avaliar os potenciais mecanismos envolvidos. Foram considerados os seguintes intervalos de frequência de 0.05 a 2Hz: cardíaca de 0.6 Hz a

2 Hz; respiratória de 0.145 Hz a 0.6 Hz; miogénica de 0.052 Hz a 0.145 Hz; simpática de 0.021 Hz a 0.052 Hz; endotelial dependente de Óxido Nítrico (endoDON) de 0.0095 Hz a 0.021 Hz; e endotelial independente de Óxido Nítrico (endoION) de 0.005 a 0.0095 Hz. Para validação da TO nos sinais de FPG, usamos outra ferramenta de refinamento analítico, a análise de textura (AT) e dados de um grupo com 12 voluntários (26.0 ± 5.0 anos, 5 homens e 7 mulheres), submetidos a dois protocolos, um de massagem conforme descrito acima e também a um protocolo de RVA, em que os participantes realizaram um procedimento de 10 minutos em supina com as pernas estendidas, Fase I (baseline); 10 minutos com um membro pendente a 50 cm em relação ao nível do coração, Fase II (teste); e 10 minutos após retorno à posição inicial, Fase III (recuperação). O membro de teste foi aleatorizado e o contralateral permaneceu imóvel e serviu como controlo. Nesta validação da complexidade do sinal de FPG, analisamos ainda a textura dos escalogramas da TO, usando como comparação a entropia multiescala (EME), procedimento padrão, tendo sido confirmada a utilidade da AT na análise da complexidade em sinais de FPG, pese embora ter sido verificada uma menor sensibilidade desta técnica nas zonas espectrais de menores frequências em relação à entropia multiescala.

Foram usados ainda neste estudo, dois grupos de indivíduos saudáveis, mas mais velhos, sujeitos à aplicação de mesmos protocolos, de massagem e de RVA, para avaliação e comparação das respostas identificadas com os grupos de jovens.

O nosso projeto foi estruturado em quatro marcos, sendo o primeiro a definição do procedimento experimental, o qual permitiu observar de imediato que o impacto da massagem na perfusão se observava em ambos os membros, apesar de aplicada em apenas uma dos membros. O segundo marco, em que pretendemos aprofundar a análise da perfusão, verificamos também que a análise de correlação dos sinais brutos de LDF e FPG confirmou existirem correlações significativas, em especial nas fases I e III, existindo ainda correlações fracas a moderadas na análise de correlação dos componentes de ambos os sinais obtidos com a TO, sendo a direção da resposta dos sinais e das suas componentes em geral coincidentes, embora com distintas intensidades de registo. Com este marco, ainda confirmamos a utilidade da análise de textura na análise da complexidade em sinais de FPG, embora exista uma menor sensibilidade desta técnica nas zonas espectrais de menores frequências em relação à

entropia multiescala. No terceiro marco confirmamos a adequação das tecnologias de medição não invasivas escolhidas e verificamos que o espectro médio dos perfis dos sinais de FLD e de FPG possuem diferentes amplitudes, independentemente dos estímulos que os fazem variar, tendo observado que o FLD parece ser mais sensível para os componentes de baixa frequência, enquanto o FPG parece possuir maior capacidade de discriminação. Este estudo confirmou a vantagem da utilização simultânea das duas tecnologias para avaliar, complementarmente, eventos que envolvam a alteração da perfusão microcirculatória de forma mais abrangente, já que as tecnologias óticas utilizadas interagem de modo diferente com os tecidos. No último marco, confirmámos que, nas presentes condições experimentais, a massagem envolve mais do que uma alteração da microcirculação local que designamos por Resposta Imediata de Adaptação Hemodinâmica (ou PAHR *Prompt Adaptive Hemodynamic Response*) que impacta toda a hemodinâmica cardiocirculatória sistémica.

Esta resposta foi abordada complementarmente em indivíduos igualmente saudáveis, mas mais velhos, constituindo o último manuscrito desta coleção, recentemente submetido para publicação. Aqui demonstramos que, embora os tipos de respostas obtidas sejam semelhantes às obtidas nos participantes jovens, esta PAHR é dependente da idade e por isso menos evidente embora presente, reforçando o interesse da utilização deste potencial indicador em meio clínico.

Com base nos resultados obtidos nestas publicações, pudemos então fundamentar as seguintes principais conclusões:

- que o procedimento experimental que desenhamos, demonstrou ser rigoroso, facilmente reproduzível e totalmente adequado aos propósitos do estudo, assegurando ainda o total conforto, e bem-estar dos participantes.
- que as tecnologias escolhidas para avaliar a microcirculação, nomeadamente a FLD e a FPG, demonstraram ser adequadas ao estudo, pois os resultados permitiram descrever quantitativamente com rigor, a microcirculação periférica *in vivo*, em condições dinâmicas como as envolvidas na massagem, tendo sido verificado que a utilização de ambas as tecnologias, fornece uma análise mais sustentada sobre os fenómenos em causa.

Contribución a un enfoque no convencional de la Función Vascular Periférica
- estudio del impacto fisiológico del masaje en la circulación del miembro inferior

- que a manobra de massagem quando aplicada no membro inferior de indivíduos jovens e saudáveis, permite aumentar o fluxo microcirculatório no membro inferior massajado, mas igualmente no membro contralateral, sendo este efeito, independente do sentido em que é aplicada a massagem, influenciando a hemodinâmica em geral, afetando a frequência cardíaca e a pressão arterial.
- que esta resposta hemodinâmica à massagem que identificamos neste estudo (PAHR), embora seja semelhante, é claramente dependente da idade.

Palavras-chave: massagem; cardiovascular; hemodinâmica; FLD; FPG; PAHR

Abstract

Massage is a long-known technique, applied with your hands for multiple purposes associated to health and well-being, namely for rehabilitation and symptoms relief in osteo-myo-articular and vascular contexts. Recognized as beneficial in many health conditions and well accepted by all age patients, from infants to the elderly, the demonstration of its physiological impact and mechanisms is still limited, and sometimes controversial or inconclusive. Some studies suggested a positive contribution of massage in pain modulation fatigue anxiety relief and stress control regardless of other underlying pathologies. The use of different massage protocols, varied assessment technologies, and the absence of results comparison are some of the difficulties that justify these complexities.

Our main objective was to study the physiological impact of massage in the human lower limb and the involved adaptive mechanisms in previously selected and characterized healthy groups. We used well known reliable non-invasive optical techniques to register microcirculation events with total comfort and safety of participants. Laser Doppler flowmetry (LDF), one of the most recognized gold standards and photoplethysmography (PPG) were our chosen technologies to quantify microcirculation in vivo. In addition, we also used other mathematical strategies for signal processing such as the Wavelet transform (WT), applied to the decomposition of these signals and texture analysis.

The present project involved 98 participants (32.6 ± 15.5 years) of both sexes (47 male and 51 female). In the papers here described, we used four groups of participants. The first one for massage with 32 participants (19.8 ± 1.5 years), with 16 male and 16 female, the second group, for the VAR (Veno-Arteriolar Reflex) procedure, with 12 participants (26.0 ± 5.0 years), 5 male and 7 female; . The last two groups, with older individuals, were one for massage (52.6 ± 6.2 years), with 11 male and 11 female, and a subset of this group submitted to VAR procedure (50.3 ± 5.6 years), involving 5 male and 5 female. All selected participants were confirmed to be normotensive and without signs of vascular impairment, with an ankle brachial index (ABI) > 1.0 . The project was previously approved by the institutional Ethics Committee (CE 03/2013.12) and all procedures followed the principles of good clinical practice established by the Declaration of Helsinki and subsequent amendments. The massage protocol was evaluated in two of

its variants in terms of the direction of application: proximal or ascending and distal or descending. In all cases the procedure was applied to one of the lower limbs (leg and foot) previously randomized, with the other limb serving as control. The experimental massage procedure involved three phases of continuous recording with participants lying down in supination - Phase I corresponding to the resting record (baseline) for 10 minutes, Phase II, provocation with the application of the procedure for 5 minutes and Phase III, recovery for 10 minutes. Blood perfusion was evaluated simultaneously by PPG (PLUX Biosystems, Portugal) and by LDF (PF 5010, Perimed, Sweden), with the probes applied to both feet, on the plantar surface of toes 1 and 2, respectively. Blood pressure, measured in the arm, was also recorded in all phases of the protocol. The application of the two protocol variants, whose application order was previously randomized, was intermediated by a washout period of thirty minutes.

For “analytical refinement” of the LDF signals, a massage subgroup was also analyzed, with 29 volunteers (19.9 ± 1.6 years, 13 male and 16 female), as described above and using the WT, the LDF signals were decomposed into their oscillatory components, to assess the potential mechanisms involved. The following frequency ranges between 0.05 and 2Hz were considered: heart rate from 0.6 Hz to 2 Hz; respiratory from 0.145 Hz to 0.6 Hz; myogenic from 0.052 Hz to 0.145 Hz; sympathetic from 0.021 Hz to 0.052 Hz; endothelial dependent of Nitric Oxide (endoNOD) from 0.0095 Hz to 0.021 Hz; and endothelial independent of Nitric Oxide (endoNOI) from 0.005 to 0.0095 Hz.

To validate WT in PPG signals, another analytical refinement tool was used, texture analysis (TA), with data from a group of 12 volunteers (26.0 ± 5.0 years, 5 male and 7 female), submitted to two protocols, one of massage as described above and also to a VAR protocol, where the participants performed a 10-minute supine procedure with the legs extended, Phase I (baseline); 10 minutes with a pending limb 50 cm in relation to the heart level, Phase II (test); and 10 minutes after returning to the initial position, Phase III (recovery). The test limb was randomized while the contralateral remained immobile serving as control. In this validation of the complexity of the PPG signal, we also analyzed the texture of the WT scalograms, using multiscale entropy (MSE), a standard procedure, as a comparison, and the usefulness of TA in the analysis of the complexity of PPG signals was confirmed, although a lower sensitivity of this technique was found in the spectral zones of lower frequencies in relation to multiscale entropy.

In this study, two groups of healthy, but older individuals were also used, subjected to the application of the same protocols, massage and VAR, to evaluate and compare the responses identified with the groups of young individuals.

Our project was structured in four milestones, the first being the definition of the experimental procedure, which allowed us to immediately observe that the perfusion impact of massage was observed in both limbs, despite being applied to only one of the limbs. The second milestone, in which we intended to deepen the data analysis we confirmed by correlation analysis of raw LDF and PPG signals the existence of significant proximity of these signals in phases I and III. Weak to moderate correlations still existing in the correlation analysis of the components of both signals obtained with WT, with the direction of the response of the signals and their components in general coincidental, although with different recording intensities. We also confirm the usefulness of texture analysis in the analysis of PPG signals complexity, although there is less sensitivity of this technique in the lower frequency spectral zones in relation to multiscale entropy. In the third milestone, we confirm the adequacy of the chosen non-invasive measurement technologies and we verify that the average spectrum of the LDF and PPG signal profiles have different amplitudes, regardless of the stimuli that make them vary, having observed that the LDF seems to be more sensitive to the low frequency components, while the PPG seems to have a greater capacity for discrimination. This study confirmed the advantage of the simultaneous use of the two technologies to complementarily assess events that involve altering the microcirculatory perfusion in a more comprehensive way, since the used optical technologies interact differently with the tissues.

In the last milestone, we confirmed that in the present experimental conditions, massage involves more than a change in the local microcirculation that we named as the Prompt Adaptive Hemodynamic Response (PAHR) impacting the entire systemic cardiocirculatory hemodynamics. This answer was addressed in a complementary way in equally healthy individuals, but older, constituting the last manuscript of this collection, recently submitted for publication. Here we demonstrate that, although responses here obtained are similar to those obtained in young participants, this PAHR

is age dependent and therefore less obvious, suggesting its potential interest to be used as a clinical indicator.

Based on the results obtained in these four milestones, we were able to substantiate the following main conclusions:

- that the experimental procedure we designed, proved to be rigorous, easily reproducible and totally adequate to the purposes of the study, while also ensuring the total comfort and well-being of the participants.
- that the technologies chosen to evaluate microcirculation, namely LDF and PPG, proved to be suitable for the study, as the results allowed to quantitatively and accurately describe the peripheral microcirculation in vivo, under dynamic conditions such as those involved in massage, having been verified that the use of both technologies provides a more sustained analysis of the phenomena in question.
- that the massage maneuver when applied to the lower limb of young and healthy individuals, allows to increase the microcirculatory flow in the massaged lower limb, but also in the contralateral limb, being this effect, regardless of the direction in which the massage is applied, influencing the hemodynamics in general, affecting heart rate and blood pressure.
- that this hemodynamic response to massage that we identified in this study (PAHR), although similar, is clearly dependent on age.

Keywords: massage; cardiovascular; hemodynamic; LDF; PPG; PAHR

Lista de abreviaturas

AT – Análise de Textura (TA – Texture Analysis)

DVP – Doença vascular periférica (PVD – Peripheral Vascular Disease)

EndoDON – endotelial dependente de Óxido nítrico (EndoDON - endothelial NO-dependent)

EndoION – endotelial independente de Óxido nítrico (EndoNOI - endothelial NO-independent)

EME – Entropia multiescala (MSE - Multiscale Entropy)

FLD – Fluxometria de laser Doppler (LDF - Laser Doppler flowmetry)

FPG – Fotopletismografia (PPG - Photoplethysmography)

IMC – Índice de massa corporal (BMI - Body mass index)

ITB – Índice tornozelo-braço (ABI - Ankle brachial index)

ON – Óxido nítrico (NO - Nitric Oxide)

OMS – Organização Mundial de Saúde (WHO – World Health Organization)

PAHR – (Resposta Imediata de Adaptação Hemodinâmica) Prompt Adaptive Hemodynamic Response

RVA – Reflexo Veno-Arteriolar (VAR – Veno Arterial Reflex)

TRF – Transformada rápida de Fourier (FFT - *Fast Fourier transform*)

TIVI – (Sistema de Imagem de viabilidade de tecidos) *Tissue viability imager*

TO – Transformada de ondeleta (WT - Wavelet transform)

UA – Unidades arbitrárias (AU – Arbitrary Units)

Capítulo I: Enquadramento e Hipóteses

Enquadramento

As doenças cardiovasculares, de acordo com dados do último relatório da OMS [1,2], representam a principal causa de morte prematura a nível mundial, uma posição consistentemente reforçada na última década. Nestas a disfunção vascular representa já a terceira causa de acidente cérebro-vascular [1,2].

Em Portugal, um estudo recente sobre a prevalência da doença arterial periférica, revelou que a doença aterosclerótica oclusiva do membro inferior tem uma prevalência de 3-10% na população em geral, que sobe para 15-20% numa população com mais de 70 anos [3,4]. Este crescimento parece estar associado ao estilo de vida mais sedentário e ao aumento da esperança de vida da população nas sociedades mais desenvolvidas em que Portugal se enquadra. A doença vascular periférica (DVP), sendo uma patologia multifatorial, com grande variabilidade de expressão clínica, está associada a uma disfunção da unidade microcirculatória que reduz progressivamente a perfusão, não apenas nas regiões mais distais do nosso corpo, mas sobretudo nas regiões especialmente sensíveis de maior fluxo. Na sua origem identificamos vários determinantes, como a profissão, a alimentação e estilos de vida desadequados. O desconforto, a dor, o cansaço, a sensação de pernas pesadas, prenunciam o aparecimento de lesões varicosas que posteriormente, podem evoluir para acidente vascular mais superficiais (tromboflebites) comprometendo gradualmente a qualidade de vida dos pacientes [5-13], ou para fenómenos mais graves e potencialmente mais

perigosos como acontece com as flebotromboses, fonte frequente de tromboembolismo pulmonar e de morte [14].

A aterosclerose, é um dos principais fatores causadores da DVP e surge associada a múltiplos fatores de risco, incluindo hábitos de vida (consumo excessivo de tabaco e álcool) e outras co-morbilidades (obesidade, diabetes) [6,8]. O mecanismo deste processo é complexo e multifactorial, resultando na redução do lúmen dos vasos afetados, o que significa uma diminuição do aporte de nutrientes aos tecidos, o que origina múltiplas perturbações sobretudo nos territórios mais sensíveis (alto fluxo / baixa resistência) como é o caso do sistema nervoso central, retina, rim [5,10,11] e claro as regiões mais distais dos membros inferiores [5,6].

As reconhecidas dificuldades de controle terapêutico destas complexas condições fisiopatológicas têm promovido a procura de formas alternativas de minorar alguns dos sintomas associados à DVP. Entre estas salientam-se as técnicas manipulativas incluídas nas chamadas terapêuticas não-convencionais, algumas de uso milenar e profundamente enraizadas na maioria das culturas um pouco por todo o mundo, como acontece com a massagem.

O termo “massagem” genericamente utilizado para designar estes procedimentos manipulativos, consiste na mobilização (com as mãos) dos tecidos moles do corpo humano efetuada por um terapeuta especializado. A sua prática não envolve riscos relevantes nem efeitos secundários nocivos, pelo que a sua utilização é muito bem aceite, associada ainda a uma sensação de bem-estar e relaxamento [15-17]. Na verdade, a sua utilização como técnica complementar de múltiplas terapêuticas tem-se expandido na atualidade [15,18], mesmo nas sociedades mais conservadoras e em indivíduos muito jovens (prematturos, recém-nascidos e crianças) [19,20].

Uma das técnicas mais populares é a *effleurage* uma variante da massagem Sueca aplicada com inúmeras finalidades, desde o relaxamento [15], ao tratamento estético, alívio de dor [20], recuperação muscular [18,21,22], melhoria de fluxo sanguíneo [23,25], ou em contextos mais específicos como na recuperação de prematuros [19]. Tal significa uma boa experiência de utilização e de obtenção de resultados que, em grande parte, não são acompanhados senão por esta evidência clínica onde a explicação de base científica está muitas vezes ausente. Este contexto proporciona uma oportunidade interessante para investigar estas evidências clínicas, de forma a compreender o impacto do procedimento e assim melhor suportar a prática e garantir a eficácia da intervenção [27,29,30]. A DVP constitui uma das principais áreas de intervenção da massagem, desde as formas mais leves/moderadas como a síndrome das “pernas pesadas” até às formas mais obvias de insuficiência vascular [5,6]. Contudo, o impacto desse procedimento no sistema cardiovascular está longe de ser esclarecido, apesar das múltiplas revisões que sobre este tema têm sido publicadas nos últimos anos [9-13] evidenciando mesmo alguma controvérsia e até oposição sobre a natureza dos efeitos encontrados [22,31]. A generalidade dos estudos sugere que a massagem facilita a perfusão local promovendo a diminuição da pressão arterial e da frequência cardíaca [22,28]. No entanto, a variedade de procedimentos, os procedimentos experimentais e os meios de quantificação utilizados são de tal forma diversificados que a comparação de resultados se torna uma tarefa impossível de conseguir.

Neste contexto o presente estudo está sustentado num desenho experimental sólido e consistente, centrado num estudo clínico complexo, considerando em primeiro lugar o superior interesse e proteção do participante. Conduzido de acordo com princípios de Boas Práticas Clínicas e princípios bioéticos universais - a Declaração Universal sobre

Bioética e Direitos Humanos da UNESCO (19/10/2005); A Carta dos Direitos Fundamentais da UE, 2000; Princípios éticos para a investigação médica envolvendo sujeitos humanos - Declaração de Helsínquia e consecutivas emendas, as diretrizes de Boas Práticas Clínicas e a Diretiva da UE sobre Ensaios Clínicos, tendo ainda em atenção a legislação nacional "Lei nº 12/2005", "Decreto-Lei nº 131/2014" ao Regulamento Geral de Proteção de Dados (UE) 2016/679 do Parlamento Europeu e do Conselho [32-35]. Neste contexto, assumimos utilizar a massagem Sueca por ser a mais conhecida e utilizada nos países ocidentais para uma enorme variedade de condições [18,28,29,37-39]. Esta massagem envolve diversas abordagens e combinações – a *effleurage*, mais superficial, ao longo do eixo principal com pressão constante da mão para relaxar os tecidos; a *petrissage*, envolvendo amassar e elevar para atingir tecidos mais profundos; o *tapotement*, um procedimento destinado a revigorar; a fricção, exercendo maior pressão e, a vibração que se espera poder preparar os músculos para uma atividade e desempenho mais intensos [18,22,25,45]. Para a avaliação do impacto deste procedimento sobre a hemodinâmica, escolhemos tecnologias que correspondem ao estado da arte das tecnologias de base ótica, não-invasivas e de fácil acesso e, por isso, das mais conhecidas e utilizadas desde a investigação aplicada à prática clínica, e cuja experiência de utilização nos permite melhor explorar as suas potencialidades [46-50]. Nestas incluímos a fluxometria por laser Doppler (FLD) considerada um *golde standard* [51-55], e a fotopletismografia de reflexão (FPG), uma outra técnica, que também tem vindo mais recentemente a conquistar espaço na avaliação da microcirculação [55-59].

Hipóteses

Considerando o enquadramento antes descrito, entendemos ser possível quantificar com rigor a perfusão vascular periférica sob o efeito de massagem, em condições tão próximas quanto possível do estado fisiológico normal.

Para tal necessitaremos de:

1. Escolher e demonstrar a adequabilidade das nossas escolhas no que respeita:
 - Às tecnologias não invasivas selecionadas para quantificação da perfusão
 - À definição do desenho experimental que garanta a reprodutibilidade e ao mesmo tempo a exequibilidade do estudo, tendo em consideração o conforto e bem-estar dos participantes em experimentação, bem como a manutenção da homeostase circulatória
2. Assumir que a manobra de massagem quando aplicada no membro inferior tem impacto na perfusão local e provavelmente na hemodinâmica em geral

Resultam assim como questões de investigação principais:

- I. Como avaliar, o mais próximo possível do estado fisiológico normal, o impacto hemodinâmico da massagem no membro inferior humano?
- II. De que modo a massagem localizada no membro inferior afeta a fisiologia vascular e microcirculatória? e quais os mecanismos potencialmente envolvidos?

Capítulo II: Objetivos

De acordo com os pontos anteriores e, sendo a massagem a técnica manipulativa mais utilizada no contexto das terapêuticas não-convencionais, com incidência na circulação vascular periférica [17,18,23], pretendemos contribuir para o estudo do impacto fisiológico da massagem no membro inferior humano e os mecanismos de adaptação envolvidos, focando-nos em populações saudáveis, previamente selecionadas e caracterizadas. Sendo certo que muitas revisões têm sido produzidas acerca dos fundamentos e conceitos base, definições e caracterização geral de muitas destas técnicas [26-30], muito existe ainda por definir para que se consiga uma normalização de procedimentos que permitam a comparação e análise rigorosa de dados.

Resulta assim que, para além da identificação da resposta e obtenção de evidências dos efeitos e mecanismos fisiológicos envolvidos na massagem, pretendemos contribuir para a normalização de procedimentos, desde logo ao nível da definição do desenho experimental, envolvendo diferentes aplicações da técnica de manipulação e diferentes tecnologias de abordagem. Estes dados serão analisados em detalhe, utilizando diversas estratégias de análise quantitativa. O protocolo experimental assim proposto, será aplicado em pacientes humanos saudáveis, de diferente idade e sexo, sem patologia clinicamente discernível (é expectável que os indivíduos mais velhos, apresentem já alteração da função vascular resultante do próprio processo de envelhecimento). Esta abordagem deverá ainda permitir obter indicações sobre o impacto da massagem na melhoria das condições e qualidade de vida dos pacientes em geral, bem como sobre o seu interesse na promoção e na prevenção da saúde vascular, de forma a demonstrar o interesse da exploração clínica desta metodologia.

Para tal, propusemo-nos percorrer o trajeto secuenciado nos seguintes marcos (*milestones*):

Marco 1 - Definição do procedimento experimental para quantificação da resposta fisiológica associada à massagem *in vivo*.

A inexistência de procedimentos experimentais previamente testados e padronizados determinou como prioridade a definição do procedimento experimental envolvendo a massagem a aplicar *in vivo*, de modo a assegurar a sua reprodutibilidade e rigor que, de acordo com as dificuldades antes apontadas, nos permitisse obter resultados comparáveis em idênticas circunstâncias experimentais, tão próximas quanto possível do estado fisiológico normal (modelo fisiológico). O protocolo original que desenhámos com registos contínuos, a) antes da massagem (*baseline*), b) durante a massagem num membro aleatorizado (servindo o outro como controlo) e c) depois da massagem (fase de recuperação), foi pensado para proporcionar uma visão dinâmica, tão detalhada quanto possível, sobre todo o processo de adaptação ao procedimento, e ao mesmo tempo, permitindo registar as variáveis hemodinâmicas que classificámos como representativas das alterações em análise.

Quanto à quantificação da resposta fisiológica associada à massagem no membro inferior, utilizámos como pressuposto tecnológico, a utilização de técnicas não invasivas, amplamente testadas e reconhecidas. As tecnologias de base ótica que escolhemos gozam de ampla demonstração da sua aplicabilidade para este tipo de avaliação, podendo ser diretamente aplicadas na pele *in vivo*, um órgão especialmente adequado para o estudo da microcirculação [46]. A fluxometria por laser Doppler (FLD) é a

tecnologia de referência [51-55], o *golde standard* e, por isso, a nossa primeira escolha. Contudo, conhecida a instabilidade deste tipo de sinais, não-estacionários e pulsáteis, entendemos utilizar simultaneamente uma outra tecnologia, também ela ótica e não invasiva de há muito conhecida e recentemente melhorada com a mais moderna eletrónica – a fotopletismografia (FPG) [55-59]. Ambas as tecnologias foram comparadas de modo a garantir a adequabilidade da sua escolha como tecnologias de referência para a obtenção destes objetivos.

Neste enquadramento geral foi possível evidenciar um dos dados experimentais tão relevante quanto inesperado – o impacto da massagem na perfusão de ambos os membros, apesar de aplicada em apenas um dos membros. Apesar de se tratar de um achado cuja origem e significado fisiológico teriam que ser analisados em passos subsequentes, trata-se desde já de um achado que merece especial referência neste marco. Os resultados, incluindo a definição do protocolo-base, encontram-se relatados no:

Artigo I – “About the in vivo discriminatory capacity of photoplethysmography versus laser Doppler flowmetry”

Marco 2 - Levando mais longe a análise de perfusão em função da massagem.

Os dados de perfusão obtidos através destes métodos de base ótica (FLD e FPG) revelam sinais complexos, tipicamente pulsáteis e não-estacionários em resultado da contribuição dos múltiplos fatores que asseguram a hemodinâmica vascular e o seu controle. Estes envolvem como elementos centrais da função “hidráulica” o bombeamento cardíaco e a respiração, a resistência dos vasos periféricos resultante da atividade miogénica e endotelial da sua parede e a atividade autonómica (simpática) que aqui se faz sentir. A circulação nas unidades mais pequenas de cada tecido e território – a microcirculação, resulta da articulação em cada momento de todos estes fatores produzidos pela permanente interação entre sensores e efetores centrais e locais.

As tecnologias que escolhemos na origem do nosso projeto possuem uma base biofísica semelhante e utilizam luz com diferentes comprimentos de onda, a referência, FLD e mais utilizada e a solução mais moderna, FPG, cada vez mais utilizada pela melhor qualidade de sinal adquirido face à pletismografia original (menos ruído graças à separação das fontes de alimentação de aquisição e de transmissão, também pela ausência de cabos, custo mais baixo, entre outras). Apesar das similitudes, foi essencial confirmar a adequabilidade desta escolha. Os nossos resultados permitiram evidenciar que ambas as tecnologias foram capazes de detetar o mesmo tipo de fenómeno, mostrando o mesmo impacto da massagem em ambos os membros, apesar de aplicada apenas num dos lados aleatoriamente escolhido.

Neste trabalho comparámos ainda o mesmo procedimento de massagem orientado em direções opostas (proximal e distal) de forma a perceber a importância deste condicionante por vezes referido como tal em literatura mais especializada. Os resultados permitiram confirmar que o FLD e FPG são tecnologias adequadas para descrever quantitativamente a microcirculação periférica *in vivo* em condições dinâmicas como as envolvidas na massagem, aparentemente mais evidentes com o procedimento proximal (do pé para o joelho), para além de ficar demonstrado que a utilização de ambas as tecnologias, fornece uma análise mais sustentada sobre os fenómenos em causa.

As semelhanças conceptuais destas duas tecnologias originam padrões de registo semelhantes, pulsáteis e não-contínuos, mas de difícil leitura e interpretação. Como antes se refere, esta complexidade resulta da contribuição simultânea do coração da respiração e das atividades simpática, miogénica e endoteliais (dependente de NO e independente de NO) na parede vascular. Estes diferentes componentes fazem parte do espectro das ondas de perfusão obtidas por FLD e podem ser detetados numa gama de frequências entre 0,05 e 2Hz [62]. Para o processamento deste tipo de sinais microcirculatórios e identificação destes componentes parece ser especialmente adequada a Transformada de Ondeleta (TO ou Transformada Wavelet de Morlet, WT), [61-66] oferecendo bons níveis resolução, sobretudo quando comparada com a Transformada de Fourier, pois é reconhecida como podendo preservar melhor a relação tempo/frequência, que é essencial face a fenómenos que variam em frequências muito baixas e ao longo do tempo, como no presente caso.

Resulta assim de forma clara, que esta análise de componentes do sinal de perfusão obtido com FLD encerra um enorme potencial no que respeita à contribuição de cada um daqueles componentes para a resposta registada em cada contexto experimental. Entendemos assim explorar também esta análise, uma vez que estes componentes oscilatórios podem proporcionar uma visão mais detalhada sobre os mecanismos fisiológicos em curso.

Efetivamente, a análise de TO, nas suas componentes que se reconhecem estar relacionadas com a atividade de diferentes órgãos e tecidos [63], da perfusão obtida do sinal de FLD durante a massagem, mostrou o aumento dos componentes cardíaco e respiratório, a par do aumento da atividade miogénica. As componentes, simpática e endoteliais (dependente de ON e independente de ON) diminuíram durante a fase de aplicação de massagem.

Estendemos então esta estratégia de análise aos sinais de FPG, conscientes de se tratar de um exercício exploratório já que são raros os trabalhos publicados sobre este tema [66]. Nestas condições encontramos resultados de análise de evolução dos componentes FLD e FPG sobreponíveis, identificados no mesmo intervalo de frequências conhecidas para a FLD (cardíaca 0.6 Hz a 2 Hz; respiratória 0.145 Hz a 0.6 Hz; miogénica 0.052 Hz a 0.145 Hz; simpática 0.021 Hz a 0.052 Hz; endoDON 0.0095 Hz a 0.021 Hz; e endoION 0.005 a 0.0095 Hz) embora mais evidentes com o sinal de FLD.

A análise de correlação dos sinais brutos de FLD e FPG confirmou estarem significativamente correlacionados, em especial nas fases I e III, enquanto, a análise de correlação dos componentes de ambos os sinais obtidos com a TO, mostrou correlações fracas a moderadas. Desta forma confirmámos que, nestas condições experimentais, a

direção da resposta dos sinais e das suas componentes são em geral coincidentes, embora com distintas intensidades de registo, em especial na fase III.

Adicionalmente, sabendo que existem outras formas de exploração deste tipo de sinais (FLD e FPG) que recolhemos e com o intuito de preparar um método para utilização neste tipo de estudos, avançamos na aplicação de duas ferramentas analíticas para validação e teste dos resultados da aplicação da transformada de Ondeleta aos sinais de fotopletismografia. As ferramentas aplicadas neste estudo foram a análise de textura e a entropia multiescala para validação da respetiva transformada para os sinais de FPG.

Os resultados demonstraram que a análise de textura permitiu detetar uma diminuição na complexidade, que pode ser interpretada como um aumento da entropia da textura dos escalogramas obtidos do sinal de FPG durante a manobra realizada, o que sugere uma diminuição da competência dos mecanismos de regulação vascular e representa uma alteração semelhante à observada com a ferramenta de referência para este tipo de análise.

Com este estudo, foi então confirmada a utilidade da análise de textura na análise da complexidade em sinais de FPG, pese embora ter sido verificada uma menor sensibilidade desta técnica nas zonas espectrais de menores frequências em relação à entropia multiescala.

Em qualquer caso, estes estudos demonstram a vantagem da utilização simultânea das duas tecnologias e o interesse da análise de componentes destes sinais através da TO e de outras ferramentas analíticas como a análise de textura, proporcionando uma visão mecanística dos eventos em estudo.

Contribución a un enfoque no convencional de la Función Vascular Periférica
- estudio del impacto fisiológico del masaje en la circulación del miembro inferior

A demonstração da adequabilidade da utilização destas tecnologias bem como da respetiva análise de dados, encontram-se detalhadamente relatados nos:

Artigo II – “Comparing the effects of the human hind limb massage by analysis of Laser Doppler Flowmetry and Photoplethysmography signal components with the wavelet transform”

Artigo III – “Texture Analysis is a Useful Tool to Assess the Complexity Profile of Microcirculatory Blood Flow”

Marco 3 - Adequação da escolha das tecnologias de medição aos propósitos do estudo.

Tendo sido demonstrada a importância da análise espectral dos componentes dos sinais de perfusão obtidos com o FLD e com o FPG, dum ponto de vista mecanístico, quisemos avaliar as características oscilatórias de cada um destes sinais uma vez que a FPG tem sido frequentemente referido como um substituto “low-cost” da FLD. Para comparar os padrões de registo associados a estes sinais de perfusão, também conhecidos por “*flowmotion*”, obtidos por FLD e por FPG nas mesmas condições experimentais, escolhemos um estímulo capaz de aumentar a perfusão distal – a massagem, e outro conhecido por diminuir a perfusão – a hiperóxia. Como antes, os sinais de FLD e de FPG foram seguidos em todas as fases das adaptações promovidas por estes estímulos, e analisados por TO.

Os resultados obtidos nestas condições demonstraram, mais uma vez, que ambas as tecnologias detetaram as alterações de perfusão esperadas em ambos os membros, confirmando que FLD e FPG medem de facto a perfusão, embora com diferentes padrões de movimento, sugerindo que, provavelmente, medem a diferentes profundidades. Efetivamente, através da análise de TO, confirmámos que os componentes espectrais de FLD e FPG se sobrepõem nos intervalos cardíaco (1.7–0.7 Hz), respiratório (0.7–0.2 Hz), miogénico (0.2–0.05 Hz), simpático (0.05–0.027 Hz), endotelial dependente de ON (0.027–0.013 Hz), e endotelial independente de ON (0.013–0.0064 Hz), contudo, não conseguimos encontrar correlações fortes entre os componentes de FLD e de FPG. Numa análise mais detalhada, encontrámos para a massagem, correlações positivas apenas para os componentes de maior frequência (cardíaco, respiratório e miogénico), enquanto para no protocolo de hiperóxia, foram

encontradas correlações positivas apenas nos componentes endoteliais de mais baixa frequência. Estas observações sugerem que os espectros das componentes do sinal, são diferentes, o que significa distintas interações luz - tecido para as duas tecnologias, em termos de penetração, sensibilidade e reflexão. Deveremos ter em consideração que a própria compressão dos tecidos pode promover a penetração da luz, nomeadamente com a FPG.

Igualmente importante, o espectro médio de todos os participantes, demonstrou que os perfis de FLD e de FPG possuem diferentes amplitudes, independentemente dos estímulos que os fazem variar, o que nos levou a comparar a sensibilidade, a especificidade e a reprodutibilidade de ambas as tecnologias, permitindo concluir que o FLD parece ser mais sensível especialmente para os componentes de baixa frequência, enquanto o FPG parece possuir maior capacidade de discriminação.

Este estudo demonstra em qualquer caso, a vantagem da utilização simultânea das duas tecnologias para avaliar, complementarmente, eventos que envolvam a alteração da perfusão microcirculatória. Estes dados, bem como a sua análise, encontram-se detalhadamente relatados no artigo seguinte:

Artigo IV – “Different lasers reveal different skin microcirculatory flowmotion – data from the wavelet transform analysis of human hindlimb perfusion”

Marco 4 - Estudo da dinâmica microcirculatória na resposta a uma massagem do membro inferior.

Por fim, com o objetivo de caracterizar o impacto da massagem do membro inferior sobre a perfusão local e sobre a dinâmica do sangue no sistema cardiovascular, desenhamos um estudo envolvendo 32 participantes jovens, de ambos os sexos (16 homens e 16 mulheres), saudáveis. Aplicámos a este grupo de estudo as metodologias (experimental e analítica) até aqui desenvolvidas, incluindo duas variações da técnica de massagem (centrípetas ou proximais e centrífugas ou distais) até aqui usada.

A razão para o estudo destas variações na direção da aplicação da massagem, prende-se com a prática clínica da massagem ter sido empregue e aperfeiçoada de forma empírica ao longo dos tempos, sendo recomendada a sua aplicação no sentido ascendente ou proximal, não tendo sido efetivamente demonstrado de forma objetiva, qual o seu impacto na microcirculação dos membros inferiores, nem noutras aplicações em que tem sido usada.

Os efeitos descritos da massagem sobre a função cardiovascular não são consensuais, com resultados muitas vezes inconsistentes e até contraditórios [24,29]. Estão identificados a bradicardia, a bradipneia e a diminuição da pressão arterial [21,22,28]. Contudo, a diversidade de protocolos experimentais, variáveis e meios de medição tem impedido a comparação objetiva de resultados. Nas presentes condições experimentais, em que a massagem se aplica exclusivamente em um dos membros inferiores, em ambas as direções (alternadamente, após um período de *wash-out*), medindo ambos os membros (o membro massajado e o membro contralateral, não massajado) observámos, consistentemente, que a massagem evoca um aumento da perfusão no

membro massajado, assim como, semelhante resposta no membro contralateral (não massajado) embora menos intensa. A diminuição da pressão arterial e da frequência cardíaca estão também consistentemente presentes.

Estas respostas haviam já sido identificadas e publicadas pelo nosso grupo de investigação, conforme descrito, sendo igualmente detetadas com as duas técnicas usadas neste estudo (FLD e FPG).

Constatamos que este tipo de manobra, embora superficial, é capaz de promover uma resposta hemodinâmica nos dois membros, que deverá resultar de um mecanismo adaptativo neuro-endócrino envolvendo componentes centrais e periféricos. De resto, este tipo de mecanismo de adaptação hemodinâmica foi igualmente detetado em outros estudos do nosso grupo. Com humanos, no estudo da resposta hemodinâmica à hiperóxia [47,66], com o estudo do reflexo venoarteriolar (VA) [50,66,67], ou mais recentemente, no estudo do impacto do exercício isométrico em apoio unipodal [68]. Este mecanismo foi também observado no rato, durante o processo de neoangiogénese que se sucede à isquemia, cirurgicamente induzida, do membro posterior (*hind-limb ischemia*) [69,70]. Todas estas intervenções provocam mudanças agudas na microcirculação regional as quais parecem ativar o mesmo tipo de integração cardiovascular. A rede somato-sensorial responsável deve, provavelmente, envolver diferentes vias aferentes, mas evoca o mesmo tipo de resposta adaptativa hemodinâmica para restaurar a homeostasia circulatória geral e, por isso mesmo, é também detetável no membro contralateral.

A comparação dos resultados obtidos com as duas variantes da massagem no sentido ascendente (proximal) e no sentido descendente (distal), não permitiu encontrar

diferenças significativas, sugerindo que não serão diferentes entre si. Os nossos resultados sugerem que o sentido da massagem não diferencia ou altera a resposta fisiológica. Contudo as respostas à massagem ascendente parecem provocar registos mais evidentes, pelo que passámos a utilizar os resultados desta variante na análise ulterior dos nossos resultados.

A análise de componentes do sinal de FLD realizado com a TO (*flowmotion*) demonstrou que a massagem evoca aumentos substanciais nos componentes cardíacos, respiratórios e miogénicos e uma redução dos componentes simpáticos e endoteliais, especialmente no membro massajado. Alterações semelhantes, mas menos evidentes, são detetadas no membro contralateral e não massajado.

Este estudo confirmou ainda, a presença de diferenças de perfusão entre sexos, com valores mais elevados nos homens, detetados com as duas tecnologias (FLD e FPG). Estas diferenças estão descritas na literatura e notam-se em especial em repouso, parecendo desaparecer na resposta à massagem. Estes dados estão devidamente reunidos e analisados no artigo seguinte:

Artigo V – “Lower limb massage in human increases local perfusion and impacts systemic hemodynamics”

A caracterização desta PAHR tornou-se assim uma direção de investigação necessária, a fim de melhor a caracterizar e estabelecer a sua aplicabilidade, especialmente como indicador com potencial clínico. Desta forma, procurámos perceber qual o impacto do envelhecimento sobre a PARH, reproduzindo experiências antes realizadas em participantes mais jovens, num painel de indivíduos mais velhos. Utilizámos como

“modificadores” a massagem (proximal) do membro inferior e o VAR que, como antes confirmámos, produzem efeitos opostos sobre a perfusão medida em ambos os pés. Conforme esperado, o perfil PAHR que obtivemos, foi em tudo semelhante ao obtido com o grupo de participantes mais jovens, mas claramente menos expressivo, para os dois protocolos de massagem e VAR. Nestes, a massagem, constituindo o “modificador” mais ligeiro e fugaz, apenas provocou aumentos significativos da perfusão no membro massajado. Ao contrário do VAR, que provocou uma redução significativa da perfusão em ambos os membros inferiores. Esta dependência da idade do PARH parece significar uma estimulação sensorial aferente, com integração e respostas efetoras mais reduzidas do que nos indivíduos mais jovens, e que poderão ser mais expressivas nem caso de doença. Estas notas aqui resumidas, encontram-se relatados no:

Artigo VI – “The modification of skin microcirculatory perfusion evokes a Prompt Adaptive Hemodynamic Response – perspective as an endpoint to investigate integrative cardiovascular (dis)function in vivo”

Conseguimos assim comprovar que a massagem, nas presentes condições experimentais, envolve mais do que uma alteração local da microcirculação, evocando uma Resposta Imediata de Adaptação Hemodinâmica (ou PAHR - *Prompt Adaptive Hemodynamic Response*) que impacta toda a homeostase cardiocirculatória. Esta resposta, (a) não é exclusiva do membro inferior (dados não publicados) (b) não depende da variante de massagem aplicada, nem (c) do sexo, sendo ainda (d) influenciada pela idade (resultados não publicados) e (e) e expectavelmente modificada por patologia (doença vascular periférica, por exemplo), justificando a exploração da sua potencial utilização para fins de diagnóstico clínico.

Capítulo III: Bibliografia

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Capítulo IV: Publicações e Resultados

Apresenta-se de seguida a compilação dos artigos publicados no âmbito do Projeto de Investigação.

Artigo I - About the in vivo discriminatory capacity of photoplethysmography versus laser Doppler flowmetry

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About the *in vivo* discriminatory capacity of photoplethysmography versus laser Doppler flowmetry

Acerca da capacidade discriminatória in vivo da fotopletismografia versus fluxometria por laser Doppler

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Abstract

Peripheral vascular disease (PVD) comprises many pathological entities, and a variety of clinical expressions, being the 3rd most reported cause of cardiovascular disease. PVD continues to motivate more and better research to understand the many complexities involved, and to develop more effective preventive measures and therapeutics. Photoplethysmography (PPG) is a classic non-invasive technique used to explore *in vivo* circulation, progressively replaced by laser Doppler flowmetry (LDF). Recent advances enable the development of a new PPG system that seems to clearly improve the applicability of this technique. In this study, we apply and compare these two non-invasive measurement techniques after a massage manoeuvre in the human leg. This exploratory study involved seven volunteers, both sexes (19.6 ± 1.3 years old). Flow measurements took place in both limbs before (I), during (II) and after the massage manoeuvre (III). Other variables, including pulse and respiratory rates and blood pressure (BP), were also quantified. Results show that the simultaneous use of LDF and PPG enables the detection of discrete changes in blood flow that could not be detected with a single technique. Furthermore, it also shows that, under these conditions, the massage improves the lower limb perfusion in both limbs (test and control).

Keywords: Microcirculation; LDF; PPG; massage; vascular regulation

Resumo

A doença vascular periférica (DVP) compreende muitas entidades patológicas e uma variedade de expressões clínicas, sendo a terceira causa principal da doença cardiovascular. A DVP ainda continua a motivar mais e melhor investigação para compreender as muitas complexidades envolvidas, e desenvolver medidas preventivas e terapêuticas mais eficazes. A fotopletismografia (FPG) é uma técnica clássica não invasiva utilizada para explorar a circulação *in vivo*, que tem vindo a ser progressivamente substituída pela fluxometria por laser Doppler (FLD). Avanços recentes permitiram o desenvolvimento de um novo sistema FPG que parece melhorar claramente a aplicabilidade desta técnica. Neste estudo, aplicamos e comparamos estas duas técnicas de medição não-invasiva após uma manobra de massagem no membro inferior humano. Este estudo exploratório envolveu sete voluntários de ambos sexos ($19,6 \pm 1,3$ anos). As medições de fluxo foram feitas em ambos os membros antes da manobra (I), durante a massagem (II) e após a recuperação (III). Outras variáveis, incluindo as frequências de pulso e respiratória, e pressão arterial (PA) também foram quantificadas. Os resultados mostram que o uso simultâneo de FLD e FPG permite detetar alterações discretas no fluxo sanguíneo que não poderíamos detetar com apenas uma das técnicas. Além disso, também mostram que, nestas condições, a massagem melhora a perfusão do membro inferior em ambos os membros (teste e controlo).

Palavras-chave: Microcirculação; FLD; FPG; massagem; regulação vascular

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Introduction

Peripheral vascular disease (PVD) comprises many pathological entities and involves a variety of clinical expressions, from heavy legs syndrome to muscular and osteoarticular inflammatory involvement and ulcers^[1,2]. It is considered a significant public health problem, and one which may lead to poor quality of life^[2]. In Portugal, the PVD prevalence is estimated to reach 5.9% of the population, mostly affecting the elderly^[3]. Its significant expression as the 3rd most reported cause of cardiovascular disease continues to motivate more and better research on the subject in order to understand the involved complexities and develop more effective preventive measures and therapeutics.

The skin is known to be an adequate location to address a wide range of functional aspects of microcirculation^[4,5] and, for that reason, laser doppler flowmetry (LDF) and photoplethysmography (PPG) became well-known, non-invasive techniques to quantify flow variables in conditions as closely as possible from the normal physiological state^[4,6-9].

LDF has gained great popularity in the last decades as a non-invasive assessment tool in vascular research and medicine. It has been explored as a useful tool in basic research to study microcirculatory regulation mechanisms in both physiological and pathophysiological states including diabetes mellitus^[10], PVD^[11], kidney disease^[12], and coronary artery disease^[13], among others, although its usefulness as a diagnostic tool is largely limited to its low reproducibility. PPG is a much older technology commonly included in many current medical devices such as pulse oximeters, and digital beat-to-beat blood pressure systems^[8]. Although based in similar optical principles, LDF and PPG are technically very different, and also differ in terms of cost. Recent technological advances promoted a clear evolution of the classic PPG, drawing our attention to this new generation hardware and software that have evolved in terms of portability and data acquisition, showing a quantitative potential until now only expected for LDF signals^[14].

In this study we explore the impact of a discrete intervention on the local microcirculation status of the lower limb, using a massage protocol as instrument, in order to evaluate the discriminatory capacity of this new generation of PPG in the detection and quantification of peripheral vascular circulation and to compare it to the information derived by LDF.

Introdução

A doença vascular periférica (DVP) compreende muitas entidades patológicas e uma enorme variedade de expressões clínicas, desde a síndrome da “pernas pesadas” até ao envolvimento inflamatório muscular e osteoarticular, e à úlcera^[1,2]. É considerado um problema de saúde pública, com grave impacto sobre a qualidade de vida^[2]. Em Portugal, estima-se que a prevalência da DVP chegue a 5,9% da população, afetando principalmente os idosos^[3]. Constituinte a 3ª causa mais importante de doença cardiovascular, continua a motivar mais e melhor investigação, a fim de compreender as complexidades envolvidas e desenvolver medidas preventivas e terapêuticas mais eficazes.

A pele é reconhecida como um órgão adequado à abordagem de vários aspetos funcionais da microcirculação^[4,5], e por essa razão, a fluxometria por laser Doppler (FLD) e a fotopletismografia (FPG) tornaram-se técnicas não invasivas, bem conhecidas, para quantificar através da pele, variáveis de fluxo em condições tão próximas quanto possível do estado fisiológico normal^[4,6,9]. A FLD ganhou grande popularidade nas últimas décadas como uma ferramenta de avaliação em medicina vascular, e utilizada em investigação básica para estudar os mecanismos de regulação microcirculatória em diversos contextos incluindo a diabetes mellitus^[10], a DVP^[11], a doença renal^[12], a doença arterial coronária^[13], entre outras, embora a sua utilidade em diagnóstico esteja limitada à sua baixa reprodutibilidade. A FPG é uma tecnologia bastante mais antiga, aproveitada em muitos dispositivos médicos atuais, como oxímetros de pulso e sistemas de pressão arterial digitais “beat to beat”^[8]. Embora baseados em princípios óticos similares, a FLD e FPG são tecnicamente muito diferentes e, igualmente diferentes em termos de custo. Avanços tecnológicos recentes permitiram uma clara evolução da FPG clássica, chamando a nossa atenção para esta nova geração de hardware e software que evoluiu em termos de portabilidade e aquisição de dados, mostrando um potencial quantitativo até agora reservado apenas aos sinais de FLD.

Neste estudo, analisamos o impacto de uma intervenção discreta sobre o estado da microcirculação local do membro inferior, um protocolo de massagem, para avaliar a capacidade discriminatória desta nova geração de FPG na deteção e quantificação da circulação vascular periférica, comparativamente à FLD.

Materials and Methods

A group of seven young healthy volunteers of both sexes (3 females, 4 males), aged between 18 and 22 years old (19.6 ± 1.3) and selected from previously outlined inclusion and non-inclusion criteria [5], participated in the study after informed written consent. All procedures were developed in accordance with the Helsinki Declaration and subsequent amendments [15]. Volunteers were asked to refrain from consuming caffeinated and alcoholic beverages 24 hours prior to experiments. All procedures were carried out in a room with controlled temperature ($21 \pm 1^\circ\text{C}$) and humidity (40 - 60 %) conditions. Volunteers acclimatized for 20 minutes in the supine position prior to measurements. The experimental protocol consisted of a 5 minute massage session applied from the foot to the knee on one randomly selected limb. The volunteers remained lying supine (Figure 1) during three recording phases: 10 minutes resting (phase I), 5 minute upward leg and foot massage for 5 minutes (phase II), and 10 minute recovery (phase III).

Materiais e Métodos

Um grupo de sete voluntários jovens, saudáveis, de ambos os sexos (3 do sexo feminino, 4 do sexo masculino) com idades entre os 18 e os 22 anos ($19,6 \pm 1,3$), selecionados a partir de critérios de inclusão e não inclusão previamente delineados [5], participaram do estudo após darem o seu consentimento informado por escrito. Todos os procedimentos foram desenvolvidos em conformidade com a Declaração de Helsínquia e respectivas emendas posteriores [15]. Os voluntários foram instruídos para não consumir bebidas cafeinadas nem alcoólicas 24 h antes das experiências. Todos os procedimentos foram realizados em ambiente com temperatura ($21 \pm 1^\circ\text{C}$) e humidade (40-60%) controladas. Os voluntários mantiveram-se em repouso durante 20 minutos em supinação antes das medições.

O protocolo experimental consistiu em uma sessão de massagem de 5 min aplicada do pé ao joelho para um membro aleatoriamente selecionado, mantendo os voluntários em supinação (Figura 1), envolvendo 3 fases de registo - 10 min em repouso (fase I), 5 min de massagem da perna e pé em sentido ascendente durante 5 minutos (fase II), recuperação de 10 min (fase III).



Figure 1/ Figura 1 - Massage application protocol (direction indicated by the arrow)
Aplicação do protocolo de massagem (direção indicada pela seta)

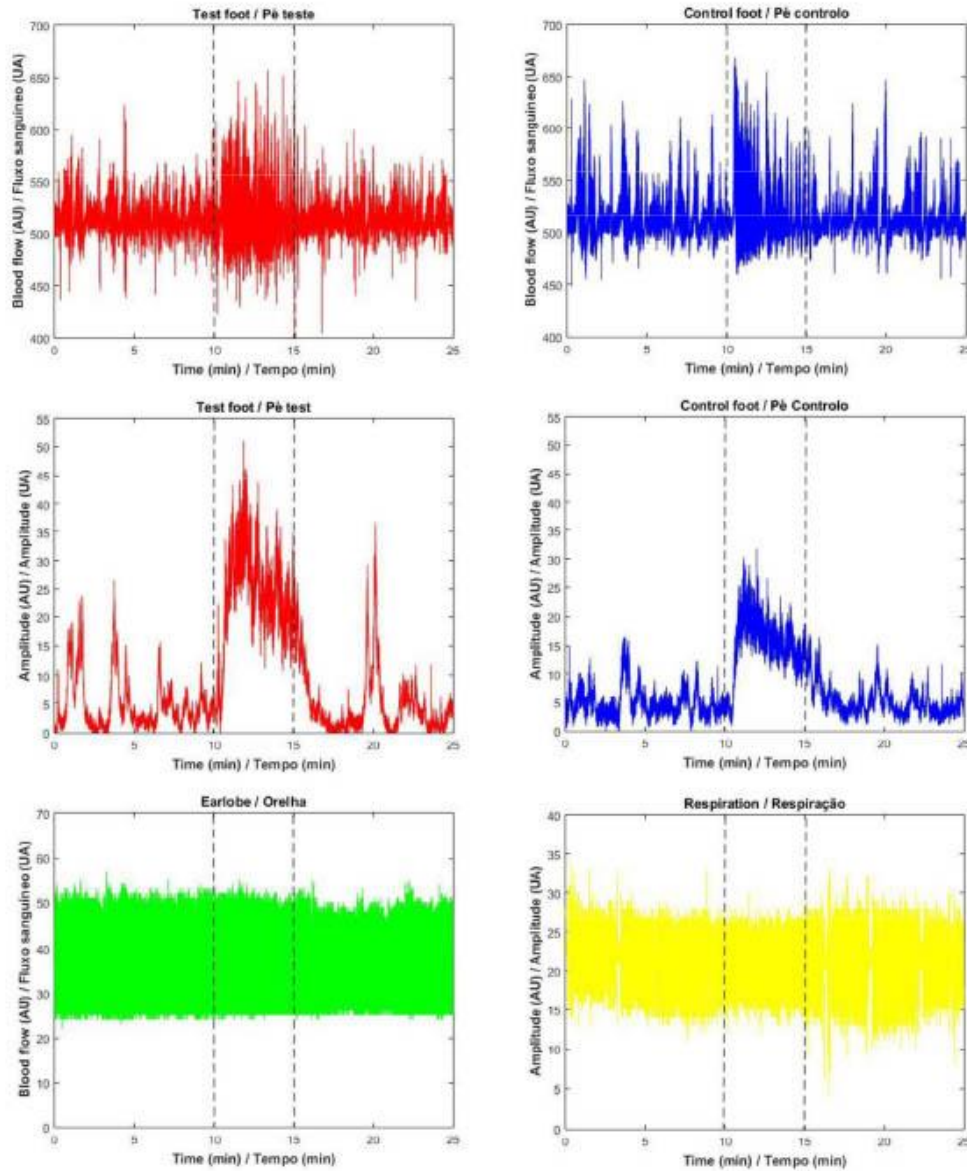


Figure 2/ Figura 2 - Recordings for a representative volunteer (female, 19 years old) of the PPG (upper left for test foot; upper right for control foot), LDF (middle left for test foot; middle right for control foot), FLD (no centro à esquerda para pé teste; no centro à direita para pé controlo; em baixo à esquerda para orelha), e respiração (em baixo à direita) de um voluntário representativo (feminino, 19 anos de idade) durante o protocolo de 25 minutos. As linhas verticais indicam as transições de fase.

Variables included local blood flow, measured by LDF (457 thermostatic small angled probe, PF5010 system, Perimed, Sweden), and by PPG (BVP sensor, Flux Biosignals, Portugal), both expressed in arbitrary units (AU). Skin temperature was also registered (457 thermostatic small angled probe, PF5010 system, Perimed, Sweden). LDF signals were acquired at a 32Hz sampling rate. Sensors were applied in the most distal areas – for PPG on the plantar surface of the first toe, in both feet (PPG_T for the test foot; PPG_C for the control foot), and on one randomly chosen earlobe (PPG_E), and for LDF on the plantar surface of the second toe in both feet (LDF_T for the test foot; LDF_C for the control foot). The pulse rate (PR) was calculated from the ear lobe PPG signal. Respiratory rate (RR) was measured with a pneumography sensor (PZT sensor, Flux Biosignals, Portugal), placed around the thorax on the projection of the sternoxiphoid joint. The PPG and PZT sensors were connected to a BITalino Plugged board (Flux Biosignals, Portugal) and signals were acquired at a 100 Hz sampling rate. The systolic (SBP) and diastolic blood pressure (DBP) were also measured in the arm by a digital device (Tensoval comfort, Hartman, Germany) on minutes 3, 5, 7 (phase I), 13, 14, 15 (phase II) and 18, 20 and 22 (phase III) of the protocol, and the mean values for each phase were calculated. Both feet and sensors were kept in the same position during the experiments.

Descriptive and non-parametric statistics were applied to data (SPSS 21.0, USA). Phase comparisons were carried out with the Wilcoxon test for paired samples for each leg, while the Mann-Whitney U test for independent samples was used for leg comparisons. The correlation between LDF and PPG signals was assessed with the Spearman test. A significance level of 95% was adopted for all statistic tests.

Results and Discussion

Table 1 resumes the mean values of the measured variables for all phases of the protocol. No significant differences between both feet perfusion were found with either LDF or PPG, suggesting a homogeneous blood distribution along the lower limbs. During massage application, statistically significant perfusion increases in both feet were detected ($p=0.018$ for LDF_T and LDF_C, $p=0.028$ for PPG_T and PPG_C), despite the fact that the response in the test foot was more pronounced in com-

As variáveis registadas foram o fluxo sanguíneo local, medido por FLD (sonda termostática 457 de ângulo curto, sistema PF5010, Perimed, Suécia), e por FPG (sensor BVP, Flux Biosignais, Portugal), ambos expressos em unidades arbitrárias (UA). A temperatura da pele foi também registada (sonda termostática 457 de ângulo curto, sistema PF5010, Perimed, Suécia). Os sinais de FLD foram adquiridos a uma frequência de amostragem de 32 Hz. Os sensores foram aplicados nas áreas mais distais – a FPG na superfície plantar do primeiro dedo do pé, em ambos os pés (FPG_T para o pé de teste, FPG_C para o pé de controlo) e num lóbulo da orelha escolhido aleatoriamente (FPG_O), na superfície plantar do segundo dedo do pé, em ambos os pés (FLD_T para o pé de teste, FLD_C para o pé de controlo). A frequência de pulso (FP) foi calculada a partir do sinal de FPG do lóbulo da orelha. A frequência respiratória (FR) foi medida com um sensor de pneumografia (sensor PZT, Flux Biosignals, Portugal), colocado em torno do tórax, na projeção da sínfise xifóesternal. Os sensores FPG e PZT foram ligados a uma placa BITalino Plugged (Flux Biosignals, Portugal) e os sinais adquiridos a uma frequência de amostragem de 100 Hz. As pressões arteriais sistólica (PAS) e diastólica (PAD) foram também medidas no braço, por um dispositivo digital (Tensoval comfort, Hartman, Alemanha) nos minutos 3, 5, 7 (fase I), 13, 14, 15 (fase II) e 18, 20 e 22 (Fase III) do protocolo, calculando os valores médios para cada fase. Ambos os pés e sensores foram mantidos na mesma posição durante as experiências.

Foram aplicadas ferramentas de análise estatística descritiva e não-paramétrica aos dados (SPSS 21.0, EUA). As comparações de fases foram realizadas com o teste de Wilcoxon para amostras emparelhadas, em cada perna, enquanto o teste U de Mann-Whitney para amostras independentes foi utilizado para comparações entre os membros. A correlação entre os sinais de FLD e FPG foi avaliada pelo teste de Spearman. Foi adotado um nível de significância de 95% para todos os testes estatísticos.

Resultados e Discussão

A Tabela 1 resume os valores médios das variáveis medidas em todas as fases do protocolo. Não foram encontradas diferenças significativas entre as perfusões de ambos dos pés com FLD ou FPG, sugerindo uma distribuição homogênea do sangue ao longo dos membros inferiores. Durante a aplicação da massagem, foram detetados aumentos estatisticamente significativos da perfusão em ambos os pés ($p = 0,018$ para FLD_T e FLD_C, $p = 0,028$ para FPG_T e FPG_C), apesar de a res-

parison to the control (LDF: 119.4% vs 51.5%; PPG: 53.4% vs 23.6%). While the test foot response seems to be due to a direct mechanical mobilization of the blood, promoted by the massage, the increase registered in the control foot suggests that this mobilization also affects the other leg, likely due to a regional regulation intended to maintain the lower limb (regarded as one unit) circulatory homeostasis.

PPG quantifies the amount of visible light reflected by microcirculatory vessels, in proportion to the blood volume perfusing the vessels. Its typical 'wave' form is explained by the variation of blood volume that occurs after a given heartbeat [8]. In contrast, LDF quantifies the velocity of circulating red blood cells (RBC), which is proportional to the frequency shift suffered by an impinging laser beam, that is, the 'Doppler effect' [16]. After multiplying this velocity by an estimated stochastic value of RBC concentration, a 'flow' value is reached. The different percent changes detected in both feet with both LDF and PPG between phases are likely attributable to the different depths of reading from the two sensors [17,18], and this difference should be further investigated. Despite these technical differences, signals from both techniques were similar in both feet, as shown by a positive significant correlation here found ($N=42$; $p=0.673$; $p<0.001$).

SBP, DBP and PR decreased significantly during massage application ($p=0,018$ for all variables), and returned to baseline values in the recovery phase (Table 1). The reduction of these haemodynamical markers during the massage has been described previously, and might be related to a related increase in parasympathetic activity [19]. RR increased significantly during the massage ($p=0,018$), which might be explained by the associated relaxing effect described by volunteers, eventually affecting central and peripheral neural activity [20,21].

Some studies have reported an increase in skin temperature during the application of a massage [22,23], however, in our study conditions, the temperature did not change significantly throughout the protocol.

posta no pé teste ter sido mais pronunciada em relação ao controlo (FLD: 119,4% vs 51,5%, FPG: 53,4% vs 23,6%). Enquanto a resposta do pé teste parece ser devida a uma mobilização mecânica direta de sangue, promovida pela massagem, o aumento registado no pé de controlo sugere que essa mobilização também afeta a outra perna, provavelmente devido a uma regulação regional destinada a manter a homeostasia circulatória no membro inferior (considerado como uma unidade). A FPG quantifica a luz visível refletida pelos vasos microcirculatórios, em proporção ao volume de sangue perfundido. A forma típica de 'onda' é explicada pela variação do volume de sangue que ocorre após um determinado batimento cardíaco [8]. Quanto à FLD, esta quantifica a velocidade dos glóbulos vermelhos (GV) circulantes, que é proporcional ao desvio de frequência sofrido pela incidência de um feixe de luz laser, isto é, o "efeito Doppler" [16]. Depois de multiplicar esta velocidade por um valor estocástico estimado da concentração de GV, é obtido um valor de "fluxo". As diferentes percentagens de alterações detetadas em ambos os pés com FLD e FPG entre as fases são provavelmente atribuíveis às diferentes profundidades de leitura dos dois sensores [17,18], que devem ser investigados. Apesar dessas diferenças técnicas, os sinais de ambas as técnicas foram semelhantes em ambos os pés, como mostrado por uma correlação significativa positiva encontrada aqui ($N = 42$; $p = 0,673$; $p < 0,001$).

A PAS, PAD e FP diminuíram significativamente durante a aplicação da massagem ($p = 0,018$ para todas as variáveis) e retornaram aos valores basais na fase de recuperação (Tabela 1). A redução destes marcadores hemodinâmicos durante a massagem tem sido descrita e pode estar relacionada com um aumento relacionado na atividade parassimpática [19]. A FR aumentou significativamente durante a massagem ($p = 0,018$), o que pode ser explicado pelo efeito relaxante associado descrito pelos voluntários, eventualmente afetando as atividades nervosa central e periférica [20,21].

Alguns estudos relataram um aumento da temperatura da pele com a massagem [22,23], no entanto, não encontramos este dado, uma vez que a temperatura não se alterou significativamente ao longo do protocolo.

Table 1/ Tabela 1 - Mean, standard deviation and percent change of the variables measured. Statistical comparison of the mean values of phases II and III regarding phase I is presented ($p < 0.05$; * - statistically significant)
Média, desvio-padrão e variação percentual das variáveis medidas. É apresentada a comparação estatística dos valores médios das fases II e III com a fase I ($p < 0.05$; * - estatisticamente significativo)

Variable / Variável		Phase I / Fase I	Phase II / Fase II	Phase III / Fase III	Δ II-I (%)	Δ III-I (%)
LDF _T (AU) / FLD _T (UA)	Mean	8.0	17.6	9.3	119.4	14.0
	sd	3.8	5.0	5.6	$p=0.018^*$	$p=0.499$
LDF _C (AU) / FLD _C (UA)	Mean	7.6	11.5	8.3	51.5	10.1
	sd	4.2	4.8	5.5	$p=0.018^*$	$p=1.00$
LDF _T vs LDF _C		$p=0.902$	$p=0.053$	$p=0.902$	-	-
Skin temperature (°C)	Mean	23.6	23.5	23.4	-0.54	-0.71
	sd	1.9	1.7	1.7	-12.7	-10.25
PPG _T (AU) / FPG _T (UA)	Mean	54.5	83.6	57.1	53.4	4.8
	sd	41.8	42.0	44.6	$p=0.028^*$	$p=0.310$
PPG _C (AU) / FPG _C (UA)	Mean	85.8	104.4	113.8	21.6	32.6
	sd	92.2	100.5	146.3	$p=0.028^*$	$p=0.499$
PPG _T vs PPG _C		$p=0.535$	$p=0.805$	$p=0.620$	-	-
PPG _R (AU) / FPG _R (UA)	Mean	16.6	17.2	15.5	3.4	-6.6
	sd	8.5	8.6	7.0	$p=0.273$	$p=0.273$
SBP (mm Hg) / PAS (mm Hg)	Mean	115.3	110.1	115.4	-4.6	0.04
	sd	11.0	9.3	10.3	$p=0.018^*$	$p=0.752$
DBP (mm Hg) / PAD (mm Hg)	Mean	71.1	64.2	68.2	-9.8	-4.3
	sd	5.0	3.6	4.6	$p=0.018^*$	$p=0.091$
PR (bpm) / FP (bpm)	Mean	69.9	66.0	69.4	-5.6	-0.7
	sd	4.5	4.7	5.2	$p=0.018^*$	$p=0.128$
RR (bpm) / FR (bpm)	Mean	18.9	20.5	19.0	8.2	0.2
	sd	2.0	2.8	2.3	$p=0.018^*$	$p=0.866$
Spearman test (LDF vs PPG) / Teste de Spearman (FLD vs FPG)		$p=0.673$; $p<0.001^*$				

Conclusions

The simultaneous use of these two technologies seems to provide a more sensitive approach to quantify peripheral microcirculation *in vivo*, allowing the detection of subtle changes in addition to other cardiorespiratory variables, thus expanding the applicability and adding value to this measurement strategy.

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Conclusões

O uso simultâneo destas duas tecnologias parece proporcionar uma abordagem mais sensível para quantificar a microcirculação periférica *in vivo*, permitindo detetar algumas alterações discretas, mas também outras variáveis cardiorrespiratórias, o que expande a sua aplicabilidade e acrescenta um novo valor a esta técnica de medição.

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Conflict of Interests

The authors declare there are no financial and/or personal relationships that could present a potential conflict of interest.

Conflito de Interesses

Os autores declaram que não há relações financeiras e / ou pessoais que possam representar um potencial conflito de interesses.

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Artigo II - Comparing the effects of the human hind limb massage by analysis of Laser Doppler Flowmetry and Photoplethysmography signal components with the wavelet transform

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Comparing the effects of human hind limb massage by analysis of Laser Doppler Flowmetry and Photoplethysmography signal components using the wavelet transform

Comparando os efeitos da massagem no membro inferior humano através da análise dos componentes dos sinais de Fluxometria de Laser Doppler e de Fotopletismografia utilizando a transformada de wavelet

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Abstract

Non-invasive optical techniques, such as LDF (laser Doppler flowmetry) and PPG (photoplethysmography) are leading technologies to assess the human microcirculation. New mathematical strategies for signal processing such as WT (wavelet transform) have been developed to enhance its quantification capabilities. We have dedicated some effort to better understand the impact of massage therapy, a non-conventional technique used to relieve symptoms of vascular failure and improve functions, using these instruments. A group of 24 young healthy volunteers (19.9 ± 1.7 years old), both sexes, was submitted to two massage protocols applied in one limb, the other serving as control. Each protocol included three phases - rest (I), massage (II), and recovery (III). LDF and PPG signals were obtained from both limbs and analyzed with the WT ($p < 0.05$).

A regional circulatory adaptation seems to take place during massage manoeuvres, affecting both test and control limbs detected with LDF and PPG. Some differences are noted regarding their oscillatory components, being clear that both sympathetic and myogenic components play a central role in the process. It is also clear that the simultaneous use of these two technologies seems to provide more detail and sensitivity in the analysis of the phenomena involved.

Keywords: hind limb massage; LDF; PPG; wavelet transform; microcirculation.

Resumo

Técnicas óticas não invasivas, como o FLD (fluxometria por laser Doppler) e a FPG (fotopletismografia), vêm sendo utilizadas na avaliação da microcirculação em humanos. Novas estratégias matemáticas para processamento de sinais, como a TO (transformada de ondeleta), foram desenvolvidas para melhorar as suas capacidades de quantificação. Temos desenvolvido vários esforços para entender melhor o impacto da massagem, uma técnica não convencional usada para aliviar os sintomas de disfunção vascular, e melhorar a função, usando estes instrumentos. Um grupo de 24 jovens voluntários saudáveis (19.9 ± 1.7 anos), de ambos os sexos, foi submetido a dois protocolos de massagem aplicados em um dos membros, servindo o outro como controlo. Cada protocolo incluiu três fases – I descanso, II massagem e III recuperação. Foram obtidos sinais de LDF e PPG de ambos os membros e analisados com a TO ($p < 0.05$).

Uma adaptação circulatória regional detetada com LDF e PPG ocorre durante as manobras de massagem, afetando ambos os membros. Algumas diferenças foram observadas em relação aos seus componentes oscilatórios, onde os componentes simpáticos e miogénicos parecem desempenhar um papel principal. Também está claro que o uso simultâneo dessas duas tecnologias parece fornecer uma análise mais sensível e apoiada dos fenómenos envolvidos.

Palavras-chave: massagem dos membros inferiores; LDF; PPG; transformada de ondeleta; microcirculação.

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Introduction

Several optical non-invasive techniques have been developed to access in vivo microcirculation, a main component of circulatory physiology^{1,2}. LDF and PPG are among the most popular technologies, sharing the same optical principles, although with different operation requirements³⁻⁵. These have been extensively applied in human studies, with different purposes^{2,4,6}. However, these technologies provide complex pulsatile signals, difficult to analyze, due to the contribution of different organs and systems^{4,7,8}.

The need to obtain a deeper analysis of such types of biological signals motivated the development of other mathematical strategies for signal processing, from which the Morlet Wavelet Transform emerged⁹. This wavelet transform (WT) has been considered a suitable method for signal decomposition in relation to more classic approaches as the fast Fourier transform (FFT), which has a low resolution and thus reduced capabilities for low frequency analysis^{8,10}. The WT provides a higher resolution and preserves the time/frequency relationship⁹ which is essential when dealing with phenomena that vary in very low frequencies and over time, as in the case of most of the biological signals, such as perfusion pulses. This strategy has been thoroughly applied to LDF, allowing its spectral decomposition in its main components, thus oscillations with different time frequencies can be linked to the activity of different tissues or organs^{11,12}. The human LDF components are identified as cardiac, respiratory, myogenic (vascular wall), sympathetic, endothelial NO-dependent activity and endothelial NO-independent, which typically correspond to spectral bands between 0.05 and 2 Hz¹³. More recently, this analysis was extended to PPG obtained with modern, more stable, systems, providing further analysis of these signals^{14,8}.

The present study takes further our focus on the effects of massage over peripheral circulation in human^{7,15}, to better characterize and understand this procedure, used in human health and also as a therapeutic tool. Although only a few studies have been published on this issue during the past decades, it is generally accepted that massage facilitates local blood perfusion while reducing heart rate and arterial blood pressure^{16,17}. Pain relief and relaxation are recurrently referred, apparently associated to parasympathetic output^{15,17,18}. Therefore, our goal was to look deeper into the LDF and PPG signals obtained during massage of the inferior limb of healthy volunteers, decomposed by WT, in order to compare their discriminative potential while having a

Introdução

Várias técnicas óticas não invasivas foram desenvolvidas para avaliar a microcirculação in vivo, um componente principal da fisiologia circulatória^{1,2}. A fluxometria por laser Doppler (FLD) e a fotopletismografia (FPG) estão entre as tecnologias mais populares, compartilhando os mesmos princípios biofísicos, embora com diferentes frequências e exigências de operação³. Estas tecnologias têm sido extensivamente usadas em humanos, com diferentes finalidades^{2,4,6}, contudo, fornecem em ambos os casos, sinais pulsáteis complexos, difíceis de analisar, devido à influência de diferentes órgãos e sistemas sobre a sua aquisição e registo^{4,7,8}. A necessidade de se obter uma análise mais profunda de tais sinais biológicos, motivou o desenvolvimento de outras estratégias matemáticas para o processamento de sinais, a partir das quais a Transformada de Ondeta de Morlet emergiu⁹. Esta transformada de ondeteta (TO) tem sido considerada um método adequado para decomposição de sinais em relação às abordagens mais clássicas, como a transformada rápida de Fourier (TRF), que possui baixa resolução, sendo menos capaz para a análise de baixa frequência^{8,10}. Assim, a TO fornece uma resolução maior e preserva a relação tempo/frequência⁹ que é essencial face a fenômenos que variam em frequências muito baixas e ao longo do tempo, como na maioria dos sinais biológicos, e como nos pulsos de perfusão. Essa estratégia foi amplamente aplicada à FLD, permitindo sua decomposição espectral nos seus principais componentes, o que significa que aquelas oscilações com diferentes frequências temporais poderiam, na verdade, estar relacionadas com a atividade de diferentes tecidos ou órgãos^{11,12}. Os componentes da FLD humana são identificados com as seguintes origens - cardíaca, respiratória, miogénica (parede vascular), simpática, endotelial dependente de NO e endotelial independente de NO, que tipicamente correspondem a bandas espectrais entre 0,05 e 2Hz¹³. Mais recentemente, esta análise foi estendida ao PPG obtido com sistemas modernos e mais estáveis, permitindo uma análise mais aprofundada destes sinais^{14,8}. O presente estudo aprofunda o nosso foco nos efeitos da massagem sobre a circulação periférica em humanos^{7,15}, para melhor caracterizar e compreender esse procedimento, também utilizado na saúde humana, até como instrumento terapêutico. Embora apenas alguns estudos tenham sido publicados sobre este tema nas últimas décadas, aceita-se que a massagem facilita a perfusão sanguínea local, enquanto reduz a frequência cardíaca e a pressão arterial^{16,17}. O alívio da dor e o relaxamento são recorrentemente referidos, aparentemente associados à

new insight into the mechanisms involved in the physiological response.

Materials and Methods

Twenty four young healthy volunteers, both sexes (19.9 ± 1.7 years old, 13 females, 11 males), body mass index (BMI) 21.8 kg/m^2 and ankle-brachial index (ABI) 1.06, were selected from previously outlined inclusion and non-inclusion criteria²⁰ after written informed consent. All procedures respected the Helsinki Declaration (and subsequent amendments) principles for human research²¹.

Volunteers abstained from consuming caffeinated and alcoholic beverages 24 hours prior to experiments. Procedures took place in a room with controlled temperature ($21 \pm 1^\circ\text{C}$) and humidity (40 to 60%). All volunteers were acclimatized for 20 minutes in the supine position prior to experiments.

Two protocols were carried out as follows - Protocol I, one session of an upward massage (upM) applied on the ascending direction in one randomly selected leg and foot, from the middle of the foot until below the knee, and Protocol II, also one session of a downward massage (dwM) in the opposite direction. Both protocols consisted of three phases, Phase I, recording during resting in supine, for 10 minutes; Phase II provocation with leg and foot massage for 5 minutes and, Phase III, recording during recovery for 10 minutes (Figure 1). The contralateral leg was used as control, keeping both feet in the same position during the experiments. Both protocols were performed in a randomly order and separated by a 30 minute 'washout' period. Blood flow was measured in the inferior aspect of the first and second toes in both feet, with PPG and LDF, respectively. LDF signal was obtained by a Perimed PF5010 system (Perimed, Sweden, expressed in arbitrary Blood Perfusion Units (BPU's) while PPG signal was obtained by a Bitalino Plugged Kit (PLUX Biosignals, Portugal), also expressed in BPU's, also allowing measurement of Blood Volume Pulse (BVP). The LDF system also recorded skin temperature in $^\circ\text{C}$. LDF signal was recorded with a frequency of 32 Hz and the PPG signal was recorded at 100 Hz and then downsampled by a factor of 3 to 33.33 Hz. LDF and PPG signals were then decomposed in their main components (cardiac, respiratory, myogenic, sympathetic, endothelial NO-depen-

ção parassimpática^{15, 17, 18}. Portanto, o nosso objetivo é olhar mais profundamente para os sinais de FLD e FPG, obtidos durante a massagem do membro inferior de voluntários saudáveis, decompostos por TO, a fim de comparar o seu potencial discriminativo e procurar um novo olhar sobre os mecanismos envolvidos na resposta fisiológica implicada.

Materiais e Métodos

Vinte e quatro jovens voluntários saudáveis, ambos sexos (19.9 ± 1.7 anos, 13 mulheres, 11 homens), índice de massa corporal (IMC) 21.8 kg/m^2 e índice tornozelo-braquial (ITB) 1.06, foram selecionados a partir de critérios de inclusão e não inclusão previamente definidos²⁰ e após obtenção de consentimento informado. Todos os procedimentos respeitaram os princípios da Declaração de Helsínquia (e emendas subsequentes) para pesquisa em seres humanos²¹.

Os voluntários abstiveram-se de consumir bebidas cafeinadas e alcoólicas 24 horas antes dos experimentos. Os procedimentos foram realizados em sala com temperatura controlada ($21 \pm 1^\circ\text{C}$) e humidade (40 a 60%). Todos os voluntários foram aclimatados durante 20 minutos na posição supina antes do início dos experimentos.

Foram aplicados dois protocolos - Protocolo I, consistindo numa sessão de massagem aplicada na direção ascendente (Ma) em uma perna e pé selecionados aleatoriamente, partindo do meio do pé até abaixo do joelho, e Protocolo II, consistindo numa sessão de uma massagem descendente (Md) na direção oposta. Ambos os protocolos incluíram em três fases, Fase I, registro em repouso em supinação, durante 10 minutos; Fase II de provocação com massagem de perna e pé durante 5 minutos e, Fase III, de recuperação com registro durante por 10 minutos (Figura 1). A perna contralateral foi usada como controle, mantendo os dois pés na mesma posição durante as experiências. Ambos os protocolos foram realizados em ordem aleatória e separados por um período de 30 min de "washout". O fluxo sanguíneo foi medido na parte inferior do primeiro e segundo dedos em ambos os pés, com FPG e FLD, respetivamente. O sinal FLD foi obtido pelo sistema Perimed PF5010 (Perimed, Suécia, expresso em unidades arbitrárias de perfusão sanguínea (UP's), enquanto o sinal FPG foi obtido pela placa Bitalino Plugged Kit (PLUX Biosignals, Portugal), também expresso em UP's, permitindo também a medição do Volume de Pulso Sanguíneo (VPS). O sistema FLD também registrou a temperatura da pele em $^\circ\text{C}$. O sinal FLD foi registrado com uma frequência de 32 Hz e o sinal FPG foi registrado em

dent (endoNOD) and endothelial NO-independent (endoNOI) with WT and their contribution to the signals was assessed, based on the already detected ²³ LDF frequency bands: (cardiac (0.6 Hz to 2 Hz), respiratory (0.145 Hz to 0.6 Hz), myogenic (0.052 Hz to 0.145 Hz), sympathetic (0.021 Hz to 0.052 Hz), endoNOD (0.0095 Hz to 0.021 Hz) and endoNOI (0.005 to 0.0095 Hz)). Descriptive and non-parametric statistics were applied to the signals and their WT components using SPSS 21.0 (IBM, USA). Phase comparisons were carried out with the Wilcoxon signed-rank test for paired samples for each leg, the Mann-Whitney U test for independent samples was used for leg comparisons, and the correlation between LDF and PPG signals was obtained with the Spearman test Rho coefficient (ρ). A confidence level of 95% ($p < 0.05$) was adopted for all tests.

100Hz e então diminuída a frequência por um fator de 3 a 33.33Hz. os sinais foram então decompostos em seus componentes principais (cardíaco, respiratório, miogénico, simpático, endotelial dependente de NO (endoNOD) e endotelial independente de NO (endoNOI)) com a TO e sua contribuição para os sinais foi avaliada, com base nas já detetadas ²³ bandas de frequência FLD: (cardíaca (0.6 Hz a 2 Hz), respiratória (0.145 Hz a 0.6 Hz), miogénica (0.052 Hz a 0.145 Hz), simpática (0.021 Hz a 0.052 Hz), endoNOD (0.0095 Hz a 0.021 Hz) e endoNOI (0.005 a 0.0095 Hz)).

Estatística descritiva e não-paramétrica foram aplicadas aos sinais e seus componentes obtidos da TO, utilizando o programa SPSS 21.0 (IBM, EUA). As comparações de fase foram realizadas com o teste de Wilcoxon para amostras emparelhadas para cada perna, reservando o teste U de Mann-Whitney para amostras independentes para comparações de perna. A correlação entre FLD e FPG foi obtida com o coeficiente Rho do teste de Spearman. Adotamos um nível de confiança de 95% ($p < 0.05$).



Figure 1/ Figura 1 - Massage protocols (upM on the left – dwM on the right).
Protocolos de massagem (ascendente à esquerda – descendente à direita).

Results

Correlating raw signals from both techniques (Table 1, Figure 2), shows for the upM protocol, a significant positive relation in all phases for both feet, in which Phase I was $\rho = 0.711$ with $p < 0.001$, Phase II was $\rho = 0.478$ with $p = 0.018$, and Phase III was $\rho = 0.697$ with $p < 0.001$ for the test foot. For the control foot, Phase I was $\rho = 0.456$ with $p = 0.025$, Phase II was $\rho = 0.484$ with $p = 0.017$, and in Phase III was $\rho = 0.490$ with $p = 0.015$. For the dwM protocol, a similar result was found, meaning a significant positive correlation in all phases, except for Phase II in the test foot, where no significant correlation was found. Phase I was $\rho = 0.659$ with $p < 0.001$, Phase II was $\rho = 0.316$ with $p = 0.133$, and Phase III was $\rho = 0.592$ with $p = 0.002$ for the test foot. For the control foot, Phase I was $\rho = 0.722$ with $p < 0.001$, Phase II was $\rho = 0.684$ with $p < 0.001$, and Phase III was $\rho = 0.693$ with $p < 0.001$.

Resultados

Correlacionando os sinais brutos das duas técnicas (Tabela 1, Figura 2), encontramos para o protocolo de Ma, uma relação positiva e significativa em todas as fases para ambos os pés, na qual a fase I foi $\rho = 0.711$ com $p < 0.001$, na fase II foi $\rho = 0.478$ com $p = 0.018$, e na fase III foi $\rho = 0.697$ com $p < 0.001$ para o pé teste. Para o pé controle, na fase I foi $\rho = 0.456$ com $p = 0.025$, na fase II foi $\rho = 0.484$ com $p = 0.017$ e na fase III foi $\rho = 0.490$ com $p = 0.015$. Para o protocolo de Md foi encontrado resultado semelhante, consistindo numa correlação positiva e significativa em todas as fases, excepto para a fase II no pé teste onde não foi encontrada correlação significativa, em que a fase I foi $\rho = 0.659$ com $p < 0.001$, na fase II $\rho = 0.316$ com $p = 0.133$ e na fase III foi $\rho = 0.592$ com $p = 0.002$ para o pé teste. Para o pé controle, na fase I foi $\rho = 0.722$ com $p < 0.001$, na fase II foi $\rho = 0.684$ com $p < 0.001$ e na fase III foi $\rho = 0.693$ com $p < 0.001$.

Table 1/ Tabela 1 - Statistical comparison between LDF and PPG for each phase of massage protocols (dwM and upM). (see text) (* $p < 0.05$; ** $p < 0.005$).
Comparação estatística entre LDF e PPG para cada fase dos protocolos de massagem (dwM e upM (ver texto) (* $p < 0,05$; ** $p < 0,005$).

	Phase I/Fase I	Phase II/Fase II	Phase III/Fase III
upM Protocol			
LDF v PPG test foot FLD v FPG pé teste	$\rho = 0.711$ $p < 0.001^{**}$	$\rho = 0.478$ $p = 0.018^{*}$	$\rho = 0.697$ $p < 0.001^{**}$
LDF v PPG control foot FPG v FPG pé controle	$\rho = 0.456$ $p = 0.025^{*}$	$\rho = 0.484$ $p = 0.017^{*}$	$\rho = 0.490$ $p = 0.015^{*}$
dwM Protocol			
LDF v PPG test foot FLD v FPG pé teste	$\rho = 0.659$ $p < 0.001^{**}$	$\rho = 0.316$ $p = 0.133$	$\rho = 0.592$ $p = 0.002^{**}$
LDF v PPG control foot FPG v FPG pé controle	$\rho = 0.722$ $p < 0.001^{**}$	$\rho = 0.684$ $p < 0.001^{**}$	$\rho = 0.693$ $p < 0.001^{**}$

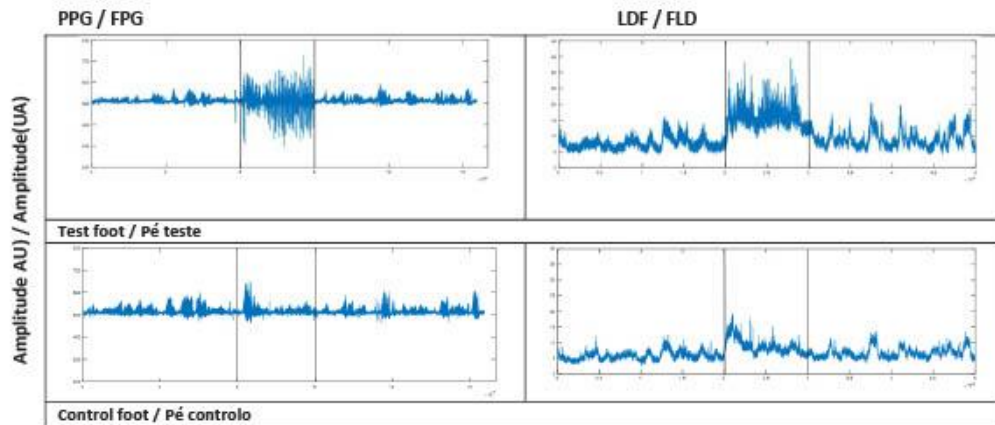


Figure 2/ Figura 2 - Illustrative example of PPG (left) and LDF (right) signals obtained in the massaged limb (test foot) and in the contralateral non-massaged foot during the execution of one of the protocols (dwM). Vertical lines indicate phase (I to III) transitions. As shown, after initiating Phase II in the massage limb, a contralateral response is detected in the resting limb, an event detected by both technologies (see text).

Exemplo ilustrativo de sinais de PPG (esquerda) e LDF (direita) obtidos no membro massageado (pé teste) e no pé não massageado (contralateral) durante a execução de um dos protocolos (dwM). As linhas verticais indicam transições de fase (I a III). Como se verifica, após o início da fase II, uma resposta contralateral é observada no membro em repouso, um evento detectado por ambas as tecnologias (ver texto).

The component analysis obtained from the application of the WT to both signals is graphically represented in Figures 3 and 4. For the upM we noticed a decrease in the cardiac components for LDF as for PPG components during Phase II, but only significant for LDF in the control foot ($p<0.001$) and in Phase III for test foot ($p=0.024$). A significant increase in the respiratory component was noted during Phase II ($p<0.001$ in both cases) for the test foot, and in PPG a significant increase was noted only in the control foot ($p=0.021$). Phase III also showed a significant increase in the respiratory component for both LDF and PPG ($p=0.022$ and $p=0.006$, respectively) only in the test foot. An increase in the myogenic component during Phase II was noted in both feet, significant for LDF ($p=0.012$) in the test foot, which contributes to the increase of the blood perfusion. At the same time, a decrease in the sympathetic components during Phase II was noted in both feet, only significant in both feet for LDF ($p<0.001$). Still, for LDF a significant decrease in the sympathetic component was shown in Phase III for both test and control feet ($p<0.001$ in both feet), and in PPG a significant decrease only in the control foot ($p<0.001$). For the endoNOD component, during Phase II a significant decrease was noted in both test (LDF, $p=0.004$ and PPG, $p<0.001$) and control feet (LDF, $p=0.046$ and PPG, $p=0.001$). In Phase III, a significant increase in this component was noted for LDF ($p=0.016$) only in the test foot, and for PPG a significant increase for both feet ($p=0.037$ and $p<0.001$) was noted. Finally, for the endoNOI component, a decrease in Phase II was noted in LDF and PPG, but only significant ($p<0.001$) in the test foot for PPG. In Phase III, a recovery was shown with a significant increase for LDF in both feet ($p<0.001$ for both feet), and for PPG a significant decrease ($p<0.001$) for the test foot.

For the oscillatory components obtained with dwM WT (Figure 4), a non-significant decrease in the cardiac components for both LDF and PPG was shown in Phase II. An increase in the respiratory component during Phase II was also noted, but only significant ($p<0.001$ in both cases) for the test foot. In Phase III, a significant decrease in this component was shown by PPG in the test foot alone. An increase in the myogenic component was noted during Phase II for both technologies and in both feet, significant for LDF in both feet ($p=0.010$ and $p=0.003$). With LDF, in Phase III this component shows a significant increase, in the control foot ($p=0.002$), and for PPG a significant decrease in both feet ($p=0.011$ and $p=0.005$). During Phase II, sympathetic components showed a decrease for both technologies in both feet, only significant in the test foot ($p=0.034$ and $p=0.005$). Still in the sympathetic component PPG in

A análise de componentes obtida a partir da aplicação da TO para ambos os sinais está graficamente representada nas Figuras 3 e 4. Para a Ma verificamos, para ambas as componentes de FLD e de FPG, uma diminuição nas componentes cardíacas durante a fase II, mas apenas significativa para a FLD no pé controle ($p<0.001$) e na fase III para o pé teste ($p=0.024$). É observado um aumento significativo na componente respiratória durante a fase II ($p<0.001$ em ambos os casos) para o pé teste, e na FPG um aumento significativo apenas no pé controle ($p=0.021$). A fase III também mostra um aumento significativo na componente respiratória tanto para a FLD quanto para a FPG ($p=0.022$ e $p=0.006$, respectivamente) apenas no pé teste. Um aumento da componente miogénica é observado durante a fase II em ambos os pés, significativo para a FLD ($p=0.012$) no pé teste, o que contribui para o aumento da perfusão sanguínea. Ao mesmo tempo, também durante a fase II, observa-se uma diminuição nas componentes simpáticas em ambos os pés, apenas significativa nos dois pés para a FLD ($p<0.001$). Ainda na componente simpática foi mostrada na fase III, para a FLD uma diminuição significativa tanto para o pé teste quanto para o controle ($p<0.001$ nos dois pés) e na FPG uma diminuição significativa apenas no pé controle ($p<0.001$). Para a componente endoNOD, durante a fase II observa-se uma diminuição significativa tanto no pé teste (FLD, $p=0.004$ e FPG, $p<0.001$) quanto no controle (FLD, $p=0.046$ e FPG, $p=0.001$). Para esta componente, na fase III houve um aumento significativo para a FLD ($p=0.016$) apenas no pé teste e para a FPG houve um aumento significativo nos dois pés ($p=0.037$ e $p<0.001$). Finalmente, para a componente endoNOI, é observada uma diminuição na fase II na FLD e na FPG, mas apenas significativa ($p<0.001$) no pé de teste para a FPG. Na fase III, uma recuperação foi verificada, com um aumento significativo para a FLD nos dois pés ($p<0.001$ para ambos os pés) e para a FPG uma diminuição significativa ($p<0.001$) no pé teste.

Para os componentes oscilatórios obtidos com a TO da Md (Figura 4), a fase II mostra uma diminuição não significativa nos componentes cardíacas, tanto para a FLD quanto para a FPG. Também é observado um aumento na componente respiratória durante a fase II, mas apenas significativo ($p<0.001$ em ambos os casos) para o pé de teste. Para esta componente na fase III é apresentada, para a FPG, uma diminuição significativa apenas no pé teste. É observado um aumento na componente miogénica durante a fase II para ambas as tecnologias e em ambos os pés, significativo para a FLD em ambos os pés ($p=0.010$ e $p=0.003$). Esta componente mostra para a FLD na fase III um aumento significativo, no pé controle ($p=0.002$), e para a FPG uma diminuição

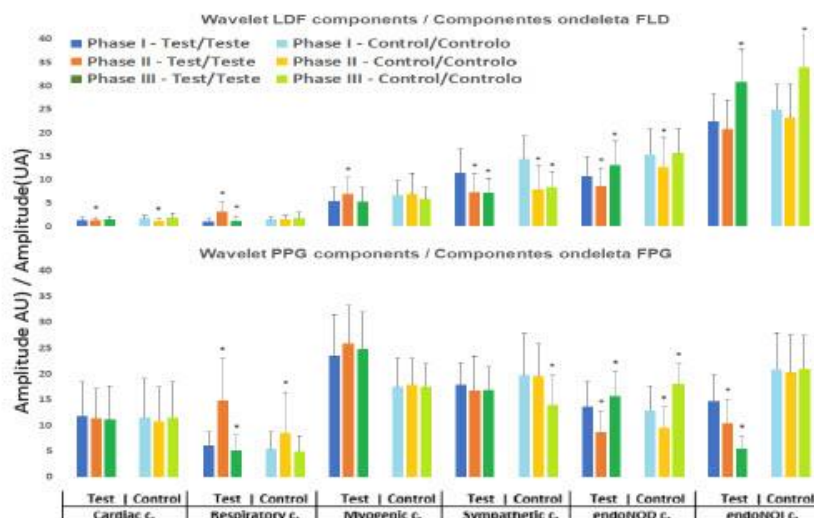


Figure 3/ Figura 3 - Component analysis of each phase of the upM protocol obtained by the Wavelet Transform of LDF and PPG signals (see text). Statistical comparison of the mean values of Phases II and III relative to Phase I is presented (* $p < 0.05$).
Análise de componentes de cada fase do protocolo upM obtido pela Transformada Wavelet dos sinais LDF e PPG (ver texto). É ainda apresentada a comparação estatística dos valores médios das fases II e III em relação à fase I (* $p < 0,05$).

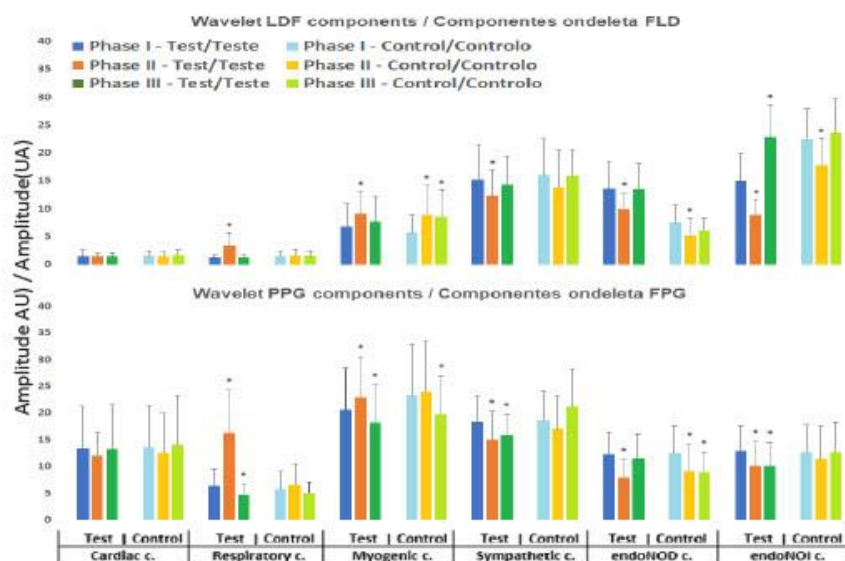


Figure 4/ Figura 4 - Component analysis of each phase of the dwM protocol obtained by the Wavelet Transform of LDF and PPG signals (see text). Statistical comparison of the mean values of Phases II and III relative to Phase I is presented (* $p < 0.05$).
Análise de componentes de cada fase do protocolo dwM obtido pela Transformada Wavelet dos sinais LDF e PPG (ver texto). É ainda apresentada a comparação estatística dos valores médios das fases II e III em relação à fase I (* $p < 0,05$).

Contribuição para uma abordagem não convencional da Função Vascular Periférica
- estudo do impacto fisiológico da massagem na circulação do membro inferior

Perfusion impact of massage in the human lower limb
Impacto da massagem na perfusão do membro inferior humano

Phase III showed a significant decrease for the test foot ($p=0.032$). In Phase II, a significant decrease in the endoNOD component was noted by LDF and PPG in both test (LDF, $p=0.002$ and PPG, $p<0.001$) and control feet (LDF, $p=0.003$ and PPG, $p=0.015$). In Phase III for this component, a significant decrease for PPG ($p=0.001$) was shown only in the control foot. Finally, for the endoNOI component, a decrease was shown in Phase II for both LDF and PPG, significant for LDF in both feet ($p<0.001$ and $p=0.001$), and for PPG in the test foot ($p=0.007$). In Phase III a significant increase was shown for this component in the test foot ($p<0.001$) for LDF while for PPG a significant decrease ($p=0.004$) was shown for the same test foot.

The correlation analysis between LDF and PPG for both protocols are shown in Table 2. Regarding the upM protocol, a significant correlation for the cardiac component ($p=0.014$) in the test foot and a non-significant correlation for the control foot was noted. A non-significant correlation for the respiratory component in the test foot and a significant correlation

significativa nos dois pés ($p=0.011$ e $p=0.005$). Durante a fase II, as componentes simpáticas mostram uma diminuição para ambas as tecnologias em ambos os pés, apenas significativa no pé teste ($p=0.034$ e $p=0.005$). Ainda na componente simpática, a FPG durante a fase III mostra uma diminuição significativa para o pé de teste ($p=0.032$). Na componente endoNOD, durante a fase II, nota-se uma diminuição significativa da FLD e FPG em ambos os pés teste (FLD, $p=0.002$ e FPG, $p<0.001$) e controlo (FLD, $p=0.003$ e FPG, $p=0.015$). Na fase III para esta componente, é apresentada uma diminuição significativa para a FPG ($p=0.001$), apenas no pé controlo. Finalmente, para a componente endoNOI, é apresentada uma diminuição na fase II, tanto para a FLD quanto para a FPG, apenas significativa para a FLD em ambos os pés ($p<0.001$ e $p=0.001$) e para FPG no pé teste ($p=0.007$). Na fase III, um aumento significativo é mostrado para esta componente no pé de teste ($p<0.001$) para a FLD, enquanto que para a FPG é mostrado um decréscimo significativo ($p=0.004$) para o mesmo pé de teste.

Table 2/ Tabela 2 - Spearman's components comparison obtained by LDF and PPG in both experimental protocols (upward and downward). Rho coefficients and p for each protocol are shown for the test and contralateral feet (* $p < 0.05$; ** $p < 0.001$)

Comparação de Spearman dos componentes obtidos por LDF e PPG em ambos os protocolos experimentais (para cima e para baixo). Os coeficientes de Rho e p para cada protocolo são mostrados para os pés teste e contralateral (* $p < 0,05$; ** $p < 0,001$)

LDF vs PPG FLD vs FPG	Test foot Pé teste	Contralateral control foot Pé controlo contralateral
Cardiac / Cardíaco upM dwM	$\rho = 0.468$; $p = 0.014^*$ $\rho = 0.559$; $p = 0.002^{**}$	$\rho = 0.359$; $p = 0.066$ $\rho = 0.426$; $p = 0.027^*$
Respiratory / Respiratório upM dwM	$\rho = 0.327$; $p = 0.096$ $\rho = 0.247$; $p = 0.223$	$\rho = 0.645$; $p < 0.001^{**}$ $\rho = 0.483$; $p = 0.011^{**}$
Myogenic / Miogénico upM dwM	$\rho = 0.533$; $p = 0.004^{**}$ $\rho = 0.699$; $p < 0.001^{**}$	$\rho = 0.410$; $p = 0.034^*$ $\rho = 0.587$; $p = 0.001^{**}$
Sympathetic / Simpático upM dwM	$\rho = 0.208$; $p = 0.298$ $\rho = 0.542$; $p = 0.004^{**}$	$\rho = 0.391$; $p = 0.044^*$ $\rho = 0.509$; $p = 0.007^{**}$
EndoNOD upM Protocol dwM Protocol	$\rho = 0.270$; $p = 0.173$ $\rho = 0.272$; $p = 0.169$	$\rho = 0.473$; $p = 0.013^*$ $\rho = 0.401$; $p = 0.038^*$
EndoNOi upM dwM	$\rho = 0.179$; $p = 0.371$ $\rho = 0.374$; $p = 0.055$	$\rho = 0.365$; $p = 0.061$ $\rho = 0.275$; $p = 0.165$

for the control foot ($p < 0.001$) was also shown. For the myogenic component, a significant correlation was detected in both feet ($p = 0.004$ for test foot and $p = 0.034$ for control foot) while for the sympathetic component, a non-significant correlation was detected in the test foot, and a significant correlation for the control foot ($p = 0.044$). Regarding the endoNOD component, a non-significant correlation was shown in the test foot and a significant correlation for the control foot ($p = 0.013$). For endoNOI component, a non-significant correlation was noted for both feet. For the dwM protocol, the cardiac component shows a significant correlation in both feet ($p = 0.002$ for tested foot and $p = 0.027$ for control foot), while the respiratory component showed a non-significant correlation in the test foot and a significant correlation in the control foot ($p = 0.011$). Regarding the myogenic component, a significant correlation was shown in both feet ($p < 0.001$ for the test foot and $p = 0.001$ for the control foot), while the sympathetic component showed a significant correlation in both feet ($p = 0.004$ for the test and $p = 0.007$ for the control). Regarding the endothelial components, the endoNOD showed a non-significant correlation for the test foot and a significant correlation for the control foot ($p = 0.038$) while, a non-significant correlation was noted for both feet (Table 2) for the endoNOI.

Discussion

Massage has long been regarded as a non-conventional procedure, supported by experience and evidence-based results. It serves, nevertheless, a broad range of applications reflecting its growing demand and popularity. Our studies on massage effects over microcirculation in vivo tend to contribute to a more mechanistic definition of the procedure by identifying the main physiological intervenients^{23, 7, 24}. We have chosen the “effleurage” technique, which consists on soft stroking with both hands¹⁶, a reference in clinical practice involving the movement application in the upward direction, and compare it with movement in the opposite direction²⁴. Additionally, we have chosen modern, easy-to-use non-invasive register systems, such as PPG, and compare it

A Tabela 2 contém a análise de correlação entre FLD e FPG para os dois protocolos. Em relação ao protocolo de Ma, observa-se uma correlação significativa para a componente cardíaca ($p = 0.014$) no pé teste e uma correlação não significativa para o pé controle. Também é mostrada uma correlação não significativa para a componente respiratória no pé de teste e uma correlação significativa para o pé de controle ($p < 0.001$). Para a componente miogénica, é detetada uma correlação significativa em ambos os pés ($p = 0.004$ para o pé teste e $p = 0.034$ para o pé controle), enquanto para a componente simpática, foi detetada uma correlação não significativa no pé teste e uma correlação significativa no pé controle ($p = 0.044$). Em relação à componente endoNOD, uma correlação não significativa é mostrada no pé teste e uma correlação significativa no pé controle ($p = 0.013$). Para a componente endoNOI, nota-se uma correlação não significativa para os dois pés. Para o protocolo de Md, a componente cardíaca apresenta uma correlação significativa em ambos os pés ($p = 0.002$ para o pé teste e $p = 0.027$ para o controle), enquanto a componente respiratória mostra uma correlação não significativa no pé teste e uma correlação significativa no pé de controle ($p = 0.011$). Em relação à componente miogénica, é demonstrada uma correlação significativa em ambos os pés ($p < 0.001$ para o pé teste e $p = 0.001$ para o pé controle), assim como a componente simpática apresenta também uma correlação significativa em ambos os pés ($p = 0.004$ para o teste e $p = 0.007$ para o controle). Em relação às componentes endoteliais, a endoNOD mostra uma correlação não significativa para o pé teste e uma correlação significativa para o pé controle ($p = 0.038$) enquanto que em relação ao endoNOI, nota-se uma correlação não significativa para os dois pés (Tabela 2).

Discussão

A massagem é há muito considerada como um procedimento não convencional, apoiado pela experiência e resultados baseados na evidência. Esta serve, no entanto, uma ampla gama de aplicações, refletindo a sua crescente procura e popularidade. Os nossos estudos sobre os efeitos da massagem na microcirculação in vivo tendem a contribuir para uma definição mais mecanicista do procedimento, identificando os principais intervenientes fisiológicos^{23, 7, 24}. Escolhemos a técnica de afluoramento, que consiste no movimento suave com as duas mãos¹⁶, uma referência na prática clínica que envolve a mobilização no sentido ascendente, e comparamos com o movimento na direção oposta²⁴. Além disso, escolhemos sistemas de leitura não invasivos,

with reference technologies such as LDF ^{4,7,6}. This new generation of PPG instrumentation and software have greatly improved the signal acquisition and readability by enhanced filtering, using separating sensor, acquisition and transmission power supplies, reducing main sources of noise such as wires ²⁵. Taken together, this improvement guarantees better signal quality (Figure 2), while apparently allowing a similar level of mathematical modelling analysis by the WT as LDF ²⁶.

As shown in Table 1, where LDF and PPG raw signals are compared in each phase of both massage protocols, we can see that these two techniques are highly and significantly correlated, especially in Phases I and III. In Phase II, the Rho decreases and for the downward protocol the statistical significance disappears. In fact, this might already suggest that these two protocols have different impacts on the regional circulation, most probably related with the different mobilizations affecting perfusion toward (downward) or away (upward maneuver) sensors. However, this analysis also confirms another intriguing, recently suggested, ²⁷ feature- that the response to the massage in the test limb also impacts the contralateral limb. This is apparent in the example shown in Figure 2 where an increased perfusion, with lower magnitude, is noted in the contralateral limb a few moments after massage begins. This likely involves a centrally mediated regulation mechanism that maintains circulatory homeostasis in the lower limb region, a mechanism that should be further investigated.

WT from both technologies allowed the decomposition of each signal in its components, revealing similar oscillatory features (Figures 2 and 3) in both protocols during the massage (Phase II). A decrease in the cardiac component and an increase in respiratory components were detected for both protocols and technologies, although not significantly in all cases. The myogenic activity increased, confirming the same observation published before related with the blood pressure decrease ^{7,24,15}. The sympathetic component also decreased in both signals, with both protocols, which could also contribute to increase perfusion. Other previous studies reported an increase in the parasympathetic activity ¹⁸ eventually related with the pulse rate and blood pressure decrease found with both LDF and PPG signals ⁷. The decrease of endoNOD and endoNOI components during massage for both technologies and protocols would presume a decrease in blood flow in the lower limb, although the increase in the myogenic component and the simultaneous decrease in the sympathetic component promotes an increase in blood flow which overcomes the physiological effect of the endothelium components decrease and explains the increase in blood flow in lower limb here observed as previously described ⁷.

modernos e fáceis de usar, como o FPG, e compará-los com tecnologias de referência, como a FLD ^{4,7,6}. Esta nova geração de FPG melhorou muito a aquisição e a legibilidade do sinal utilizando filtros, separação das fontes de alimentação de aquisição e transmissão, reduzindo as principais fontes de ruído, tais como os fios ²⁵. Em suma, garantindo melhor qualidade de sinal (Figura 2), enquanto permite aparentemente um nível semelhante de análise de modelagem matemática com a TO como a FLD ²⁶.

Conforme apresentado na Tabela 1, onde os sinais brutos de FLD e FPG são comparados em cada fase dos dois protocolos de massagem, podemos ver que essas duas técnicas estão significativamente correlacionadas, em especial nas fases I e III de ambos os protocolos. Na fase II o Rho diminui e, para o protocolo descendente, a significância estatística desaparece. De fato, isto pode já sugerir que esses dois protocolos têm impactos diferentes na circulação regional, provavelmente relacionados com as diferentes mobilizações que se aproximam (manobra descendente) ou se afastam (manobra ascendente) dos sensores de perfusão. Entretanto, esta análise também confirma outra característica intrigante, recentemente sugerida ²⁷, ou seja, que a resposta à massagem no membro teste também afeta o membro contralateral. Este efeito é muito claro no exemplo mostrado na Figura 2, onde um aumento da perfusão, com menor magnitude, é observado no membro contralateral alguns momentos após o início da massagem. Tal poderá envolver um mecanismo de regulação central, que mantém a homeostase circulatória na região inferior, que deverá ser melhor investigado.

A TO de ambas as tecnologias permitiu a decomposição de cada sinal nos seus componentes, revelando, em ambos os protocolos, características oscilatórias semelhantes (Figuras 2 e 3) durante a massagem (fase II). Uma diminuição na componente cardíaca e um aumento nos componentes respiratórios foram detectados para ambos os protocolos e tecnologias, embora não significativamente. A atividade miogénica aumentou, confirmando a mesma observação publicada anteriormente relacionada com a diminuição da pressão arterial ^{7,24,15}. A componente simpática também diminuiu em ambos os sinais, com ambos os protocolos, o que também poderia contribuir para o aumento da perfusão. Outros estudos relataram um aumento na atividade parassimpática ¹⁸, eventualmente relacionada com a frequência de pulso e diminuição da pressão arterial encontrados com os sinais de FLD e FPG ⁷. A diminuição dos componentes endoNOD e endoNOI durante a massagem para ambas as tecnologias e protocolos presumiria uma diminuição no fluxo sanguíneo no membro inferior, embora o aumento da componente miogénica e a diminuição simul-

LDF and PPG Spearman component analysis showed weak to moderate correlations although the direction of the component response is generally coincident between the two technologies (Figures 3 and 4). Therefore, comparing both technologies, by raw signals and WT components, we may conclude we found similar behaviour and responses, although a few differences prevail, especially for Phase III. The differences observed are likely related to the different light interactions occurring within the tissues^{28, 29, 30}.

Finally, regarding the massage procedures, we found similar responses with both protocols, indicating that the physiological impact of the upward and the downward massage is nearly the same. Both protocols evoked perfusion changes in the same direction, and both protocols affected the contralateral limb, although the response to the upM maneuver seems to underline the importance of the myogenic component in the test foot.

Conclusions

Our study demonstrates that a regional circulatory adaptation seems to take place even when a transitory event such as single limb massage, interferes with circulatory homeostasis in the limb pair. In this respect both massage manoeuvres show the same impact, although some differences are detected regarding the oscillatory components of LDF and PPG. The use of both technologies provide a more supported and sensitive analysis, but the modern PPG systems seem to be well-suited to quantitatively describe in vivo peripheral microcirculation.

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tânea da componente simpática promovam um aumento do fluxo sanguíneo que supera o efeito fisiológico da diminuição das componentes endoteliais, e explica o aumento do fluxo sanguíneo nos membros inferiores, aqui observado e anteriormente descrito⁷.

A análise de Spearman das componentes de FLD e FPG mostrou correlações fracas a moderadas, embora a direção da resposta da componente seja geralmente coincidente entre as duas tecnologias (Figuras 3 e 4). Portanto, comparando ambas as tecnologias, nos seus sinais brutos e nos seus componentes, podemos concluir que encontramos em geral, comportamentos e respostas semelhantes, embora algumas diferenças prevaleçam, especialmente para a fase III, provavelmente relacionada com as diferentes interações de luz que ocorrem nos tecidos^{28, 29, 30}.

Finalmente, em relação aos procedimentos de massagem, encontramos respostas semelhantes com ambos os protocolos, o que significa que o impacto fisiológico da massagem ascendente e descendente está muito próximo. Ambos os protocolos evocaram alterações de perfusão na mesma direção, ambos os protocolos afetaram o membro contralateral, embora a resposta à manobra de Ma pareça sublinhar a importância da componente miogénica no pé teste.

Conclusões

O nosso estudo demonstra que ocorre uma adaptação circulatória regional mesmo quando um evento transitório, como no caso a massagem de um membro tem lugar, interferindo na homeostase circulatória de ambos os membros. A esse respeito, ambas as manobras de massagem mostram igualmente o mesmo impacto, pese embora algumas diferenças sejam detetadas em relação aos componentes oscilatórios do FLD e do FPG. O uso de ambas as tecnologias fornece uma análise mais fina e apoiada, mas este novo sistema FPG parece ser perfeitamente adequado para descrever quantitativamente a microcirculação periférica in vivo.

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Perfusion impact of massage in the human lower limb
Impacto da massagem na perfusão do membro inferior humano

Conflict of Interests

The authors declare there are no financial and/or personal relationships that could present a potential conflict of interests.

Conflito de Interesses

Os autores declaram que não há relações financeiras e/ou pessoais que possam representar um potencial conflito de interesses.

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Artigo III - Texture Analysis is a Useful Tool to Assess the Complexity Profile of Microcirculatory Blood Flow

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Article

Texture Analysis is a Useful Tool to Assess the Complexity Profile of Microcirculatory Blood Flow

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Abstract: The quantitative assessment of cardiovascular functions is particularly complicated, especially during any physiological challenge (e.g., exercise), with physiological signals showing intricate oscillatory properties. Signal complexity is one of such properties, and reflects the adaptability of the physiological systems that generated them. However, it is still underexplored in vascular physiology. In the present study, we calculate the complexity of photoplethysmography (PPG) signals and their frequency components obtained with the wavelet transform (WT), with two analytical tools—(i) texture analysis (TA) of WT scalograms, and (ii) multiscale entropy (MSE) analysis. PPG signals were collected from twelve healthy young subjects (26.0 ± 5.0 y.o.) during a unilateral leg lowering maneuver to evoke the venoarteriolar reflex (VAR) while lying supine, with the contralateral leg remaining stationary. Results showed that TA was able to detect a decrease in complexity, viewed as an increase in texture entropy (TE), of the PPG scalograms during VAR, similarly to MSE, suggesting that a decrease in the competence of vascular regulation mechanisms might be present during VAR. Nonetheless, TA showed lower sensitivity than MSE for low frequency spectral regions. TA seems to be a promising and straightforward analytical tool for the assessment of the complexity of PPG perfusion signals.

Keywords: signal complexity; texture analysis; multiscale entropy analysis; wavelet transform; photoplethysmography

1. Introduction

The regulation of the cardiovascular system results from the adjustment of several biophysical phenomena, both electrical and mechanical. Their coordination is highly complex, depending on the continuous feedback and cross-talk between effector organs and controlling systems [1]. The heart pump and respiration are the most notorious “central” processes governing the performance of the cardiovascular system, while on a more “peripheral” level, the phenomena that control vascular tone, such as the myogenic activity of the vessel wall, the sympathetic activity and the endothelial release of vasoactive substances, play crucial roles [2–4]. Microcirculation signals are composed of both these central and peripheral components and therefore provide an “integrated” view of cardiovascular function [4,5]. The contributions from these multiple regulation systems and their intricate interplay explain the oscillatory properties of microcirculation signals. From an analytical standpoint, these oscillatory properties are increasingly considered as a means to extract more sensible information

not apparent from the general analysis of the raw signals, in particular their spectral origin and the significance of their fractal and chaotic profiles [5–7]. In recent years, much attention has been given to the study of the complexity of physiological signals, a property that reflects the adaptability of the systems that generated them. Intuitively, the term complexity is often associated with “structural richness” [8], which can be applied to the study of biological signals and images. Complexity is typically assessed as “entropy,” a general measure of “disorganization” in physical, chemical and biological phenomena [9]. In biomedical research, entropy is used as a quantitative parameter, and is typically assessed in continuous, but temporally unpredictable, physiological signals, such as electrocardiography [10], electroencephalography [11], blood pressure [12], laser Doppler flowmetry (LDF) [13], and photoplethysmography (PPG) [14]. Physiological signals may be regular (e.g., more periodic) or irregular. The higher entropy measured in irregular signals reflects the adaptation capability to changing internal and external conditions of the biological system that generated them [15]. Overall, the entropy of physiological signals tends to decrease whenever the systems that generated them lose adaptability, which can result in a compromise of their function or even in disease. Therefore, a suitable metric of complexity should assign higher values to the output signal of a “healthier” system with a rich and meaningful structure and lower values to either random dynamics or predictable systems, which are often associated with disease [8,16,17]. This has been observed in the aging process [18,19] and in cardiovascular, metabolic and neurological diseases [18], among several others. In addition to pathological states, some experimental procedures are sufficiently “challenging” to temporarily provoke tissue dysfunction, especially those that reduce perfusion [6]. For continuous signals, the multiscale entropy analysis (MSE) is considered a robust analytical tool, having shown superiority over other measures for its ability to assess entropy over different time scales [19]. Entropy can also be assessed in texturally rich biological images, reflecting their textural “disorganization.” Texturally rich images can be assessed with texture analysis (TA), an analytical tool allowing the detection of various features on a gray-level image to discriminate textural differences, such as texture entropy (TE), contrast, correlation, homogeneity, and energy. Entropy calculated from TA of images may reflect different information than that conveyed by the entropy assessment of continuous numerical series. When applied to a grayscale image, high entropy means that the pixels can adopt a high number of gray levels, expressing richer information [20]. TA has been used in medical imaging with the purpose of improving diagnostic capacity, given that human visual inspection constitutes a process that is expensive, time-consuming and prone to interpretation errors [21]. Thus far, TA in medical imaging has been more frequently applied to mammography [22], ultrasonography, computerized tomography (CT) [23], and magnetic resonance imaging (MRI) [24] techniques [25], as well as to dermatoscopy [26] and microscopy images [27]. In particular, TE has been used to explore immune competence of lymphoid tissue in microscopic images [28], to assess the onset organ (liver) disease and tumor (colorectal cancer) evolution in CT scans [29–31], and to assess bone regeneration in fractures [32] and osteoarthritis [33], among others. In medical imaging, TE does not necessarily decrease when images display abnormal features. In fact, since abnormal or dysmorphic features often increase the coarseness of smooth or uniform images from healthy subjects, increases in TE are often seen in pathological images, such as in the case of aged tissues and organs [34] as well as the case of neoplastic formations [30,31]. In these situations, a richer texture and therefore, higher entropy, is associated with dysfunction. Although currently underexplored in vascular physiology, the complexity assessment of microcirculation signals, both raw and decomposed, may deepen our knowledge of the mechanisms underlying perfusion regulation. Microcirculation signals are easily decomposed into their frequency components by the wavelet transform (WT) [4]. One of the WT main outputs is a scalogram, i.e., a multi-patterned image representation of the time evolution of all components of the decomposed signal. Given their textural richness, WT scalograms seem suitable candidates for TE analysis, however, to our knowledge this analysis has not been previously attempted. Since WT scalograms are not medical images, the interpretation of TE in this context must be necessarily different from the one performed in medical diagnosis. Given the fact that a WT scalogram is a 2D projection of

a 3D frequency spectrum, (i.e., the 3D spectrum seen from above), it is only logical to assume that a change in the entropy of the numerical series that constitute the 3D spectrum would also be observed in corresponding WT scalograms. Therefore, the assumption that similar trends in complexity would be observed with MSE and TA seems legitimate. Our objective was to compare the complexity of microcirculation PPG signals calculated with TE against MSE during a typical venoarteriolar reflex (VAR).

2. Materials and Methods

2.1. Experimental

Twelve healthy young adult subjects (26.0 ± 5.0 y.o., seven females, five males) participated in this study after giving informed written consent. All subjects were healthy, with no cardiovascular diseases, were non-smokers, and abstained from consuming alcohol and caffeine-containing beverages 24 hours prior to the experimental procedure. The protocol was approved by the School of Health Sciences and Technologies' Ethical Commission. All subjects gave written informed consent, and the study was conducted in accordance with the Declaration of Helsinki and subsequent amendments [35]. After 20 minute acclimatization to room conditions (temperature: 22 ± 1 °C, humidity: 40–60%), a postural challenge to elicit the VAR was applied, as previously described [4]—10 minutes lying supine with both legs extended (baseline, Phase I); 10 minutes with one foot lowered 50 cm from heart level (challenge, Phase II); and 10 minutes resuming the initial posture (recovery, Phase III). The contralateral foot remained stationary and served as control. Blood flow signals, expressed in arbitrary units (AU), were acquired from the first toe of both feet at a 100 Hz sampling rate with a reflection photoplethysmography Blood Pulse Volume sensor (Biosignals Flux, Lisboa, Portugal) connected to a BITalino Plugged microprocessor board (Biosignals Flux).

2.2. Numerical

The raw PPG signals were imported to Matlab (Mathworks R2012, Natick, MA, USA) and smoothed with a moving average filter.

The WT (<http://noc.ac.uk/using-science/crosswavelet-wavelet-coherence>) was then applied to the smoothed signals, which allowed the decomposition into their main frequency components. Wavelets are a family of functions constructed from translations and dilations of a single function called the “mother wavelet” $\psi(t)$, collectively defined by:

$$\psi_{a,b}(t) = \frac{1}{\sqrt{|a|}} \psi\left(\frac{t-b}{a}\right), a, b \in \mathbb{R}, a \neq 0 \quad (1)$$

The parameter a is the scaling parameter or scale, measuring the degree of compression, while parameter b is the translation parameter, determining the time location of the wavelet. If $|a| < 1$, then the wavelet in the above equation is the compressed version of the mother wavelet and corresponds mainly to higher frequencies. If $|a| > 1$, then $\psi_{a,b}(t)$ has a larger time-width than $\psi(t)$ and corresponds to lower frequencies. Thus, wavelets have time-widths adapted to their frequencies.

In this case, the central frequency of the wavelet was 6 Hz, with a discretization factor of 10 scales per octave. The detected components of the PPG signals occurred at the following frequency ranges: cardiac [1.6–0.7 Hz], respiratory [0.7–0.26 Hz], myogenic [0.26–0.1 Hz], neurogenic/sympathetic [0.1–0.045 Hz], endothelial NO-dependent (NOd) [0.045–0.015 Hz] and NO-independent (NOi) [0.015–0.007 Hz], which were in line with the ranges previously described for LDF [36]. From the WT, two main outputs were generated for each subject: (1) a scalogram and (2) a 3D periodogram. The WT scalogram (Figure 1) shows the time (here converted to sample number) evolution of the amplitude with a factor of 2^n (here in color scale) for each signal scale. The approximate frequency of a component is obtained by dividing the central wavelet frequency by the scale of the component. From a visual inspection, the WT scalogram also presents an oscillatory pattern in pixel distribution on different scales, making it a

suitable candidate for TA. The scalogram was then used as a grayscale image, where several regions of interest (ROI) were marked, each per component per phase of the protocol. Each ROI was then converted to a gray-level co-occurrence matrix (GLCM), a matrix where the number of rows and columns is equal to the number of gray levels (G) in the image, from which the TE was calculated as follows [37]:

$$\text{Entropy} = - \sum_{i=0}^{G-1} \sum_{j=0}^{G-1} P(i, j) \cdot \log[P(i, j)] \quad (2)$$

where $P(i, j)$ denotes the probability of occurrence of a given element in the matrix.

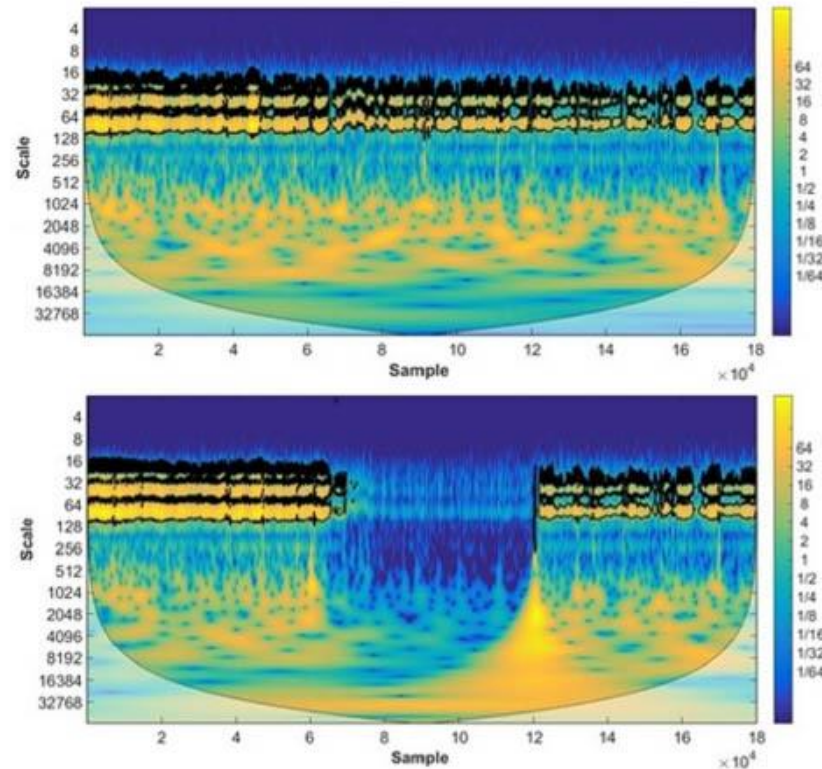


Figure 1. WT scalogram of the PPG signal from the control (top) and test (bottom) feet from a representative subject (20 y.o.).

To correctly select each ROI, a control sine wave signal was generated with multiple components occurring at known frequencies ($f_1 = 3.2$ Hz; $f_2 = 1.6$ Hz; $f_3 = 0.7$ Hz; $f_4 = 0.26$ Hz; $f_5 = 0.1$ Hz; $f_6 = 0.045$ Hz; $f_7 = 0.015$ Hz; $f_8 = 0.007$ Hz), where each frequency is close to the ones that define the borders of the PPG components' detected frequency ranges. The sine wave signal was then deconstructed with the WT, and a reference scalogram was generated (Figure 2).

From the 3D periodogram (Figure 3), a 2D time evolution of the amplitude of each component was constructed by averaging the amplitude at each time point for each spectral interval (Figure 4). Signals were acquired at a 100 Hz sampling rate, which translates to a total number of 180,000 samples for the entire 30 minute protocol. The wavelet period is a natural logarithmic representation of the wavelet scale, with the frequency in Hz exponentially related to this period. The time evolution was

analyzed for both the raw PPG signal and its components with MSE, an algorithm that quantifies the randomness/unpredictability of a signal over different time scales as follows [19]:

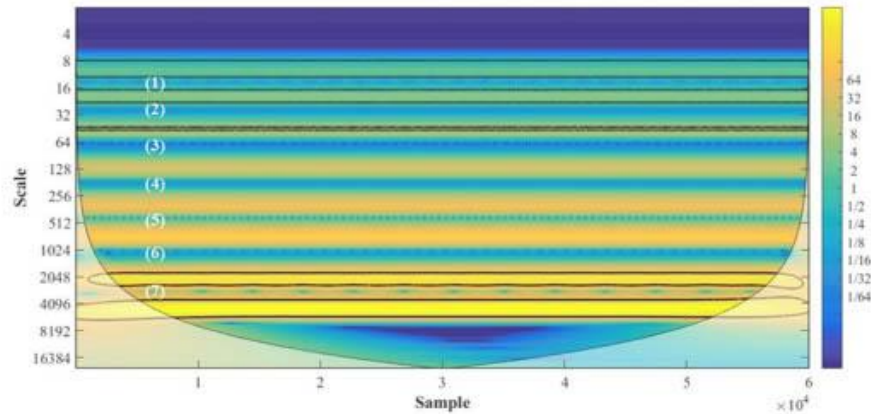


Figure 2. WT scalogram of the reference sine wave signal. The region corresponding to each component frequency interval is shown (1—first harmonic of the cardiac component; 2—cardiac; 3—respiratory; 4—myogenic; 5—sympathetic; 6—endothelial NO-dependent; 7—endothelial NO-independent).

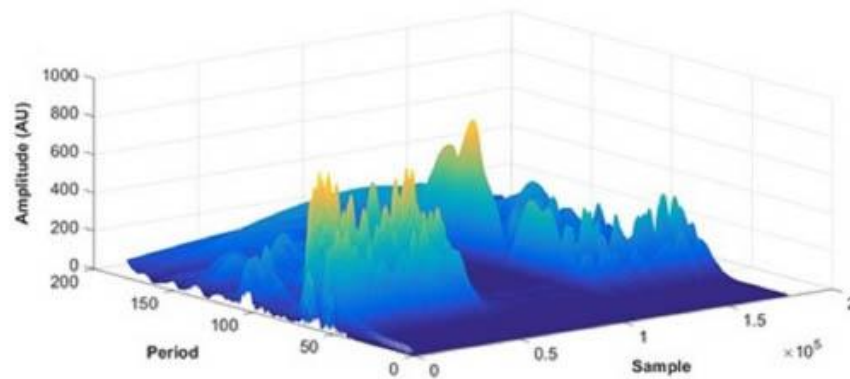


Figure 3. PPG 3D frequency spectrum (period vs. sample vs. amplitude) for the test foot of a representative subject (20 y.o.). Signals were acquired at a 100 Hz sampling rate, which translates to a total of 180,000 signal samples for a 30 minute acquisition period. Period is a natural logarithmic representation of the wavelet scale.

Given a time series $x_i = 1, \dots, N$, a consecutive coarse-grained time series $y(\tau)$ is constructed:

$$y_j^{(\tau)} = \frac{1}{\tau} \sum_{i=(j-1)\tau+1}^{j\tau} x_i \quad (3)$$

where τ represents the scale factor and $1 \leq j \leq N/\tau$. The sample entropy (*SampEn*) of each coarse-grained time series is then computed. *SampEn*(m, r, N) is the negative natural logarithm of the

conditional probability that a dataset of length N , having repeated itself with a tolerance of r points, will also repeat itself for $m + 1$ points, without allowing self matches:

$$\text{SampEn}(m, r, N) = -\ln \frac{A^m(r)}{B^m(r)} \quad (4)$$

where $A^m(r)$ is the probability that two sequences will match for $m + 1$ points and $B^m(r)$ is the probability that two sequences will match for m points. The more regular and predictable a time series is, the lower the value of SampEn . The more random a time series is, the higher the value of SampEn . Plotting the SampEn over the scale factor yields the MSE curve, which gives insight into the integrated complexity of the system over the time scales of interest, which can be of interest when comparing groups where differences in specific time scales are probable. The randomness/unpredictability of the signal can finally be straightforwardly summarized as the complexity index (CI), which corresponds to the area under the MSE curve [38]. The CI and TE were statistically compared between each phase of the protocol with the Wilcoxon signed-rank test, and were compared between feet for each phase with the Mann-Whitney independent sample test, adopting a 95% confidence interval.

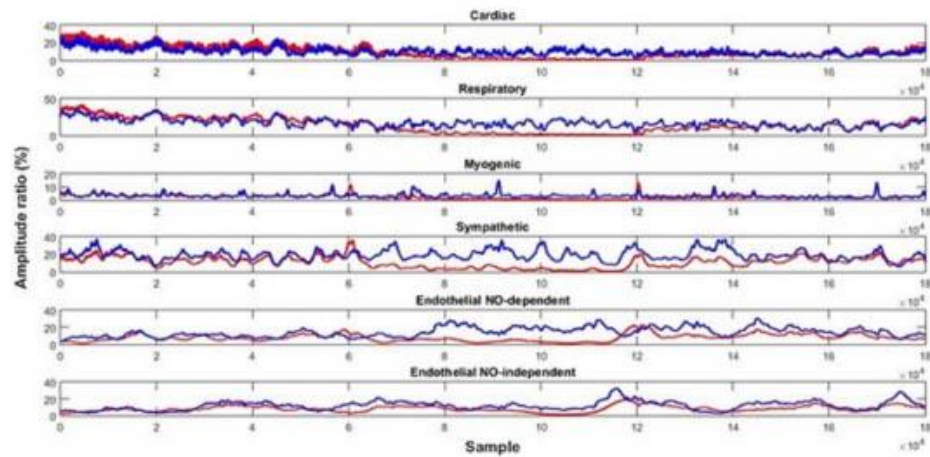


Figure 4. Time evolution of the PPG signal components' amplitude ratio for the control (blue) and test (red) feet throughout the protocol (baseline: signal samples 0–60,000; challenge: signal samples 60,000–120,000; recovery: signal samples 120,000–180,000) for a representative subject (20 y.o.).

3. Results

As previously published, during the postural challenge, perfusion decreased significantly on both the test and control feet [4]. The mean and standard deviation (SD) of the entropy parameter (CI) calculated with MSE is presented in Table 1. For the test foot, the CI of the raw PPG signal and all its components decreased significantly during the challenge (Phase II, raw signal: $p = 0.005$; cardiac: $p = 0.012$; respiratory: $p = 0.002$; myogenic: $p = 0.003$; sympathetic: $p = 0.002$; NOd: $p = 0.002$; NOi: $p = 0.002$). For the control foot, the CI of the raw PPG signal showed an increase without statistical significance. All the components' CI decreased, with the exception of the cardiac, although not so pronounced as in the test foot (respiratory: $p = 0.002$; myogenic: $p = 0.003$; sympathetic: $p = 0.002$; NOd: $p = 0.002$; NOi: $p = 0.002$). During recovery (Phase III), significant differences were still detected on both feet regarding baseline for the respiratory (test: $p = 0.002$; control: $p = 0.002$), myogenic (test: $p = 0.008$; control: $p = 0.008$), sympathetic (test: $p = 0.002$; control: $p = 0.002$), NOd (test: $p = 0.002$; control: $p = 0.002$) and NOi (test: $p = 0.002$; control: $p = 0.003$). The mean and SD of the entropy parameter (TE) calculated for the sine wave and PPG signals with TA is shown in Table 2. For the sine wave

signal scalogram, TE decreased from the cardiac component towards the low frequency components, where it increased again for the NOi. For the test foot PPG scalogram, TE decreased significantly in the cardiac ($p = 0.004$), respiratory ($p = 0.013$), and myogenic ($p = 0.008$) components, increased significantly in the NOd ($p = 0.012$) and NOi ($p = 0.010$), and showed no change in the sympathetic component. During recovery, all components' TE returned except for the NOd, which showed a significant difference regarding baseline ($p = 0.007$). For the control foot PPG scalogram, changes in TE were not so pronounced as in the test foot, with only the cardiac component showing a significant difference regarding baseline ($p = 0.001$). During recovery, only the cardiac component showed a significant difference ($p = 0.022$) regarding baseline. No significant differences in entropy were found between feet at baseline (Tables 1 and 2) either with CI or TE. During the challenge phase, significant differences were found for the raw signal ($p < 0.001$) and all components ($p = 0.001$ for the respiratory; $p < 0.001$ for the remaining) with CI. Similarly, TE showed significant differences in all components (cardiac: $p = 0.001$; respiratory: $p = 0.045$; myogenic: $p = 0.028$; NOd: $p = 0.001$; NOi: $p = 0.001$), except the sympathetic. During recovery, the CI of the sympathetic ($p = 0.012$), NOd ($p = 0.002$) and NOi ($p < 0.001$) were significantly different, while with TE no statistical differences were found.

Table 1. Mean and standard deviation (SD) of the MSE complexity index for each foot on each phase of the protocol (card—cardiac, resp—respiratory, myo—myogenic, sym—sympathetic, NOd—endothelial NO-dependent, NOi—endothelial NO-independent). Statistical comparison to Phase I is shown (*— $p < 0.05$).

Complexity Index		Test Foot		Control Foot		p-Value (T vs. C)
		Mean $\pm$ SD	p-Value	Mean $\pm$ SD	p-Value	
raw signal	Phase I	34.8 $\pm$ 6.1	-	34.2 $\pm$ 8.2	-	0.843
	Phase II	15.0 $\pm$ 11.5	0.005 *	35.9 $\pm$ 8.7	0.182	<0.001 *
	Phase III	31.4 $\pm$ 7.3	0.182	37.0 $\pm$ 6.7	0.239	0.089
card	Phase I	25.9 $\pm$ 6.1	-	29.3 $\pm$ 7.2	-	0.347
	Phase II	15.6 $\pm$ 9.0	0.012 *	28.7 $\pm$ 6.5	0.754	<0.001 *
	Phase III	23.8 $\pm$ 7.3	0.224	29.7 $\pm$ 7.8	0.875	0.089
resp	Phase I	25.9 $\pm$ 6.1	-	29.3 $\pm$ 7.2	-	0.347
	Phase II	6.7 $\pm$ 4.2	0.002 *	14.3 $\pm$ 4.8	0.002 *	0.001 *
	Phase III	10.1 $\pm$ 5.5	0.002 *	13.7 $\pm$ 4.9	0.002 *	0.160
myo	Phase I	23.2 $\pm$ 8.7	-	25.9 $\pm$ 7.5	-	0.378
	Phase II	3.9 $\pm$ 3.1	0.003 *	12.0 $\pm$ 2.8	0.003 *	<0.001 *
	Phase III	7.9 $\pm$ 5.4	0.008 *	10.9 $\pm$ 5.8	0.008 *	0.160
sym	Phase I	25.9 $\pm$ 6.1	-	29.3 $\pm$ 7.2	-	0.347
	Phase II	2.6 $\pm$ 2.4	0.002 *	10.5 $\pm$ 1.9	0.002 *	<0.001 *
	Phase III	6.8 $\pm$ 2.2	0.002 *	10.7 $\pm$ 4.0	0.002 *	0.012 *
NOd	Phase I	25.9 $\pm$ 6.1	-	29.3 $\pm$ 7.2	-	0.347
	Phase II	2.5 $\pm$ 3.5	0.002 *	11.5 $\pm$ 2.6	0.002 *	<0.001 *
	Phase III	5.5 $\pm$ 2.1	0.002 *	11.0 $\pm$ 4.8	0.002 *	0.002 *
NOi	Phase I	25.9 $\pm$ 6.1	-	29.3 $\pm$ 7.2	-	0.347
	Phase II	1.9 $\pm$ 0.9	0.002 *	10.2 $\pm$ 3.6	0.002 *	<0.001 *
	Phase III	6.2 $\pm$ 3.4	0.002 *	13.6 $\pm$ 5.0	0.003 *	<0.001 *

Table 2. Mean and standard deviation (SD) of the texture entropy for each foot on each phase of the protocol (card—cardiac, resp—respiratory, myo—myogenic, sym—sympathetic, NOd—endothelial NO-dependent, NOi—endothelial NO-independent). Statistical comparison to Phase I and between the test and control limbs are shown (*— $p < 0.05$).

Texture Entropy	Sine Wave Signal	Test Foot		Control Foot		p-Value (T vs. C)
		Mean $\pm$ SD	p-Value	Mean $\pm$ SD	p-Value	
card	Phase I	6.6 $\pm$ 0.5	-	6.8 $\pm$ 0.3	-	0.198
	Phase II	6.2 $\pm$ 0.7	0.004 *	6.9 $\pm$ 0.3	0.011 *	0.001 *
	Phase III	7.0 $\pm$ 0.2	0.563	7.0 $\pm$ 0.2	0.022 *	0.713
resp	Phase I	6.5 $\pm$ 0.2	-	6.5 $\pm$ 0.2	-	0.551
	Phase II	5.5 $\pm$ 1.3	0.013 *	6.3 $\pm$ 0.2	0.286	0.045 *
	Phase III	6.5 $\pm$ 0.2	0.638	6.4 $\pm$ 0.2	0.530	0.478
myo	Phase I	6.6 $\pm$ 0.3	-	6.7 $\pm$ 0.1	-	0.089
	Phase II	6.3 $\pm$ 0.4	0.008 *	6.6 $\pm$ 0.1	0.433	0.028 *
	Phase III	6.5 $\pm$ 0.2	0.289	6.7 $\pm$ 0.2	0.754	0.266
sym	Phase I	6.2 $\pm$ 0.3	-	6.2 $\pm$ 0.4	-	0.671
	Phase II	6.2 $\pm$ 0.2	0.147	6.2 $\pm$ 0.2	0.814	0.887
	Phase III	6.0 $\pm$ 0.2	0.594	6.1 $\pm$ 0.3	0.239	0.630
NOd	Phase I	5.8 $\pm$ 0.5	-	5.9 $\pm$ 0.4	-	0.630
	Phase II	6.4 $\pm$ 0.2	0.012 *	5.7 $\pm$ 0.5	0.272	0.001 *
	Phase III	6.1 $\pm$ 0.3	0.007 *	5.9 $\pm$ 0.3	0.307	0.060
NOi	Phase I	6.0 $\pm$ 0.4	-	5.9 $\pm$ 0.4	-	0.551
	Phase II	6.2 $\pm$ 0.3	0.010 *	5.5 $\pm$ 0.7	0.753	0.001 *
	Phase III	6.2 $\pm$ 0.3	0.594	5.8 $\pm$ 0.8	0.126	0.060

4. Discussion

In this study, the acquired PPG signals and an artificial sine wave signal were decomposed with the WT into their respective components so that TE and CI could be calculated. The relative contributions of each of the components to the overall signal, expressed as amplitude ratios, are typical WT outputs but were not considered for this study as these results were previously published [4]. During leg lowering, perfusion decrease in the test foot is explained by a constriction of arterioles secondary to the increased venous distension when the foot is in a pendent posture, which constitutes the VAR [39]. The fast perfusion decreases in the control foot (not so pronounced as in the test foot) is thought to be due to a centrally mediated neurogenic reflex initiated to maintain the vascular homeostasis in the lower limb [4]. The sine wave signal scalogram showed TE values generally invariant regarding the component, which reflects the regularity of these signals. The highest TE values were recorded for both the highest (cardiac) and lowest (NOi) frequency components, meaning a richer texture was found in the corresponding ROIs, likely explained by the particular overlap of the spectral waves that define them. Contrarily, the PPG signal scalogram showed that the higher frequency (HF) components (cardiac, respiratory, myogenic) displayed higher TE values in either foot, meaning the corresponding ROIs are more texturally rich, i.e., more complex in comparison to the lower frequency components. This suggests that TE assessment of PPG scalograms is sensitive to the underlying phenomena that explain the physiological complexity of these signals. In our data, both TE and MSE analyses showed an overall decrease in the PPG signal and in the respective components' entropies during leg lowering in both the test and control feet. This result seems to indicate a bilateral decrease in the vascular adaptation capacity to the lower perfusion levels observed during leg lowering. This apparent decrease in adaptation capacity is further reinforced by the observation that several PPG components' entropies show significant differences between the recovery and baseline phases, suggesting that full recovery was not attained at the end of the protocol. In our view, these results indicate that that VAR creates a state of reduced perfusion that, while not compromising tissue viability, destabilizes the evoked regulatory mechanisms. When considering the test foot, MSE detected a significant decrease in the entropy of all components during VAR. TE, however, detected an entropy decrease in the cardiac,

respiratory, and myogenic components, an increase in both endothelial components and no change in the sympathetic. Regarding the control foot, an overall entropy decrease was observed with both tools. MSE recorded a decrease in the complexity of all components, although not so pronounced as in the test foot, which again is likely attributable to the magnitude of the perfusion decrease. Again, TE showed more subtle changes, only significant for the respiratory and NOi components. Overall, MSE and TE showed similar responses in the high frequency components (cardiac, respiratory, myogenic), and dissimilar responses in the low frequency components (sympathetic, NOd, NOi), suggesting that TE loses analytical sensitivity in the latter regions. This loss of sensitivity may be attributed to an incomplete visual resolution of the scalogram bands of the low frequency components, leading to a less precise identification of the respective ROIs. Nevertheless, TE was able to differentiate the entropies of most components during VAR between the test and control limbs. Overall, although both TE and MSE were able to detect differences in entropy, MSE was consistently more sensitive. One should keep in mind that MSE and TE ensure different scale evaluations—frequency for TE and time for MSE, which are two aspects of the same reality (i.e., perfusion signal). Thus, both analytical strategies provide different views into the same physiological event, which can also help to explain the differences in sensitivity found. MSE detected changes in entropy between limbs during both VAR and recovery phases, while TE was only able to detect the more pronounced changes that occurred during VAR. Furthermore, MSE was sensitive to changes in entropy for a greater number of components in comparison to TE. Although the performance of the reference MSE method was superior under the conditions studied, these results suggest that TE is an interesting and suitable tool for assessing the complexity of PPG microcirculation signals considered as WT scalograms, in particular for the analysis of high frequency components.

5. Conclusions

In this paper we present for the first time the use of texture entropy (TE) for the quantification of complexity in microcirculatory perfusion signals, using MSE as a reference analytical tool. Our results show that TE was able to detect a decrease in complexity of the PPG scalograms during VAR, similarly to MSE. This complexity decrease suggests that a decrease in the competence of vascular regulation mechanisms might be present during VAR and should be further investigated. Results from both tools were aligned for the high frequency components, but not for the low frequency components, suggesting a decrease in sensitivity of TE in the latter spectral regions. Recognizing the value of increasing the sample size (and heterogeneity) in future studies, our results have shown Texture Analysis (TA) to be a promising method to assess the complexity of PPG perfusion signals.

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Ethics Statement: This study was carried out in accordance with the recommendations of Ethical Principles for Medical Research Involving Human Subjects, Declaration of Helsinki. The protocol was approved by the School of Health Sciences and Technologies' Ethical Commission. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

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Artigo IV - Different lasers reveal different skin microcirculatory flowmotion – data from the wavelet transform analysis of human hindlimb perfusion

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Different lasers reveal different skin microcirculatory flowmotion - data from the wavelet transform analysis of human hindlimb perfusion

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Laser Doppler flowmetry (LDF) and reflection photoplethysmography (PPG) are standard technologies to access microcirculatory function *in vivo*. However, different light frequencies mean different interaction with tissues, such that LDF and PPG flowmotion curves might have distinct meanings, particularly during adaptative (homeostatic) processes. Therefore, we analyzed LDF and PPG perfusion signals obtained in response to opposite challenges. Young healthy volunteers, both sexes, were assigned to Group 1 ($n = 29$), submitted to a normalized Swedish massage procedure in one lower limb, increasing perfusion, or Group 2 ($n = 14$), submitted to a hyperoxia challenge test, decreasing perfusion. LDF (Periflux 5000) and PPG (PLUX-Biosignals) green light sensors applied distally on both lower limbs recorded perfusion changes for each experimental protocol. Both techniques detected the perfusion increase with massage, and the perfusion decrease with hyperoxia, in both limbs. Further analysis with the wavelet transform (WT) revealed better depth-related discriminative ability for PPG (more superficial, less blood sampling) compared with LDF in both challenges. Spectral amplitude profiles consistently demonstrated better sensitivity for LDF, especially regarding the lowest frequency components. Strong correlations between components were not found. Therefore, LDF and PPG flowmotion curves are not equivalent, a relevant finding to better study microcirculatory physiology.

Laser Doppler flowmetry (LDF) and imaging (LDI) are the gold standard technologies in vascular medicine, although high cost (including operation-time cost), oversensitivity, and multiple difficulties in data interpretation limit their usefulness^{1–3}. Photoplethysmography (PPG) is also a practical reference, also non-invasive, made popular by its low cost (acquisition and operation) and portability^{4–7}. Better sensors and more effective wireless communications have renewed its interest such that PPG is currently no longer only an alternative to electrocardiography (ECG) to measure heart rate, but rather an innovative breakthrough in the wearable health monitoring technologies era, with multiple applications in sight^{7–10}.

These two technologies, LDF and PPG, are based in similar biophysics but use different laser frequencies, such that the respective perfusion related information is obtained at different depths, resulting in different discrimination capacities. The perfusion estimation provided by LDF is assumed to be linearly related to the velocity and concentration of moving erythrocytes^{11–13}. Our instrument uses a probe with a standard fiber separation (0.25 mm) and a 780 nm wavelength. The typical measurement depth, according to the manufacturer, is 0.3–0.5 millimeter, with a portion of the signal able to penetrate as deep as 1 mm^{13,14}. For reflection PPG this depth discrimination depends on the light wavelength used (green and red being the most common) and the distance between the light source and the photo detector^{15–17}. Some studies have conducted depth discrimination only based on wavelength^{18,19} while others were based on both wavelength and source-to-detector distance². A recent paper demonstrated that the penetration depth increases with the wavelength of light used on skin¹⁴. The same computational simulation used by these authors suggests that increasing the beam width also increases the light penetration depth.

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Figure 1. Schematic diagram of the experimental setup to illustrate the application of LDF (1) and PPG (2) probes for the distal assessment of perfusion changes in the human lower limb (see text).

The green (530 nm) wavelength light is one of the most frequent choices as it is very resistant to motion related artifacts. It is also considered appropriate for measurements of superficial (0.2–0.4 mm) perfusion^{1,2}. However, it is a challenge to ensure the signal is arterial in origin as to identify all other hemodynamic influencers^{4,20,21}.

Pulse wave analysis is very susceptible to motion artifacts, such that the Fast Fourier (FF) or the Wavelet Transform (WT) has been used to decompose^{22,23} and “de-noise”^{24,25} the spectra of these complex oscillatory signals. The application of WT to the PPG signal led researchers in the field to propose the “augmentation index (AI)” and the “b/a ratio,” which seem to help characterize vascular ageing^{26,27}. More recently, the second derivative wave of the original PPG signal (accelerometer data) was referred to contain important health-related information^{4,25}.

The true significance of LDF and PPG spectra are important issues from the mechanistic point of view, especially since PPG is often referred as a low-cost replacement for LDF. In previous studies, WT analysis of PPG signals revealed an oscillatory pattern (flowmotion) close to that of LDF^{2,28}. A pilot study has shown a good correlation between PPG and LDF for specific physiological frequency bands²⁹.

In the present study, we are trying to establish how equivalent are the flowmotion patterns of LDF and PPG signals distally obtained (Fig. 1) in healthy human volunteers, under the same reproducible experimental conditions. Two well-known opposite challengers were applied – a standard (Swedish) massage procedure, known to increase perfusion^{30,31}, and hyperoxia, which evokes a decrease in perfusion^{28,32}. LDF and PPG signals were followed in all phases of the evoked adaptative responses to each challenge and analyzed by WT. This approach, comparing two gold standards used in vascular medicine instrumentation, as close as possible from the normal physiological state, are meant to help develop more precise approaches to quantitatively assess microcirculatory function, a crucial step to improve patient prognosis^{33,34}.

Results

Perfusion changes obtained during the experimental protocols developed under these two approaches (massage and hyperoxia) are displayed in Fig. 2. Some perfusion differences between limbs are immediately noted in Phase I, with LDF as with PPG, no matter the group. Such differences have been previously reported^{25–27} in assessments made by an optical probe, and have been attributed to the existing spatial vascular variability between feet.

Comparison of all limbs in all individuals (Wilcoxon sign rank test) revealed no significant differences in perfusion (data not shown). Comparing all limb pairs in each group (Mann-Whitney U test) confirmed this finding (Fig. 2).

These two challenges induce opposite responses (Fig. 3). Massage increases perfusion in Phase II of the massaged limb but also in the contralateral limb (as discussed ahead). In the test foot, this perfusion increase is statistically significant both with LDF ($p < 0.001$) and PPG ($p = 0.013$). On the other hand, hyperoxia evokes a significant decrease of perfusion in Phase II in both limbs, detected with both technologies (LDF $p < 0.001$ on right and left feet; PPG $p < 0.05$ on right and left feet). The intense perfusion reduction is not fully recovered in Phase III in all individuals (Figs 2 and 3).

Examining each signal components’ evolution in response to different challengers (Table 1, Fig. 4), the effects of massage on LDF show a significant increase in the cardiac ($p < 0.001$), respiratory ($p < 0.001$) and myogenic ($p = 0.016$) components during Phase II, compared to baseline. These differences persist in Phase III for the cardiac and respiratory components ($p = 0.016$ and $p = 0.012$, respectively). PPG components show a similar significant increase in the cardiac ($p = 0.003$) and respiratory ($p < 0.001$) components, and furthermore, a significant decrease in the sympathetic ($p < 0.001$), NOd ($p < 0.001$) and NOi ($p < 0.001$) components. The cardiac ($p = 0.010$), myogenic ($p < 0.001$) and sympathetic ($p < 0.001$) components of PPG signal show significant differences in Phase III compared with baseline.

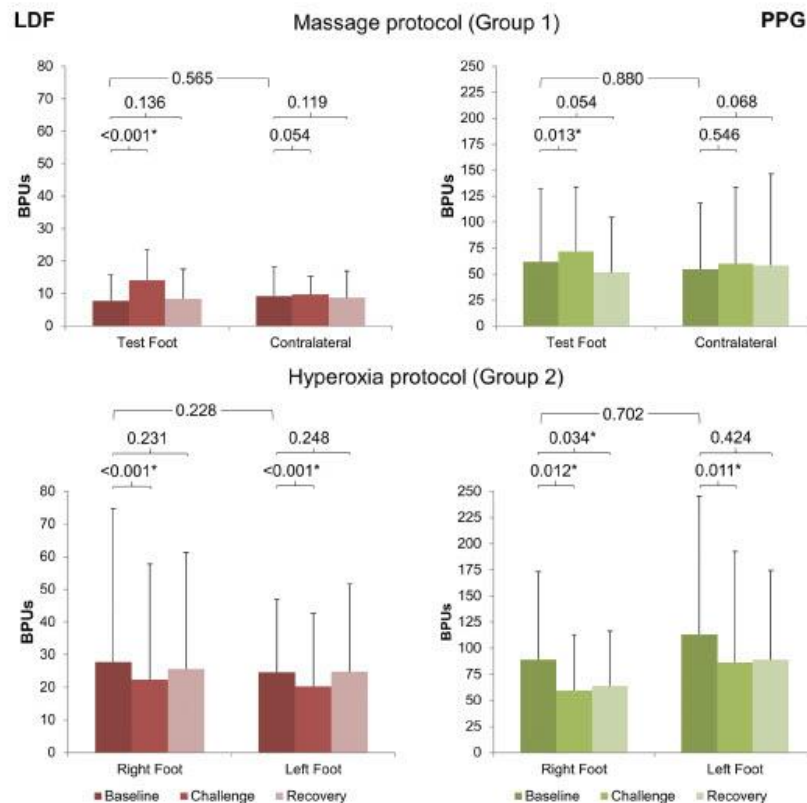


Figure 2. Graphic representation of the evolution of LDF and PPG perfusion changes detected in Group 1 (n = 29) and Group 2 (n = 14) in the distal lower limb, as a result of two opposite vasoactive challenges – massage, known to improve perfusion in the lower limb, and hyperoxia, known to evoke a perfusion reduction. Results (X mean and sd standard deviation) are shown for both limbs. Statistical comparisons between both feet at baseline top line, Mann-Whitney test) and between phases in each foot (Wilcoxon test) are also shown (*p < 0.05).

Regarding hyperoxia, LDF cardiac and respiratory components increased in Phase II, but no statistical significance was found when compared with baseline. The myogenic, sympathetic, NOd, and NOi activities decreased during challenge, but only the sympathetic was significantly different (p = 0.017) regarding Phase I. During recovery some differences were still observed, but only NOi was significantly higher (p = 0.023) than baseline. PPG signal components during hyperoxia show a decrease of cardiac (non-significant), sympathetic, and NOd activities (p = 0.011 and p = 0.012, respectively) while the respiratory and myogenic activities increased significantly (p < 0.001 and p = 0.013, respectively). During Phase III all components' activities returned to baseline except for the cardiac and respiratory, still showing significant differences (p = 0.045 and p = 0.009, respectively) to baseline. In Group 1, positive correlations for LDF and PPG components were found for the cardiac (R = 0.589, p < 0.001), respiratory (R = 0.562, p < 0.001), and myogenic (R = 0.279, p = 0.034) components (Table 2). For Group 2 positive correlations could only be found for NOd (R = 0.388, p = 0.023) and NOi (R = 0.378, p = 0.028) components.

Kurtosis and skewness were also calculated as statistical measures of LDF and PPG spectral distributions (Table 3). Kurtosis of the normal distribution as a value of 3.0. Kurtosis lower than 3.0, also called platykurtic, means the distribution produces fewer and less extreme outliers than does the normal distribution, e.g., low and broad central peaks and short and thin tails. Skewness is a measure of the symmetry in a distribution and essentially measures the relative size of the two tails. A normal distribution will have a skewness of 0 although it may increase depending on the sample size.

Kurtosis in Group 1 was found to be lower than 3.0 for all LDF and PPG components except respiratory (Table 3). LDF components' kurtosis were higher than PPG except for the NOi. The cardiac and sympathetic kurtosis were significantly higher in LDF (p < 0.001 and p = 0.007) and a positive skewness was present except

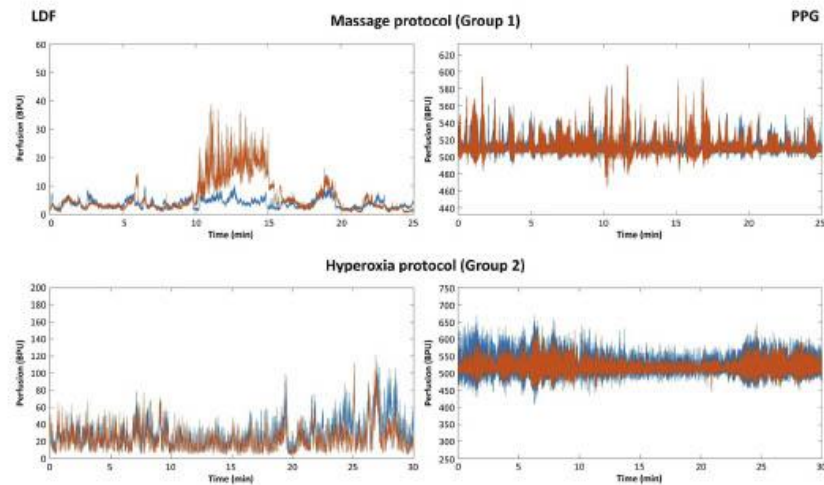


Figure 3. Illustrative examples of LDF (left column) and PPG (right column) obtained in a representative subject. Both limbs are represented in distinct colors – In Group 1 the massage limb is orange and the contralateral is blue; in Group 2 left limb is orange and right limb is blue. As illustrated, massage evokes a perfusion increase detected in both limbs in Phase 2, more obvious in the massaged limb with LDF. Hyperoxia evokes a perfusion decrease also in both limbs in Phase 2, clearly visible with both techniques (see text).

for NOd in PPG and NOI in LDF, showing negative values. Cardiac component skewness was found to be significantly higher in PPG, while the myogenic skewness was significantly higher with LDF. In Group 2, all LDF and PPG components exhibited a kurtosis lower than 3.0 with higher values for LDF, except for the NOI, and significant differences in the myogenic and sympathetic components ($p = 0.001$ and $p = 0.003$, respectively). In turn, skewness profiles were highly variable. The cardiac, respiratory, myogenic, and NOd components had skewnesses > 0 with both LDF and PPG. The sympathetic component had skewnesses > 0 with LDF and a skewnesses < 0 with PPG. The NOI had a skewnesses < 0 with both techniques. The cardiac skewness was significantly lower with LDF ($p = 0.040$), while the myogenic and sympathetic were significantly higher ($p = 0.016$ and $p = 0.050$) with LDF.

Discussion

LDF and PPG share a common biophysical basis. These techniques produce multi-scaled, non-linear, oscillatory signals, integrating several related physiological influences (cardiac, respiratory, autonomic, among others) that result in a periodic oscillatory pattern also known as the skin (microcirculatory) flowmotion^{38–40}. The recognized susceptibility to artifacts (movement, environmental noise, light, etc.), partially explains the multiple difficulties in its interpretation (Fig. 3). LDF uses a laser beam that penetrates the skin producing the “Doppler effect,” which has been long known^{41,42} and is extensively applied to vascular research. Its pulsatile nature, synchronous with the heartbeat together with other respiratory, myogenic, sympathetic, and endothelial contributions, explains these oscillations that produce this non-continuous signal^{36,40,42}. Reflection PPG uses visible light to measure the volumetric variations of blood circulation and is composed of pulsatile (AC) and superimposed (DC) components⁹. The AC component is related with the blood (believed to be mostly arterial) volume synchronous variations resulting from the heartbeat which, in turn, relates with the cardiac events^{20,43}. The DC component is determined by respiration, autonomic nervous system activity, and thermoregulation⁴⁵. Pulse Rate Variability (PRV), extracted from PPG signal, has been proposed to relate with autonomic activity, showing a promising potential to follow up cardiovascular pathophysiology^{6,44}. In the present paper we are measuring perfusion changes at different depths, with opposite challenges and under controlled conditions, expecting to find different flowmotion expressions at different depths.

Both technologies detected the perfusion increase following massage, also in the contralateral non-massaged limb, and the perfusion decrease, again in both limbs, following hyperoxia (Fig. 2). These dual (both limbs) observations were originally reported elsewhere, in human as in mice^{29,30,32}. This research indicates that LDF and PPG measure perfusion directly, even at different tissue levels.

In order to look deeper into LDF and PPG flowmotion, we analyzed changes occurring under challenge by the wavelet transform (WT), a well-known analytical instrument mostly applied to LDF analysis refinement. For LDF it is accepted that those oscillations, cardiac (0.6–2.0 Hz), respiration (0.15–0.6 Hz), myogenic (~0.05–0.15 Hz), neurogenic (~0.02–0.05 Hz), and endothelial (~0.0095–0.02 Hz), determining skin flowmotion, represent the influence of heart rate, respiration, myogenic^{36,42}, autonomic^{39,45}, and endothelial dependent and independent vascular smooth muscle relaxation^{35,40,46}.

Component	LDF signal			PPG signal		
	Baseline	Challenge	Recovery	Baseline	Challenge	Recovery
Massage protocol (Group 1)						
<i>Cardiac</i>	<i>X</i>	8.2	11.5	8.3	10.4	12.0
	<i>sd</i>	8.3	7.9	8.4	6.3	4.2
	<i>p</i>	—	<0.001*	0.016*	—	0.003*
<i>Respiratory</i>	<i>X</i>	1.2	1.7	1.3	6.1	18.5
	<i>sd</i>	0.5	0.7	0.7	2.9	8.0
	<i>p</i>	—	<0.001*	0.012*	—	<0.001*
<i>Myogenic</i>	<i>X</i>	3.5	5.4	4.0	21.5	22.6
	<i>sd</i>	1.4	2.8	2.1	8.0	6.4
	<i>p</i>	—	0.016*	0.092	—	0.052
<i>Sympathetic</i>	<i>X</i>	7.7	8.4	8.5	16.3	12.5
	<i>sd</i>	3.6	4.0	4.1	4.1	3.9
	<i>p</i>	—	0.136	0.853	—	<0.001*
<i>Endothelial NOd</i>	<i>X</i>	13.8	12.8	14.0	17.8	13.6
	<i>sd</i>	5.3	5.7	5.2	6.1	4.1
	<i>p</i>	—	0.128	0.877	—	<0.001*
<i>Endothelial NOI</i>	<i>X</i>	23.0	21.6	21.7	13.7	9.6
	<i>sd</i>	5.6	6.6	5.9	4.8	3.3
	<i>p</i>	—	0.082	0.088	—	<0.001*
Hyperoxia protocol (Group 2)						
<i>Cardiac</i>	<i>X</i>	1.1	1.2	1.1	18.4	15.8
	<i>sd</i>	0.7	0.5	0.4	9.2	6.5
	<i>p</i>	—	0.085	0.866	—	0.095
<i>Respiratory</i>	<i>X</i>	3.0	3.5	2.8	9.5	13.1
	<i>sd</i>	1.9	1.9	1.3	3.2	4.3
	<i>p</i>	—	0.123	0.442	—	<0.001*
<i>Myogenic</i>	<i>X</i>	9.0	8.9	9.3	22.6	25.1
	<i>sd</i>	4.1	4.4	4.3	8.2	8.5
	<i>p</i>	—	0.309	0.952	—	0.013*
<i>Sympathetic</i>	<i>X</i>	8.0	6.5	7.9	15.5	13.7
	<i>sd</i>	3.7	2.2	3.0	4.8	4.1
	<i>p</i>	—	0.017*	0.544	—	0.011*
<i>Endothelial NOd</i>	<i>X</i>	20.1	18.6	19.4	11.4	9.6
	<i>sd</i>	6.4	6.4	7.5	3.5	3.0
	<i>p</i>	—	0.102	0.321	—	0.012*
<i>Endothelial NOI</i>	<i>X</i>	12.8	12.0	14.2	9.6	10.4
	<i>sd</i>	4.3	5.7	4.1	4.9	3.7
	<i>p</i>	—	0.278	0.023*	—	0.993

Table 1. LDF and PPG spectral component changes resulting from the application of massage and hyperoxia to Group 1 (n = 29) and Group 2 (n = 14) obtained by the Wavelet Transform (see text). Challenge and recovery p-values result from each phase comparisons with baseline (X mean, sd standard deviation, *p < 0.05).

Knowing that PPG signals are obtained at a different wavelength, the same rationale was applied to PPG signals comprised of common pulsatile (e.g. cardiac) and superimposed (e.g. respiratory) components.

As mentioned previously, WT has been used as a de-noising tool to remove Gaussian-like noise from the PPG signal^{23,24}, increasing the interest and utility of this pulse waveform. Pulse wave analysis is being currently used to study diabetes, atherosclerosis and cardiovascular disease^{8,24,27}, far beyond the original oxygen saturation and heart rate determination.

Our approach is in line with this recent effort to further investigate new information and applications for the PPG signal⁸. LDF and PPG components ranges were found to overlap in cardiac (1.7–0.7 Hz), respiratory (0.7–0.2 Hz), myogenic (0.2–0.05 Hz), sympathetic (0.05–0.027 Hz), endothelial NO-dependent (NOd) (0.027–0.013 Hz), and endothelial NO-independent (NOI) (0.013–0.0064 Hz) ranges, as published previously^{28,47}. Table 1 depicts some differences that seem to result directly from the different depth discriminative capacities of LDF and PPG. The few hundred micrometer (<1 mm) depth assumed for LDF and PPG means that both technologies provide estimations in a small superficial vascular volume^{1,12,13} where the high frequency components are still the main determinants of flowmotion. However, the superficial mobilization of blood seems to affect PPG penetration. It is known that in compressed tissues, light penetrates more deeply into the tissue, reaching deeper and larger vessels, which increases PPG signal⁸. Deeper penetration allows access to higher volumes of blood

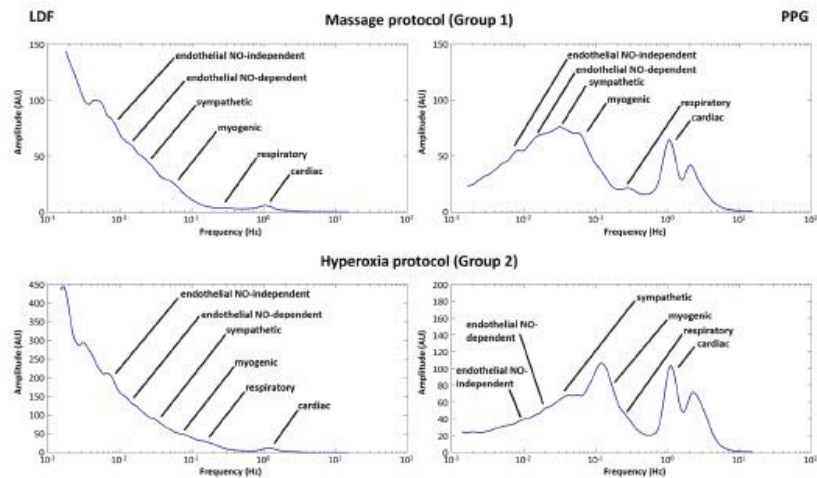


Figure 4. Frequency spectra showing the mean amplitude profiles changes evoked by massage and hyperoxia in LDF (left column) and PPG (right column) ($n = 43$, data from both limbs). As shown, the lowest frequency range components show wider amplitudes for LDF, determining this characteristic profile which likely results from the higher sensitivity and specificity attributed to this technology. Regarding PPG, wider variations are noted with higher frequency components, primarily cardiac and myogenic (see text).

Component		Massage (Group 1)	Hyperoxia (Group 2)
Cardiac	R	0.589	-0.093
	P	<0.001*	0.599
Respiratory	R	0.562	0.057
	P	<0.001*	0.750
Myogenic	R	0.279	0.134
	P	0.034*	0.451
Sympathetic	R	0.066	0.278
	P	0.622	0.112
Endothelial NOd	R	0.127	0.388
	P	0.341	0.023*
Endothelial NOi	R	0.172	0.378
	P	0.197	0.028*

Table 2. Spearman's R coefficient for the linear correlation of the perfusion percent change (provocation-baseline) for both LDF and PPG techniques in both protocols (* $p < 0.05$).

perfusing the area perfusion. For Group 1, LDF only detects significant changes in the high frequency components. However, PPG consistently detects all component changes in the expected direction. For Group 2 the reduced perfusion decomposition observed as a result of central and peripheral interactions of hyperoxia is unevenly described by LDF and PPG. In fact, the high variability from the LDF signal only allows a significant detection for the sympathetic activity reduction, while PPG gives a detailed description for all components according with our expectations (Table 1). Contrary to previously published work⁴², correlations between components from LDF and PPG changes are not strong (Table 2). For Group 1, significant positive correlations are found for the high-frequency cardiac, respiratory, and myogenic components. For Group 2, significant positive correlations are found only for the low-frequency endothelial components.

Analysis of the complete (25 minutes) frequency spectra of LDF and PPG depicts another important feature. Figure 4, representing the mean spectra of all subjects, shows different amplitude profiles for both signals, irrespective of the challenge. As shown, for LDF higher amplitude changes mostly occur in the lowest frequency regions, with myogenic, sympathetic, and endothelial components designing this descendent profile. In contrast, PPG amplitudes vary mostly in the higher amplitude ranges with major contributions from cardiac and myogenic components (high respiratory variability) and much more discrete contributions from the lower frequencies range, especially for Group 2. These observations complement our view resulting, in our opinion, from the high sensitivity of LDF regarding PPG. Although not extensively documented, a few studies have compared sensitivity,

Component		Massage (Group 1)		Hyperoxia (Group 2)	
		KT	SK	KT	SK
cardiac	LDF	1.82 (0.26)	0.13 (0.08)	1.92 (0.20)	0.30 (0.13)
	PPG	1.74 (0.12)	0.17 (0.07)	1.90 (0.50)	0.40 (0.30)
	p	<0.001*	0.001*	0.054	0.040*
respiratory	LDF	4.17 (1.06)	1.10 (0.41)	2.95 (0.76)	0.66 (0.35)
	PPG	4.03 (0.87)	1.12 (0.30)	2.80 (0.60)	0.60 (0.30)
	p	0.217	0.866	0.300	0.251
myogenic	LDF	2.19 (0.34)	0.18 (0.17)	2.46 (0.45)	0.41 (0.26)
	PPG	2.15 (0.18)	0.10 (0.16)	2.30 (0.20)	0.30 (0.20)
	p	0.095	0.012*	0.001*	0.016*
sympathetic	LDF	2.17 (0.30)	0.07 (0.18)	2.19 (0.24)	0.04 (0.24)
	PPG	2.12 (0.24)	0.03 (0.23)	2.10 (0.20)	-0.10 (0.20)
	p	0.007*	0.384	0.003*	0.050*
endothelial NOd	LDF	2.17 (0.37)	0.03 (0.22)	2.17 (0.36)	0.03 (0.32)
	PPG	2.16 (0.31)	-0.01 (0.26)	2.10 (0.30)	0.00 (0.20)
	p	0.407	0.401	0.375	0.315
endothelial NOi	LDF	2.11 (0.38)	-0.11 (0.31)	2.07 (0.34)	-0.06 (0.32)
	PPG	2.25 (0.42)	0.01 (0.33)	2.20 (0.50)	-0.100 (0.30)
	p	0.125	0.061	0.510	0.244

Table 3. Mean (SD) kurtosis (KT) and skewness (SK) of the LDF and PPG signal spectral bands obtained in both groups. Statistical comparison between feet and between techniques is presented (T – test foot, C – control foot, *p < 0.05).

specificity, and reproducibility of this technique with others, suggesting that LDF should be regarded as a reference for tissue vitality testing^{48,49}. Again, this is in opposition to recent remarks suggesting that PPG could replace LDF for endothelial studies⁴⁷. We should stress that our protocol practically eliminated main variability sources, namely by registering LDF and PPG signals from both limbs³², reinforcing the robustness of our data and analysis.

Finally, kurtosis was generally higher in LDF than in PPG spectral bands, especially for high frequency components, suggesting less power distribution in each frequency range (Table 3). Globally, no matter the protocol, differences in kurtosis and skewness were observed more frequently for the myogenic, cardiac, and sympathetic components, with the respiratory and endothelial components consistently showing no differences.

Globally, LDF and PPG seem to be composed of different hemodynamic oscillations, showing different sensitivities to these flowmotion components. The simultaneous use of LDF and PPG seems to complement and detail our view on microcirculatory flowmotion.

Methods

Subjects. A convenience set of 43 healthy individuals, both sexes (20.0 ± 2.0 y.o.) was gathered according to previously defined inclusion and non-inclusion criteria. Selected subjects had no relevant clinical history, were non-smokers, not taking any vasoactive medication, and were asked to refrain from consuming caffeine containing beverages 24 hours prior to their participation in the experimental procedures. Female subjects were at different phases of their fertility cycle and not taking oral contraception. All individuals gave their written informed consent prior to the beginning of the study. Two experimental groups were defined and allocated to two perfusion challenge procedures. Group 1 included 29 subjects (19.9 ± 1.6 y.o., 13 males, 16 females) submitted to a massage protocol and Group 2 included 14 subjects (20.3 ± 2.9 y.o., 6 males, 8 females) submitted to a hyperoxia challenge test. Specific conditions to observe in each protocol have been published elsewhere^{3,30,37}.

The experimental protocol was previously approved by the Universidade Lusófona's School of Health Sciences Ethical Commission (code reference: CE03/2013.12) and carried out in accordance with the Declaration of Helsinki and respective amendments, observing good clinical practice for medical research involving human subjects⁵⁰.

Experimental. Before the application of procedures, subjects were left to acclimatize to room conditions (23 ± 1 °C, 40–60% humidity) for 20 minutes in the seated position. Perfusion signals from LDF and PPG were simultaneously measured on the inferior aspect of the second and third toes in both feet (Fig. 1). This was a practicality-based option since not every first toe is large enough to accommodate both probes in adequate measuring conditions. In addition, large areas of skin on the first toe are frequently thick enough to alter the light penetration. We found no significant differences in perfusion measured by LDF or PPG in these conditions among any of the toes (data not shown). For the massage protocol, subjects were positioned supine, both legs slightly flexed, with one of the legs, randomly chosen, used as control. For the hyperoxia protocol subjects remained seated, both feet flat on the floor, knees and shoulders aligned.

Measurement technologies and signal processing. LDF signals were acquired with a Periflux 5000 device (Perimed, Sweden) and PF5010 units. Two small angled probes were used, placed with double-sided adhesive strips (PF105–3, Perimed, Sweden) on the toe's inferior aspect (Fig. 1). Signals were acquired at a 32 Hz sampling

rate with a time constant of 0.2. The LDF probe had a 0.25 mm fiber separation and emitted a laser beam of 780 nm wavelength.

PPG signals were acquired by reflection photoplethysmography (PPG) with a Blood Volume Pulse (BVP) sensor attached to a BITalino plugged microprocessor board (PLUX Wireless Biosignals, Portugal). The sensor emitted a green light of 530 nm wavelength (2.3 mm separation between LED and receptor) and was adhered to the inferior aspect of the toe using a double-sided adhesive strip (Jurasstic High Bond Tape, UK). PPG signals were acquired with a 100 Hz sampling rate, and were pre-smoothed by a moving average filter here described as:

$$y_i(t) = \frac{1}{2N+1}((y(t+N) + y(t+N-1) + \dots + y(t-N))) \quad (1)$$

where $y_i(t)$ is the smoothed value for the i -th data point, N is the number of neighboring data points on either side of $y_i(t)$ and $2N+1$ refers to the data span. Signals were then down-sampled to 32 Hz before further processing.

The Wavelet Transform (WT), a well-known tool for refined spectral analysis²³, was applied to all signals to assess their main activity components. For each signal, a mean spectrum of all phases was calculated for each protocol. For Group 1, the mean LDF and PPG spectra from the massaged foot were compared with the ones from the control foot. In Group 2, LDF and PPG spectra are mean values from both feet.

PPG spectra were found to be similar to LDF, showing closely related frequency ranges for all components, in line with other reports^{28,47}. From these frequency spectra, the frequency range, obtained by visual analysis, kurtosis and skewness of each spectral band were also calculated. Signal pre-processing and WT analysis were performed using Matlab software (Mathworks R012, USA).

Challenge procedures. **Massage:** After acclimatization to the laboratory environmental conditions, Group I individuals were submitted to a massage protocol ("effleurage") performed by a specialized therapist. This reference technique^{31,51} was applied to a randomly chosen leg in the upwards direction (from the ankle towards the knee), and consisted of the application of a series of light compression strokes with the palms of both hands along the leg's longitudinal axis, in a rhythmic and repetitive way. Almond oil was used as a lubricant to reduce discomfort⁵². The contralateral leg remained in the same position and was used as control. The protocol included three phases – a 10 min baseline resting register (Phase I), a 5 min massage challenge (Phase II), and a 10 min recovery (Phase III).

Hyperoxia: After acclimatization to the laboratory environmental conditions, a special facemask (AGA MedControl 45 Bar Oxygen Demand Valve facemask, AGA AB, Sweden) was applied to Group 2 subjects to guarantee a fully controlled breathing with a 100% oxygen (Medical oxygen, AirLiquide, PT) saturated atmosphere. The protocol included three phases – a 10 min baseline resting register with the volunteer breathing room atmosphere (Phase I), after applying the mask, a 10 min challenge by breathing a 100% oxygen atmosphere (Phase II), and a 10 min recovery (Phase III) again breathing room atmosphere³⁷.

Variables and statistics. For each phase of both protocols, perfusion changes were calculated as the mean value of the LDF signal and as the mean amplitude of the PPG signal, both expressed in arbitrary units (AU). For the component analysis, the activity of each component was calculated as the percent ratio of the area under the curve (AUC) of that component on the average signal frequency spectrum (30 minutes) divided by the AUC of the entire frequency spectrum. For statistical purposes, the components' activities were calculated in three analysis periods selected inside the cone of influence (COI) of the WT scalogram. For the massage protocol, 4.00 min to 9.00 min corresponds to Phase I, 12.00 min to 15.00 min corresponds to Phase II and 17.00 min to 25.00 min corresponds to Phase III. For the hyperoxia protocol, 4.00 min to 9.00 min corresponds to Phase I, 12.00 min to 17.00 min corresponds to Phase II and 21.00 min to 26.00 min corresponds to Phase III. To assess statistical equivalency between subjects at baseline, the mean perfusion and the components' activities were compared between the different subjects with the Wilcoxon signed rank test for one sample. To assess physiological changes in the signals' components with each challenge, the mean perfusion and the components' activities were compared between phases with the Wilcoxon signed rank test for paired samples. For the massage protocol, these variables were compared between massaged and control feet by the Mann-Whitney U test for independent samples. Linear correlations of the percent variation of LDF and PPG signals between Phases I and II were tested with Spearman's R coefficient. All statistical tests were carried out with SPSS 22 (IBM, USA) and a $p < 0.05$ value adopted.

Conclusions

LDF and PPG use different light wavelengths, with different penetration, sensitivity, and reflection capacities. Even accepting that some of the pulsatile and superimposed determinants might be the same, it is not granted that each frequency component in LDF and PPG have the same origin. In fact, the not so obvious correlation between both spectral profiles obtained under the same conditions reinforces that view, meaning that the oscillations registered with both signals are different. PPG revealed higher depth discrimination than LDF to detect hemodynamically-imposed changes in the contribution of the different components, irrespective of the challenge, which seems to be an additional value to explore. Furthermore, the simultaneous use of LDF and PPG offers a complementary view on the observed perfusion events, where the WT spectral signal decomposition greatly improves their quantification and opens new mechanistic directions of skin microcirculatory flowmotion to explore.

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Conceptualization and study design, L.M.R. Performance Research, C.R., H.S. Data curation and Analysis, H.F., H.S., C.R. Drafting, Editing and Reviewing L.M.R., H.S. Supervision and Final Writing, L.M.R.

Competing interests

The authors declare no competing interests.

Additional information

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Artigo V - Lower limb massage in human increases local perfusion and impacts systemic hemodynamics

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RESEARCH ARTICLE

Lower limb massage in humans increases local perfusion and impacts systemic hemodynamics

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¹CBIOS Research Center for Biosciences and Health Technologies, Universidade Lusófona, Lisbon, Portugal; ²Faculty of Pharmacy and Pharmacology, Science Department, Universidade de Lisboa, Lisbon, Portugal; and ³Faculty of Sciences, Institute for Biophysics and Biomedical Engineering, Universidade de Lisboa, Lisbon, Portugal

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Rodrigues LM, Rocha C, Ferreira HT, Silva HN. Lower limb massage in humans increases local perfusion and impacts systemic hemodynamics. *J Appl Physiol* 128: 1217–1226, 2020. First published March 19, 2020; doi:10.1152/jappphysiol.00437.2019.—Massage is commonly used as a complementary therapy for many different conditions. Demonstration of its physiological impact and understanding of its therapeutic mechanisms is still insufficient and often inconclusive. This study aims to characterize the physiological effects of effleurage, one of the most popular techniques, on human in vivo microcirculation and its impact on cardiovascular function. Two differently oriented variations of the technique, referred to influence physiological outcomes, were applied to 32 young (mean 19.8 ± 1.6 yr old) healthy volunteers of both sexes in a single, randomly chosen limb after informed written consent. Each protocol included a 10-min baseline (Phase I), a 5-min massage (Phase II), and a 10-min recovery (Phase III) register. A 30-min washout period separated both protocols. Perfusion was assessed by laser Doppler flowmetry (LDF) and reflection photoplethysmography (PPG), with their sensors applied distally in both feet. Blood pressure and pulse were also obtained. LDF signals were further analyzed in their components by the (Morlet) wavelet transform to probe the mechanisms involved. Results showed that effleurage consistently evoked a significant ($P < 0.001$) perfusion increase in the massaged limb, also visible in the contralateral limb (not significant) independently from the orientation (variant) used. No matter the perfusion differences known between sexes, the adaptive response was equivalent in both sexes. The component analysis of the LDF curves also suggests that these procedures, although brief and superficial, do modify multiple components of cardiovascular integration, with cardiac, respiratory, and myogenic components appearing to play a major role in reestablishing distal microcirculatory homeostasis.

NEW & NOTEWORTHY The impact of effleurage, a well-known massage procedure used in human rehabilitation, in the lower limb hemodynamics, is demonstrated. When applied in a sole limb, massage increases skin microcirculatory flowmotion not only locally but also beyond, affecting systemic hemodynamics. This observation is an interesting example of the efficacy of cardiovascular integration mechanisms involving distal microcirculatory homeostasis. The proposed methodology allows a mechanistic view over skin flowmotion regulation, being applicable to further explore massage and its impact on microcirculatory physiology.

cardiovascular effects; effleurage; LDF; PPG; wavelet transform

INTRODUCTION

Massage is an ancient procedure, likely used for thousands of years by practitioners with multiple purposes related to health and well-being. The mobilization of soft tissue during massage involves “a set of manipulations and/or maneuvers performed with the hands from a specialized therapist and applied to the human body with preventive, hygienic, therapeutic, rehabilitating, and psychological objectives in a methodical, orderly, and reasoned manner” (16, 49). More complex definitions can be found for different purposes (e.g., sports, muscle performance, rehabilitation), and various types of massage therapies are available, sometimes performed in combination or associated with devices (e.g., needles, bands) (13, 15, 18, 63).

A wide variety of massage techniques are accessible, all intended to favor the individual’s well-being. Demonstration of its physiological impact and mechanisms is still limited and often inconclusive, although it is recognized as beneficial to many health conditions and is clearly well accepted by the patient (11, 13, 15, 23, 60). Many studies have demonstrated the positive contribution of massage in pain modulation, relief of fatigue, and management of anxiety and stress (7, 21, 26, 33, 59), in chronic conditions as in terminal pathologies, and also in children (12, 45). Recent data have associated massage, and the way the procedure is applied, to complex immunomodulation mechanisms (58–60) and significant blood pressure reduction, suggesting a potential integration effect of complex central and local psychophysiological mechanisms (12, 21, 34, 60, 64).

The hemodynamic expressions of massage in the lower limb are particularly interesting, considering its multiple potentials (preventive, palliative, therapeutic) as well as the current prevalence of peripheral vascular disease in cerebral-vascular stroke (64). However, solid science-based information on the cardiovascular and hemodynamic impacts of massage is scarce and often controversial. Lack of randomized control trials, inadequate quantification and interpretation of variables, procedure variety (massage type, application form, duration, frequency), and too descriptive, less mechanistic views on outcomes help to explain these difficulties. In previous publications, the primary effect of one of these techniques on the in vivo perfusion of the human distal lower limb has been explored (20, 38, 39).

Our group has been particularly interested in microcirculatory modeling control and adaptive mechanisms (31, 32, 52).

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Thus, in the present study, we aimed to better characterize the impact of a popular type of massage, the effleurage technique, on human in vivo microcirculation. Effleurage was applied in two variations, proximal and distal, to assess how "orientation" might influence outcomes. Perfusion changes were assessed with simultaneous measurements by laser Doppler flowmetry (LDF) and reflection photoplethysmography (PPG). Both signals are known to be pulsatile in nature due to multiple contributions of flow, such as cardiac, respiration, myogenic, autonomic, and endothelial (6, 24). The wavelet transform (WT) analysis of these signals depicts the frequency and amplitude variation of each of those components, providing a mechanistic view on the registered events.

METHODS

Subjects. A convenient sample of healthy male ($n = 16$, mean age 19.3 ± 1.1 yr) and female ($n = 16$, mean age 20.3 ± 1.8 yr) participants was selected mostly among the university's student population. Specific inclusion/noninclusion criteria were applied, having in mind this type of research (52). All participants were normotensive with no signs of vascular impairment, confirmed by the ankle-brachial index (ABI) > 1.0 (2). The combined ABI was 1.06 ± 0.04 for male participants, and 1.06 ± 0.05 for female participants. All 32 participants had a normal body mass index (BMI), with mean BMIs of 23.5 ± 3.3 kg/m² for males and 20.9 ± 2.4 kg/m² for females.

All participants were nonsmokers, free of any regular medication or food supplementation, and had equivalent levels of physical activity. All female participants were taking oral contraceptives and had regular menstrual cycles.

Experimental procedures were previously submitted and approved by the school's Ethics Commission and carried out in accordance with the Declaration of Helsinki and respective amendments, observing good clinical practice for medical research involving human participants (62). All participants provided informed, written consent and were asked to refrain from consuming caffeinated or any other vasoactive beverages within 24 h before the experiments.

Experimental: massage. Experiments took place with controlled temperature and humidity ($21 \pm 1^\circ\text{C}$ and 40–60%, respectively). Participants were allowed to acclimatize for 20 min lying in the supine position before experimentation. Massage was applied to one randomly chosen leg, with the contralateral leg, in the same position, serving as control. Procedures involved the application of a sequence of two massage (effleurage) protocols to all individuals, executed by an experienced licensed therapist (15, 17, 26, 56). The effleurage technique implies the application of a series of short, rhythmic, and repetitive strokes with light compression, with the palms of both hands gliding along the leg's longitudinal axis, to soften and lengthen muscle fibers. Almond oil, known to affect only the most superficial epidermal layers, was used as a lubricant to reduce discomfort (3, 36, 39). Two variations of this technique were applied in a randomized sequence: one centripetal or proximal, developed in the upward direction (from the ankle to the knee), and the other centrifugal or distal, in the downward direction starting at the knee. The orientation factor of application has been referred as potentially affecting the blood flow variations associated with massage (further information below). Each protocol included three phases: a 10-min baseline resting register (*Phase I*), a 5-min massage challenge (*Phase II*), and a 10-min recovery (*Phase III*). Both protocols were applied to the same leg and were separated by a 30-min washout period.

Measurement technologies and signal processing. Blood perfusion was assessed through LDF and PPG, two optical technologies using different laser wavelengths, with different depth capacities (4, 5, 19, 35, 43). LDF and PPG signals were obtained from sensors applied in

the plantar aspects of the second and first toes, respectively, in both feet. This option also proved to substantially reduce variability when measuring in the distal inferior limb. LDF signal, expressed in arbitrary blood perfusion units (BPUs), was obtained with a Perimed PF5010 system (Perimed Sweden), which also allowed the continuous monitoring of skin surface temperature. The blood volume pulse (BVP), also expressed in BPUs, and the cardiac rate were obtained from the PPG signal, registered using a Bitalino Plugged Kit (PLUX Biosignals Portugal). Blood pressure (systolic and diastolic) was measured in the arm during all three phases with a Tensioval Comfort system (Hartman, Germany).

Using a MATLAB-based toolbox, LDF signals (32 Hz sampling rate) were further decomposed by the WT analysis (using the Morlet wavelet) into their main frequency components (cardiac, respiratory, myogenic, sympathetic, endothelial NO-dependent (endoNOD), and endothelial NO-independent (endoNOI)) to assess their contributions to the obtained signal.

The reference frequency bands used were 0.6–2 Hz for cardiac, 0.145–0.6 Hz for respiratory, 0.052–0.145 Hz for myogenic, 0.021–0.052 Hz for sympathetic, 0.0095–0.021 Hz for endoNOD, and 0.005–0.0095 Hz for endoNOI (6, 24, 30).

Statistics. For each phase of both protocols, perfusion was calculated as the mean value of the LDF signal and as the mean amplitude of the PPG signal. For the component analysis, the activity of each component was calculated on each phase as the percent ratio of the area under the curve (AUC) of that signal component in the frequency spectrum divided by the AUC of the entire frequency spectrum for that phase. Descriptive and comparative statistics were applied to LDF and PPG signals and WT components using SPSS v.22.0 software (IBM Corp.). As in previous publications (38–41), normality requirements were not present, which herein we also confirmed by the Shapiro-Wilk test. A nonparametric approach was applied using the Wilcoxon signed rank test for paired samples for phase comparisons and the Mann-Whitney *U* test for independent samples. Repeated measures of the same subject at different times (before, during, and after massage) involving multiple testing and comparisons might increase the risk of type 1 error, raising the issue of adjustment for multiple testing. This is particularly relevant in clinical trials, although methods to adjust for multiple testing in studies collecting repeated measurements are few and their application controversial. Our study adopts a confidence level of 99.9%, meaning that any $P > 0.001$ should be considered as nonsignificant, which suitably accounts for multiple comparisons.

RESULTS

To assess the lower limb microcirculatory function, sensors were applied in both feet, an option that we (32) demonstrated to substantially reduce variability of results. An illustrative example of the global perfusion changes obtained during massage is shown in Fig. 1. As depicted, the massage procedure in one limb simultaneously impacts the contralateral (nonmassaged) limb. This observation could not be so clearly perceived without the simultaneous recording of perfusion in both limbs (31, 32).

As shown in Table 1, both technologies detected the perfusion increase in both limbs as a consequence of massaging one limb (*Phase I* to *Phase II*) in both the proximal (upward) and the distal (downward) procedures. Significant differences were present in the massage limb in *Phase II* with LDF. In the contralateral foot, LDF still detected some increases, but these were not significant. Recovery (*Phase III*) showed the return to reference values with both LDF and PPG in the massaged limb as in the contralateral limb. The distal procedure (Table 1) evoked comparable but less pronounced perfusion changes in

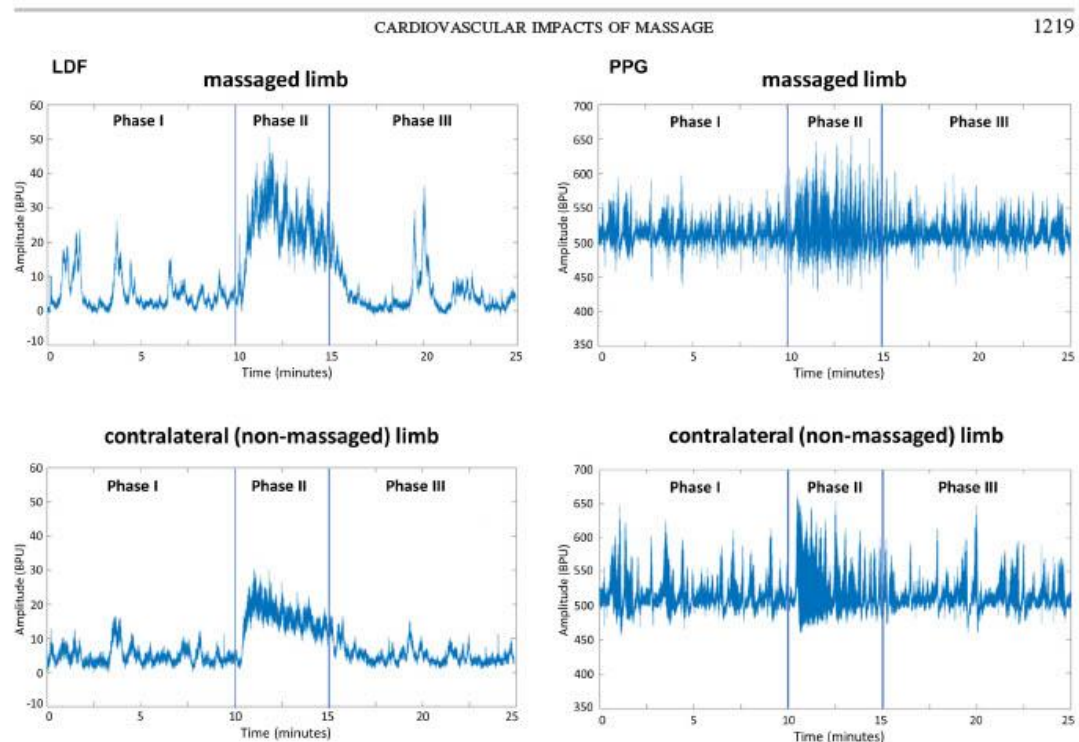


Fig. 1. Illustrative example (data from one representative participant) of a typical recording of laser Doppler flowmetry (LDF) and reflection photoplethysmography (PPG) [expressed in arbitrary blood perfusion units (BPUs)] obtained in both limbs under the current experimental setting using the proximal massage procedure. Following the baseline resting phase (*Phase I*), a clear increase in blood perfusion is depicted (*Phase II*) also in the contralateral nonmassaged limb. These events are detected by both measurement technologies. After massage (*Phase III*), perfusion returns to baseline reference values in most of the participants (see RESULTS).

both limbs. Again, in *Phase II*, LDF detected a perfusion increase in both limbs but significant ($P < 0.001$) only in the massaged limb, while PPG detected a consistent increase only in the massaged limb ($P = 0.002$). In *Phase III*, perfusion was always lower than in *Phase II* as measured by LDF or PPG and not significantly different from *Phase I* in both limbs. However, PPG detected higher reductions in both limbs compared with *Phase I* and *Phase II*, particularly in the contralateral limb ($P = 0.040$). Skin surface temperature was noted to be relatively stable in all study participants (data not shown), although a small, nonsignificant reduction was registered in *Phase III* in the nonmassaged limb.

Both massage procedures evoked similar reductions in the systolic blood pressure ($P < 0.001$) and pulse, returning to baseline values in *Phase III*.

Comparing perfusion data from all participants (Mann-Whitney U test) obtained from LDF and PPG in each phase of the experimental procedures (Table 2), we found no differences between limb pairs in any participant for *Phase I*. Therefore, *Phase II* differences between limbs can be reasoned only as a consequence of massage, no matter the procedure involved (Table 2). Furthermore, comparing the impact of both massage procedures (Table 3) in absolute values, or as variations of variables in each phase of the experimental protocol, we could

not find any significant differences between proximal and distal variants of this technique.

Relevant perfusion differences were detected, by LDF as well as by PPG, between male and female participants in all phases of the experimental procedures (Table 4). These sex-related differences have been previously described and recently explained in terms of microcirculatory reactivity (42, 57). We calculated the perfusion means from both limbs (massaged and nonmassaged) for both procedural variants, in each experimental phase, and compared them by sex (Table 4). Alterations attributed to massage ($\Delta\text{II-I}$, $\Delta\text{III-II}$, and $\Delta\text{III-I}$) were analogous in male and female participants, suggesting a parallel sex-independent adaptive behavior following massage (Table 4).

Finally, the WT analysis of LDF signals obtained during the experimental procedures has shown the evolution of the different flow components described previously. Figure 2 illustrates the mean changes registered in both limbs with both effleurage variations. LDF components evolved in a similar way in each procedure in both limbs, always more obvious in the massaged limb. High-frequency components (cardiac, respiratory, and myogenic) seem to be the most relevant statistically, still significant ($P < 0.001$) during recovery (*Phase III*) with the proximal maneuver.

Contribuição para uma abordagem não convencional da Função Vascular Periférica
- estudo do impacto fisiológico da massagem na circulação do membro inferior

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CARDIOVASCULAR IMPACTS OF MASSAGE

Table 1. Mean lower limb perfusion changes expressed in BPUs obtained by LDF and PPG in all participants' left and right lower limbs during the experimental protocol

	LDF, BPU		PPG, BPU		Systolic Pressure, mmHg	Diastolic Pressure, mmHg	Pulse (bpm)
	Massaged	Contralateral (nonmassaged)	Massaged	Contralateral (nonmassaged)			
Proximal procedure							
Phase I	6.3 ± 3.4	7.0 ± 3.2	50.3 ± 50.5	45.1 ± 49.0	111.6 ± 11.5	70.6 ± 6.1	68.1 ± 6.2
	5.8	6.3	34.9	30.6	110.2	70.8	67.9
Phase II	12.7 ± 7.3	8.3 ± 3.9	62.8 ± 46.0	49.3 ± 54.9	108.8 ± 10.8	67.8 ± 7.4	65.7 ± 6.3
	10.6	7.7	47.7	36.0	106.8	65.8	65.8
Phase III	6.6 ± 4.2	7.0 ± 3.7	43.9 ± 37.8	44.2 ± 58.8	112.5 ± 10.8	72.2 ± 11.8	68.4 ± 6.1
	5.1	6.3	30.0	29.2	112.5	70.8	69.0
ΔII-I, %	114.8 ± 101.8	24.1 ± 45.7	43.6 ± 49.7	11.5 ± 37.1	-2.5 ± 3.0	-4.0 ± 5.1	-3.6 ± 3.3
	94.7	10.3	32.9	2.6	-2.1	-3.1	-3.7
	<0.001*	0.003	0.001	0.304	<0.001*	<0.001*	<0.001*
ΔIII-I, %	2.3 ± 18.4	-1.4 ± 15.5	-5.8 ± 20.6	-1.9 ± 44.6	0.8 ± 3.2	1.2 ± 8.6	0.5 ± 3.6
	-1.3	-4.6	-5.3	-11.9	0.3	0.3	-0.1
	0.953	0.035	0.140	0.013	0.126	0.784	0.822
Distal procedure							
Phase I	6.6 ± 4.2	7.8 ± 4.7	50.7 ± 51.5	49.2 ± 43.4	111.3 ± 10.1	69.9 ± 7.1	68.4 ± 6.6
	5.2	6.8	31.9	29.1	111.0	70.8	67.7
Phase II	12.0 ± 7.3	8.4 ± 4.3	63.5 ± 45.4	45.1 ± 38.3	108.4 ± 10.3	68.2 ± 6.1	65.4 ± 6.6
	10.8	7.0	45.4	28.6	108.4	67.3	65.2
Phase III	7.2 ± 6.7	7.9 ± 5.9	52.4 ± 56.6	43.4 ± 38.3	111.4 ± 10.5	70.6 ± 6.8	68.0 ± 6.3
	4.7	6.4	29.0	23.7	110.0	71.7	67.7
ΔII-I, %	99.5 ± 104.6	14.6 ± 34.6	49.1 ± 76.4	1.7 ± 29.5	-2.8 ± 3.1	-2.7 ± 6.9	-4.3 ± 3.7
	70.6	1.7	32.0	0.0	-2.8	-2.6	-4.4
	<0.001*	0.228	0.002	0.955	<0.001*	0.059	<0.001*
ΔIII-I, %	2.9 ± 38.8	0.8 ± 27.0	2.5 ± 36.4	-5.9 ± 25.2	-0.1 ± 3.7	0.8 ± 5.2	-0.5 ± 2.8
	-1.0	-5.0	-6.2	-5.8	0.0	2.1	-0.5
	0.852	0.070	0.525	0.040	0.787	0.073	0.254

Data are expressed as means ± SD and medians, followed by *P* values where applicable; (*n* = 32). Mean lower limb perfusion changes (*n* = 32) expressed in arbitrary blood perfusion units (BPUs) obtained by laser Doppler flowmetry (LDF) and reflection photoplethysmography (PPG) in all participants' left and right lower limbs during the experimental protocol (see RESULTS). Statistical comparison (Wilcoxon sign rank test) between lower limbs is shown in each phase of the protocol. **P* < 0.001.

DISCUSSION

Research on the effects of massage has been centered in specific conditions as a consequence of the multiplicity of benefits and safety of this "complementary and alternative medicine practice" (11, 13, 15, 51). From the various types or modalities of massage included in some reviews, the Swedish massage is the most common reference used in the Western countries for a wide variety of clinical conditions, including athletic performance and rehabilitation (15, 17, 18, 26, 56, 63). The Swedish massage technique encompasses five main approaches, which might be

performed with different combinations (18): effleurage, more superficial, along the main axis with constant pressure by the heel of the hand to relax the tissues; petrissage, involving kneading and lifting to reach deeper tissues; tapotement, a procedure (slapping, cupping) meant to invigorate; friction, involving more powerful pressure to reach deeper tissues; and vibration, expected to excite and prepare muscles for more intense activity and performance (18, 29, 33, 61).

The effectiveness of massage regarding hemodynamics and cardiovascular function has been unclear, and results have

Table 2. Statistical comparison of perfusion changes between massaged and contralateral nonmassaged limbs registered in all participants

	<i>Phase I</i>		<i>Phase II</i>		<i>Phase III</i>		Δ II-I		Δ III-I	
	Mean	Median	Mean	Median	Mean	Median	Mean	Median	Mean	Median
<i>Proximal procedure</i>										
LDF, massaged vs. nonmassaged	6.3 vs. 7.0	5.8 vs. 6.3	12.7 vs. 8.3	10.6 vs. 7.7	6.6 vs. 7.0	5.1 vs. 6.3	114.8 vs. 24.1	94.7 vs. 10.3	2.3 vs. -1.4	-1.3 vs. -4.6
	<i>P</i> = 0.334		<i>P</i> = 0.020		<i>P</i> = 0.390		<i>P</i> < 0.001*		<i>P</i> = 0.237	
PPG, massaged vs. nonmassaged	50.3 vs. 45.1	34.9 vs. 30.6	62.8 vs. 49.3	47.7 vs. 36.0	43.9 vs. 44.2	30.0 vs. 29.2	43.6 vs. 11.5	32.9 vs. 2.6	-5.8 vs. -1.9	-5.3 vs. -11.9
	<i>P</i> = 0.638		<i>P</i> = 0.043		<i>P</i> = 0.555		<i>P</i> = 0.003		<i>P</i> = 0.390	
<i>Distal procedure</i>										
LDF, massaged vs. nonmassaged	6.6 vs. 7.8	5.2 vs. 6.8	12.0 vs. 8.4	10.8 vs. 7.0	7.2 vs. 7.9	4.7 vs. 6.4	99.5 vs. 14.6	70.6 vs. 1.7	2.9 vs. 0.8	-1.0 vs. -5.0
	<i>P</i> = 0.229		<i>P</i> = 0.039		<i>P</i> = 0.175		<i>P</i> < 0.001*		<i>P</i> = 0.550	
PPG, massaged vs. nonmassaged	50.7 vs. 49.2	31.9 vs. 29.1	63.6 vs. 45.1	45.5 vs. 28.6	52.4 vs. 43.4	29.0 vs. 23.7	49.1 vs. 1.7	32.0 vs. 0.0	2.5 vs. -5.9	-6.2 vs. -5.8
	<i>P</i> = 0.722		<i>P</i> = 0.013		<i>P</i> = 0.301		<i>P</i> = 0.001*		<i>P</i> = 0.524	

Statistical comparison (Mann-Whitney *U* test) of perfusion changes between the massaged and contralateral (nonmassaged) limb registered in all participants (*n* = 32) regarding laser Doppler flowmetry (LDF) and reflection photoplethysmography (PPG) signals with both massage procedures (see RESULTS). **P* < 0.001.

Table 3. Statistical comparison of the impact of the 2 effleurage variations on perfusion and hemodynamics of all participants in massaged and nonmassaged limbs

	Phase I	Phase II	Phase III	ΔII-I	ΔIII-I
<i>Proximal vs. distal procedure</i>					
LDF					
Massaged	6.3 vs. 6.6 5.8 vs. 5.2 0.973	12.7 vs. 12.0 10.6 vs. 10.8 0.653	6.6 vs. 7.2 5.1 vs. 4.7 0.768	114.8 vs. 95.5 94.7 vs. 70.6 0.390	2.3 vs. 2.9 -1.3 vs. -1.0 0.677
Contralateral	7.0 vs. 7.8 6.3 vs. 6.8 0.877	8.3 vs. 8.4 7.7 vs. 7.0 0.883	7.0 vs. 7.9 6.3 vs. 6.4 0.809	24.1 vs. 14.6 10.3 vs. 1.7 0.280	-1.4 vs. 0.8 -4.6 vs. -5.0 0.825
PPG					
Massaged	50.3 vs. 50.7 34.9 vs. 31.9 0.936	62.8 vs. 63.5 47.7 vs. 45.4 0.872	43.9 vs. 52.4 30.0 vs. 29.0 0.732	43.6 vs. 49.1 32.9 vs. 32.0 0.768	-5.8 vs. 2.5 -5.3 vs. -6.2 0.663
Contralateral	45.1 vs. 49.2 30.6 vs. 29.1 0.851	49.3 vs. 45.1 36.0 vs. 28.6 0.697	44.2 vs. 43.4 29.2 vs. 23.7 0.867	11.5 vs. 1.7 2.6 vs. 0.0 0.638	-1.9 vs. -5.9 -11.9 vs. -5.8 0.390
Systolic pressure	111.6 vs. 111.3 110.2 vs. 111.0 0.979	108.8 vs. 108.4 106.8 vs. 108.4 0.930	112.5 vs. 111.4 112.5 vs. 110.0 0.648	-2.5 vs. -2.8 -2.1 vs. -2.8 0.372	0.8 vs. -0.1 0.3 vs. 0.0 0.340
Diastolic pressure	70.6 vs. 69.9 70.8 vs. 70.8 0.510	67.8 vs. 68.2 65.8 vs. 67.3 0.510	72.2 vs. 70.6 70.8 vs. 71.7 0.888	-4.0 vs. -2.7 -3.1 vs. -2.6 0.320	1.2 vs. 0.8 0.3 vs. 2.1 0.209
Pulse rate	68.1 vs. 68.4 67.9 vs. 67.7 0.835	65.7 vs. 65.4 65.8 vs. 65.2 0.968	68.4 vs. 68.0 69.0 vs. 67.7 0.804	-3.6 vs. -4.3 -3.7 vs. -4.4 0.280	0.5 vs. -0.5 -0.1 vs. -0.5 0.340

Data are expressed as means $\pm$ SD and medians, followed by *P* values (*n* = 32). Statistical comparison (Mann-Whitney *U* test for independent samples) on the impact of the 2 effleurage variations on perfusion and hemodynamics of all participants in both massaged and nonmassaged limbs. Data from both limbs obtained by proximal massage are compared with data obtained from the distal procedure (see RESULTS).

often been inconsistent (11, 15, 33, 56). Bradycardia, bradypnea, and a decrease in blood pressure are well documented and recently reviewed (13, 14, 34, 51). In recent years, significant evidence has suggested that massage increases blood flow (15, 34), although a clear demonstration of the mechanisms involved is still lacking.

A primary factor to consider is the applied mechanical pressure, likely promoting the local release of vasodilator mediators (e.g., nitric oxide, histamine, adenosine) and/or reducing the sympathetic activity, justifying this flow-mediated

dilation hyperemia (15, 23, 27). This mechanical pressure involving a compression/squeezing effect over the venous compartment was referred to facilitate venous return to the heart (10, 27), but no clear data supported this view. On the contrary, recently published data point to a rather superficial effect of this technique on the skin, without the mobilization of those quantities of blood from the peripheral venous compartment (23, 43).

Our results have consistently shown that effleurage evokes an increased perfusion in the massaged limb. Moreover, it has

Table 4. Sex-related differences detected on perfusion means (LDF and PPG, expressed in BPUs) from all participants in each phase of the applied experimental protocol involving these 2 variations of the effleurage technique

	Phase I		Phase II		Phase III		ΔII-I	ΔIII-I
	Mean $\pm$ SD	Median	Mean $\pm$ SD	Median	Mean $\pm$ SD	Median		
Proximal procedure								
LDF								
Male	8.0 $\pm$ 3.0	7.2	11.5 $\pm$ 4.6	10.1	8.3 $\pm$ 4.4	7.0	<i>P</i> = 0.323	<i>P</i> = 0.491
Female	5.3 $\pm$ 2.0	4.9	9.5 $\pm$ 5.2	7.1	5.2 $\pm$ 1.7	4.9		
PPG								
Male	67.2 $\pm$ 49.7	47.0	77.0 $\pm$ 52.6	57.2	60.5 $\pm$ 48.9	39.5	<i>P</i> = 0.696	<i>P</i> = 0.361
Female	28.2 $\pm$ 14.5	23.0	35.1 $\pm$ 17.4	26.9	27.6 $\pm$ 15.8	24.2		
Distal procedure								
LDF								
Male	8.5 $\pm$ 4.5	6.7	10.7 $\pm$ 4.9	9.8	8.0 $\pm$ 4.2	6.2	<i>P</i> = 0.039	<i>P</i> = 0.224
Female	5.9 $\pm$ 3.8	4.3	9.7 $\pm$ 5.9	7.8	7.1 $\pm$ 7.7	4.3		
PPG								
Male	69.3 $\pm$ 56.6	56.2	68.3 $\pm$ 47.7	44.5	62.3 $\pm$ 54.6	40.9	<i>P</i> = 0.305	<i>P</i> = 0.752
Female	30.6 $\pm$ 15.2	27.7	40.4 $\pm$ 21.3	34.9	33.4 $\pm$ 28.7	25.4		

Data are expressed as means $\pm$ SD. Sex-related differences (Mann-Whitney *U* test for independent samples) detected on perfusion means (LDF and PPG, expressed in BPUs) from all participants (*n* = 32) in each phase of the applied experimental protocol involving these 2 variations of the effleurage technique. Significant differences between male and female participants are present, in absolute value, but massage evokes adaptive responses with equivalent magnitude (see RESULTS).

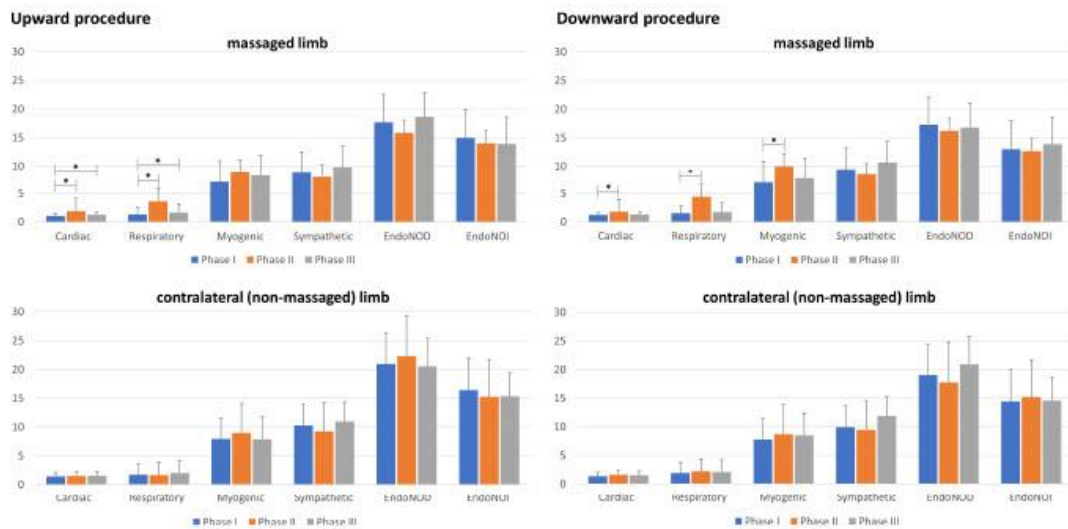


Fig. 2. Component analysis of laser Doppler flowmetry (LDF) signals (means $\pm$ SD) obtained during application of the experimental protocol involving these 2 effleurage massage variants, proximal or centripetal, developed in the upward direction from the ankle to the knee, and distal or centrifugal, in the downward direction, from the knee to the ankle (see RESULTS). Amplitude variation is shown from high-frequency (cardiac) to lowest frequency components [reference frequency bands used were cardiac (0.6–2 Hz), respiratory (0.145–0.6 Hz), myogenic (0.052–0.145 Hz), sympathetic (0.021–0.052 Hz), endoNOD (0.0095–0.021 Hz), and endoNOI (0.005–0.0095 Hz)]. endoNOD, endothelial NO dependent; endoNOI, endothelial NO independent. Statistical analysis of variation between *Phases II and I* and *III and I* are also indicated (* $P < 0.001$).

shown that such a superficial intervention is also able to evoke a similar response in the contralateral (nonmassaged) limb, a peripheral microcirculatory adaptive mechanisms we found in mice following a hindlimb ischemia (HLI) procedure during recovery neovascularization (31, 32), and also in humans by studying the venoarteriolar (VA) reflex (39, 40, 54) or under hyperoxia (52, 55).

Assessment technology is another key factor to consider. LDF and PPG are likely to be the most frequently used technologies, exploring interactions between light and tissue to calculate blood flow. However, both signals are expressed in arbitrary (perfusion) units, meaning that blood flow is not measured directly. In fact, these signals are typically pulsatile, combining multiple contributions (heart rate and contractility, respiration, and autonomic, myogenic, and endothelial activities) present in these perfusion signals, also known as the microcirculatory flowmotion (22, 43, 46, 47). LDF and PPG operate with different wavelengths, which means different penetration and discrimination capacities. LDF is assumed to be linearly related to the velocity of moving erythrocytes (35, 43), operating with a 780-nm wavelength red/near-infrared light providing a measurement depth of ~ 0.3 – 0.5 mm, although some light might reach 1 mm or more (4, 35). PPG can use different light wavelengths, the green and the red being the most popular (50). The green (530 nm) wavelength light is considered relatively free from noise artifacts, providing more superficial (2 mm) perfusion measurements compared with red light (5, 19). Therefore, it is clear that we cannot be completely certain about the origin of the pulse to identify the multiple contributory hemodynamic influencers (1, 43). As shown in Table 1, both technologies revealed the same type of perfusion events as a consequence of massage no matter the effleurage variant con-

sidered. The differences detected between phases (Table 1) are not only due to the different penetration/discriminative capacities of both lasers, but likely are also affected by the compression exerted on the tissue that is referred to deepen PPG penetration (5). Reductions in skin temperature, even if discrete, should also be considered, as it might potentially mask the expected effect, especially in the contralateral nonmassaged limb, and this draws our attention to improving protocols to preserve homeothermy.

Therefore, *Phase II* perfusion adjustments, detected by either LDF and PPG, can be attributed only to the massage procedure. The concomitant contralateral responses would result from a centrally mediated reflex, likely related to the bilaterality of the sensorimotor network evoked by the acute changes in regional microcirculation (8, 9). From our experience, the stimulation of one limb, no matter if brief as the VA reflex or the effleurage, or more durable, as in neovascularization, seems to activate the same cardiovascular integration through a functional network that also involves the contralateral equivalent to restore distal circulatory homeostasis. Recently, we (44) found this integration to be present in weight transfer in the one-legged stance test. In fact, our studies suggest that multiple detectors and neuroregulatory effectors cooperate, centrally and peripherally, to reestablish the systemic hemodynamic homeostasis modified by different stressors. Thus, we decided to look deeper into the skin microcirculatory flowmotion with the WT analysis of both massage variants. As mentioned previously, the type of stroke as the applied technique, including the massage orientation, were mentioned to potentially affect outcomes (34, 56). Centrifugally oriented massage was referred to favor immunomodulation and improve production of blood cells in children's acute lymphoblastic leukemia (11, 12, 45) as well as to facilitate weight gain in very-low-weight neo-

nates (28, 37). This component analysis of LDF signals reveals that effleurage evokes (Fig. 2) substantial increases in cardiac, respiratory, and myogenic components and a reduction of sympathetic and endothelial components specially in the massaged limb. Similar (although much less evident) changes are detected in the contralateral nonmassaged limb, also with the distal massage. These should explain the primary increase of local perfusion, followed by a consistent reduction of blood pressure and heart rate. During recovery, these activities are reduced, more obviously in the massaged limb, restoring the vascular homeostasis for both distal limbs as one. Therefore, it is clear that, in our experimental conditions, these direction variants do not influence outcomes, producing instead comparable perfusion changes in both limbs and decreasing blood pressure and pulse with similar magnitudes (Table 1 and Table 3).

In conclusion, the present study was centered on characterizing the impact of effleurage on in vivo perfusion and hemodynamics as closely as possible to the normal physiological state. Our option for nonparametric statistical analysis, also used by other authors and in particular with optical measurement technologies (25, 46, 48), was based on our methodological choices. Nevertheless, we adopted a more stringent significance level ($P < 0.001$) in determining the effect of massage on microcirculatory perfusion and its impact on cardiovascular function. Therefore, it is clear that this short, superficial intervention considerably increases skin microcirculatory flowmotion in the massaged area, and beyond, in the contralateral nonmassaged limb, and that the massage orientation variants tested have had an equivalent impact under these conditions.

To our knowledge, the impact of localized massage on other parts of the body, including a nonhomologous limb such as the arm or hand, has not been described. We might conjecture that it would not be very different in nature and duration, reflecting the sensitivity of cardiovascular integration to restore physiological hemodynamics. Nevertheless, future studies on this theme should take into account some specific considerations: 1) the choice of measurement technologies is critical, keeping in mind that when using optical methods, we are not assessing blood flow but rather perfusion or flowmotion at different depths, depending on the chosen wavelengths (1, 41, 43, 47), and the use of a sole technology will bring major limitations in data interpretation; 2) the mathematical modeling of these signals is but one way to look deeper into its components, as we did for the LDF data, with WT emphasizing myogenic (by reduction of the sympathetic output?) rather than endothelial components. Other strategies (detrended fluctuation, entropy, texture analysis) might be used (53, 55), or these BPU's might be compared with the calculated perfusion (the concentration of red blood cells $\times$ velocity) as means to potentially improve data quality; 3) the impact of frequency and duration of effleurage in healthy individuals has not been studied, nor do we know how other groups of individuals (e.g., older, obese, diabetic) will respond to these experimental conditions. However, our approach seems to be appropriate to study these mechanisms regulating skin flowmotion and applicable for further exploration of massage and its impact on microcirculatory physiology.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.M.R. conceived and designed research; L.M.R., C.R., H.T.F., and H.N.S. analyzed data; L.M.R., C.R., and H.N.S. interpreted results of experiments; L.M.R. and C.R. drafted manuscript; L.M.R. and H.T.F. approved final version of manuscript; C.R. and H.N.S. performed experiments; C.R. prepared figures; H.N.S. edited and revised manuscript.

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Artigo VI - The modification of skin microcirculatory perfusion evokes a Prompt Adaptive Hemodynamic Response – perspective as an endpoint to investigate integrative cardiovascular (dis)function *in vivo*

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Em Submissão

Abstract

Cardiovascular homeostasis is a notable example of integrative physiology regulation involving the interaction of multiple players to ensure a permanent adaptation to each organ's needs, without a single unique “maestro.” This prompt adaptive hemodynamic response (PAHR) to transitory changes in skin microcirculation was our focus, with the aim to better understand its characteristics and potential use as a clinical endpoint. Twenty-two healthy adults (both sexes, 52.5 ± 6.1 y.o.) were selected. Procedures were approved by the institutional Ethical Commission and complied with principles of good clinical practice for human research. We used two optical technologies to evaluate perfusion - laser Doppler flowmetry (LDF) (Perimed PF5010, Perimed, Sweden) and photoplethysmography (PPG) (Bitalino-PLUX Biosignals, Portugal). Sensors were applied in both feet (2nd and 3rd toes, inferior) to record perfusion at baseline (i) and during a (single limb) challenge (ii) and recovery (iii). Two challenges with opposite impact on perfusion were applied - massage (n=22) and a venoarteriolar reflex (VAR) (n=10) procedure. Descriptive and comparative statistics were performed, and a 95% level of confidence adopted. As in our previous study, PAHR was detected by LDF and PPG in both limbs. A perfusion increase was observed with massage (only significant in the test limb) and a perfusion decrease (statistically significant in both limbs) with VAR, accordingly altering ($p < 0.05$) blood pressure and pulse. LDF signals decomposition analysis (wavelet transform) was similar to those obtained in younger participants, although more discrete. VAR produced a clear reduction ($p < 0.05$) in all components in the test foot. The apparent age dependency of PAHR suggests that pathology might compromise its efficiency. PARH seems to be the basis of all hemodynamical adaptive homeostasis, illustrating a complex interaction between peripheral and central sensors and effectors and suggesting potential utility as an endpoint with predictive value to investigate human *in vivo* cardiovascular function.

Keywords: Prompt Adaptive Hemodynamic Response - PAHR; cardiovascular; microcirculation; homeostasis; LDF; PPG

Introduction

From the physiological point of view, microcirculation is regarded as the most important component of the cardiovascular system. It is essential to preserve cell viability and all organ functions and involves a remarkable capacity to adapt local perfusion to global haemodynamical requirements (1-4). Endothelium and microvascular walls are not passive structures reacting to blood pressure. Perfusion biophysics (flow, stress, pressure, stretch, and resistance) originating from heart contraction and tempered by large arteries condition microcirculation. The permanent crosstalk between microcirculation and higher circulatory structures is manifested in various ways (2,4-8). In fact, recent evidence demonstrates that these forces resulting from the pulsatile pressure and flow, known for many years as the Windkessel effect (9), trigger chemical signaling and transduction from microcirculation (7-9). The permanent management of different sensors and effectors, central as peripheral, involves multiple neuroendocrine regulation mechanisms for prompt vascular adjustment - down to the microcirculatory level - to ensure a continuous balance between the cell needs and cardio-respiratory performance. Therefore, under normal physiological conditions, this balance between macro and microcirculation results in a hemodynamical homeostasis which requires structural integrity to respond to neuroendocrine output affecting cardiorespiratory performance, systemic vascular resistance, and local microcirculatory (endothelial and myogenic) activity (5-10). When this balance cannot be maintained, disease develops. Microcirculatory dysfunction is related to high blood pressure and atherosclerosis, both of which increase pressure and flow pulsatility. The latter condition is an indicator of microcirculatory damage especially relevant to sensitive high flow / low resistance organs such the retina and the central nervous system and the kidney (10-12) . Recently, microcirculatory dysfunction has also been associated with many other non-cardiovascular pathophysiological processes,

including metabolic, neurodegenerative, and neoplastic processes, among others (13-17). However, detailing the terms of the cross-talk between regulators and effectors and identifying early indicators of dysfunction (and disease) are still uncertain objectives, and overcoming this limitation could improve prevention and prognosis for many disease states .

Our research group has been dedicated to the physiological modeling of microcirculatory function to identify the basic mechanisms contributing to the regulation and maintenance of local flow and cardiovascular homeostasis (6, 18-23). Our observations of microcirculatory perfusion, as quantified by non-invasive optical-based systems, may have clinical relevance and utility. By challenging local skin microcirculation with different (intensity/duration) hemodynamical modifiers (e.g., reactive hyperemia, hyperoxia, massage), we can identify and reproduce the same endpoint integrating various reflexes, what we named as a *“Prompt Adaptive Hemodynamic Response”* (PAHR), which immediately attempts to reestablishes a (regular) circulatory homeostasis, independent of the type of circulatory challenge triggering modification, as we have previously published in studies with young healthy adult participants (20,22). Our goal in the present study was to characterize and further explore this PAHR in aged participants submitted to two different challengers – massage (effleurage) and a leg-dropping procedure to evoke the venoarteriolar reflex VAR, with the expectation to observe an age-dependency which may help us to interpret its physiological significance and applicability.

Methods

Subjects

A convenience sample of twenty two healthy participants (52.5 ± 6.1 years old) both sexes ($n=11$ for each sex) was selected after informed written consent. Specific inclusion / non-inclusion criteria previously defined and applied in similar research (19) were applied. All participants were normotensive with no signs of vascular impairment, confirmed by the Ankle-Brachial Index ($ABI\ 1.1 \pm 0.1$) (24), normal body mass index ($BMI\ 24.8 \pm 2.1$), and (self-reported) equivalent levels of physical activity. Further, participants were non-smokers and free of any regular medication or food supplementation. All female participants were menopausal without hormone-replacement therapy. Study participants were asked to refrain from consuming caffeinated and/or any other vasoactive beverages within 24 hours prior to the experiments.

All procedures were previously approved by the institutional Ethical Commission and carried out in accordance with the Declaration of Helsinki and respective amendments, observing good clinical practice for medical research involving human participants (25).

Experimental

Experiments took place with controlled temperature and humidity ($21 \pm 1^{\circ}C$; 40-60%) and light. Participants were allowed to acclimatize to room conditions (~ 20 min) lying in the supine position.

Massage

Massage was applied to one randomly chosen leg with the contralateral leg, in the same position, serving as control. This procedure, also known as *effleurage* was applied to all individuals in the ascending direction by an experienced licensed therapist (26,27) and involved the application of short, rhythmic and repetitive strokes with light compression, with the palms of both hands gliding along the (challenged) leg's longitudinal axis, in the

proximal direction (from the ankle to the knee) to soften and lengthen muscle fibers. Almond oil, known to affect only the most superficial epidermal layers, was used as a lubricant to reduce any friction discomfort (28,29). The protocol, as registered by LDF and PPG (see below), included three phases – a 10-minute baseline resting register (Phase I), a 5-minute massage challenge (Phase II), and a 10-minute recovery (Phase III).

The Veno-arterial Reflex (VAR)

From the original participant pool, five men and five women (mean 50.3 ± 5.64 years old, ABI 1.1 ± 0.1 and BMI 24.9 ± 2.2) volunteered to participate in a standard VAR procedure (30). The experiment involved a 10 minute baseline recording phase, in a supine position, both legs lying parallel to the body axis (Phase I); a 10 minute challenging recording phase with one randomly chosen leg pending approximately 50 cm below the heart level (Phase II); and a 10-minute recovery (Phase III), resuming the initial position.

Measurement technologies and signal processing

In both procedures, blood perfusion was assessed through laser Doppler flowmetry (LDF) and photoplethysmography (PPG), two optical technologies based on related principles but with different skin depth capacities (31-33). LDF and PPG sensors were applied in the plantar aspects of the second and first toes, respectively, in both feet. This strategy substantially reduces variability when measuring in the distal inferior limb as previously published (6,20,22). LDF signal, expressed in arbitrary Blood Perfusion Units (BPUs), was obtained with a Perimed PF5010 system (Perimed, Sweden), which also allowed the continuous monitoring of skin surface temperature. PPG signal, also expressed in BPUs, was obtained by a Bitalino Plugged Kit (PLUX Biosignals, Portugal), which also recorded Blood Volume Pulse (BVP) and heart rate. Blood pressure (systolic and diastolic) was

measured in the arm during the procedure (Tensoval Comfort device, Hartman, Germany).

LDF signals (32 Hz sampling rate) were further decomposed by the Wavelet transform (WT) analysis by a MATLAB-based toolbox (Mathworks Inc., 151 Natick, MA, USA) into their main frequency components (cardiac, respiratory, myogenic, sympathetic, endothelial NO-dependent (endoNOd) and endothelial NO-independent (endoNOi)) to assess their contribution to the signal obtained. Reference frequency bands for cardiac (0.6 Hz to 2 Hz), respiratory (0.145 Hz to 0.6 Hz), myogenic (0.052 Hz to 0.145 Hz), sympathetic (0.021 Hz to 0.052 Hz), endothelial NO-dependent (endoNOd - 0.0095 Hz to 0.021 Hz) and endothelial NO-independent (endoNOi - 0.005 to 0.0095 Hz) (34-36) components were used.

Statistics

Descriptive and comparative statistics were applied to LDF and PPG signals and to the WT components using IBM SPSS v22.0 software (IBM Corporation, NY, USA). Perfusion changes were calculated as the mean value of the LDF signal and as the mean amplitude of the PPG signal. In the absence of a normal distribution, a non-parametric approach was adopted using the Wilcoxon signed-rank test for paired samples for phase comparisons and the Mann-Whitney U test for independent samples. For the wavelet analysis, the activity of each component was calculated for each phase as the percent ratio of the area under the curve (AUC) of that signal component in the frequency spectrum divided by the AUC of the entire frequency spectrum for that phase. A confidence level of 95% ($p < 0.05$) was adopted.

Results

The application of the effleurage procedure to a single limb modifies perfusion in both the challenged and the contralateral non-challenged limbs (6,20-23) (Figure 1, Table 1). This integrated reflex, which our group has named PAHR is consistently detected by both LDF and PPG. PAHR is statistically significant in the foot of the challenged limb, and disappears in both limbs in recovery (Phase III). Significant reductions ($p < 0.05$) in systolic and diastolic pressures (the latter persisting in the Phase III) and pulse are also detected (Table 1).

Illustrative examples of these observations are shown in Figure 1.

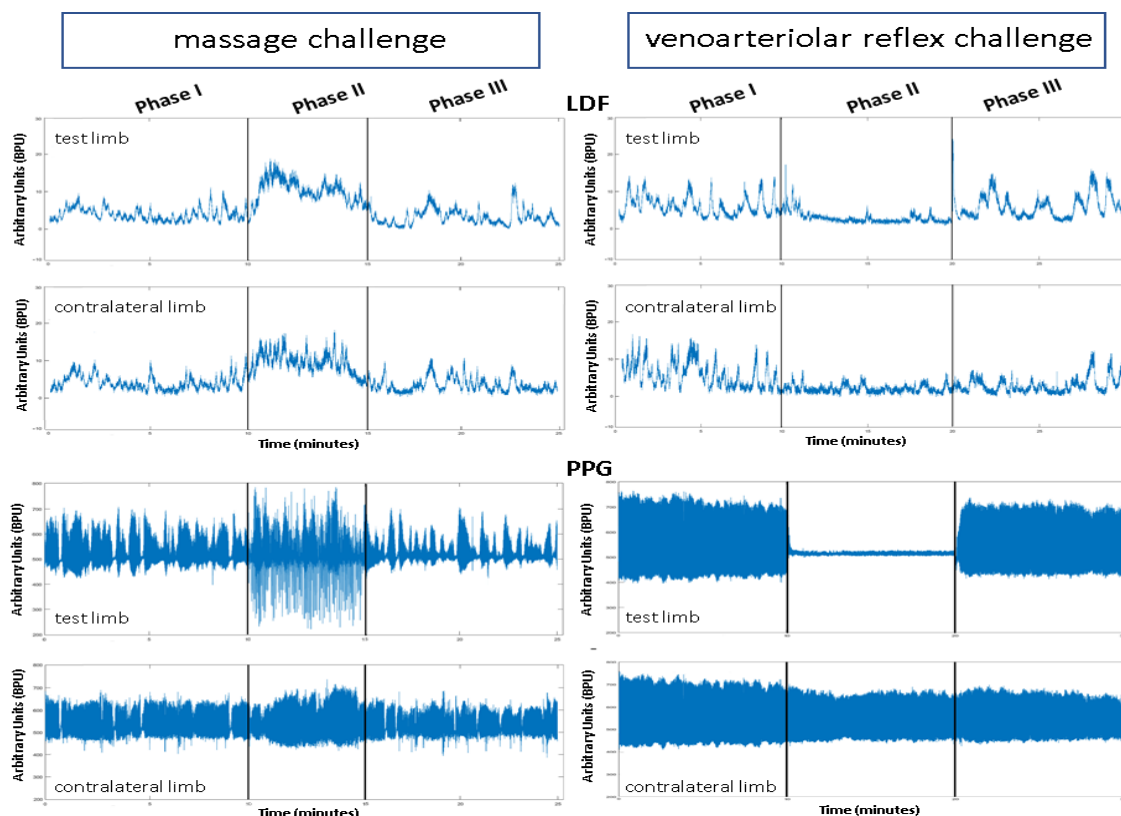


Figure 1 - Illustrative example of the mean lower limb perfusion changes expressed in arbitrary blood perfusion units obtained by laser Doppler flowmetry (LDF) and reflection photoplethysmography (PPG) in all participants' test and contralateral lower limbs after application of the proximal massage (effleurage) procedure (left side) and the venoarteriolar reflex (right side) respectively.

Table 1 - Mean lower limb perfusion changes expressed in arbitrary blood perfusion units obtained by laser Doppler flowmetry (LDF) and reflection photoplethysmography (PPG) in all participants' left and right lower limbs after application of the proximal-ascending massage (effleurage) procedure (see text). Blood pressure (systolic and diastolic, mm Hg) and pulse (bpm) are also shown. Data are expressed as means $\pm$ SD and medians, followed by p values where applicable (see RESULTS). Statistical comparison (Wilcoxon sign rank test) between massaged and contralateral (non-massaged) limbs is between shown in each phase of the protocol. * $p < 0.05$; ** $p < 0.005$.

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	Phase I		Phase II		Phase III		Δ II-I (%)			Δ III-I (%)		
	mean $\pm$ sd	median	mean $\pm$ sd	median	mean $\pm$ sd	median	mean	p	median	mean	p	median
LDF (BPU's)												
massaged	19.6 $\pm$ 27.2	10.6	35.2 $\pm$ 43.3	19.5	23.3 $\pm$ 31.7	14.2	79.8	0.001**	83.9	18.8	0.159	34.4
contralateral	25.8 $\pm$ 45.9	12.9	30.6 $\pm$ 49.0	13.2	23.4 $\pm$ 37.2	13	18.5	0.322	2.5	-9.2	0.39	1.2
PPG (AU's)												
massaged	148.7 $\pm$ 141.3	89.1	208.8 $\pm$ 152.8	157.2	147.8 $\pm$ 128.6	103.5	40.5	0.007*	76.5	-0.6	0.355	16.2
contralateral	138.5 $\pm$ 129.9	88.7	156.8 $\pm$ 137.3	104.7	129.6 $\pm$ 131.7	88.1	13.2	0.338	18.1	-6.4	0.131	-0.7
Systolic pressure												
(mmHg)	121.5 $\pm$ 12.5	121.5	119.5 $\pm$ 12.8	119.5	123.0 $\pm$ 12.2	123	-1.6	0.020*	-1.6	1.2	0.06	1.2
Diastolic pressure												
(mmHg)	80.3 $\pm$ 7.4	80.3	77.2 $\pm$ 5.3	77.2	81.0 $\pm$ 7.1	81	-3.9	0.001**	-3.9	0.9	0.022*	0.9
Pulse												
(bpm)	62.1 $\pm$ 10.8	60.3	60.3 $\pm$ 10.5	59.7	62.0 $\pm$ 11.0	60.3	-2.9	0.001**	-1	-0.1	0.758	0

Results also reveal intraindividual differences in baseline perfusion between both feet and interindividual sex-related perfusion differences. These baseline and gender-related asymmetries have been previously described, although they are still poorly understood (see ahead) (37,38). Comparison of LDF and PPG perfusion data obtained in all participants for each phase of the experimental procedures (Table 2) revealed no differences between limb pairs in any phase of the procedure except for Phase II values obtained with LDF and PPG which can only be attributed to the challenge procedure.

The application of WT analysis to the LDF signals offers a mechanistic view of this PAHR (Figure 2). Results from this analysis are in line with a similar approach in our previously published study (22) with younger subjects, with a comparable evolution of each component mean in both limbs, although much more discrete. No statistically significant differences could be detected in our current study with older healthy adults.

Table 2 - Statistical comparison (Mann-Whitney U test) of perfusion changes between the massaged and contralateral (non-massaged) limb registered in all participants (n =22) regarding laser Doppler flowmetry (LDF) and reflection photoplethysmography (PPG) signals following the application of the proximal-ascending massage procedure *p <0.05.

	Phase I		Phase II		Phase III		Δ II-I		Δ III-I	
	mean	median	mean	median	mean	median	mean	median	mean	median
LDF	19.6 vs 25.8	11.0 vs 12.9	35.2 vs 30.6	19.5 vs 13.2	23.3 vs 23.4	14.2 vs 13.0	79.8 vs 18.5	83.9 vs 2.5	18.8 vs -9.2	34.4 vs 1.2
massaged vs contralateral	p=0.622		p=0.372		p=0.944		p<0.001*		p=0.060	
PPG	148.7 vs 138.5	89.1 vs 88.7	208.8 vs 156.8	157.2 vs 104.7	147.8 vs 129.6	103.5 vs 88.1	40.5 vs 13.2	76.5 vs 18.1	-0.6 vs -6.4	16.2 vs -0.7
massaged vs contralateral	p=0.796		p=0.146		p=0.557		p=0.041*		p=0.526	

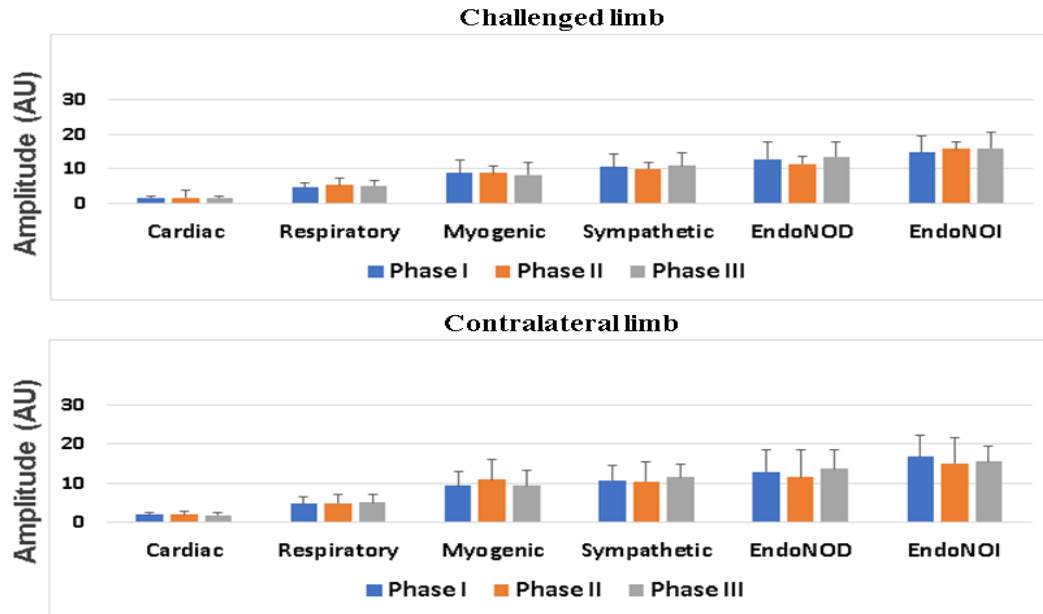


Figure 2 - Component analysis of laser Doppler flowmetry (LDF) signals (means $\pm$ SD) obtained with the application of the proximal effleurage massage (see RESULTS). Amplitude variation (AU's) is shown from high- to lowest frequency components. Frequency bands used were cardiac (0.6–2 Hz), respiratory (0.145–0.6 Hz), myogenic (0.052– 0.145 Hz), sympathetic (0.021– 0.052 Hz), endoNOd (0.0095– 0.021 Hz), and endoNOi (0.005– 0.0095 Hz)]. Statistical significance of variation between Phases II and I and between Phases III and I were also calculated (*p <0.05).

Table 3 summarizes the perfusion and pulse changes obtained with the VAR procedure. A similar PAHR with variation in the opposite direction (of the massage challenge) was detected. That is, in contrast to the massage challenge, where perfusion was increased in both limbs, a significant reduction in perfusion was detected in Phase II with both LDF and PPG in the pending limb and in the contralateral (static) limb. After resuming the initial position, perfusion recovers in both limbs, although perfusion remained lower than baseline at the end of Phase III. No differences between sexes were found in any of the experimental phases.

Figure 3 represents the spectral analysis of the LDF signals obtained with the VAR challenger. The intensity of the PAHR response generated by the VAR in both limbs enables a more clear vision of LDF signal components evolution. Results are in accordance with recently published information obtained from PPG signal decomposition

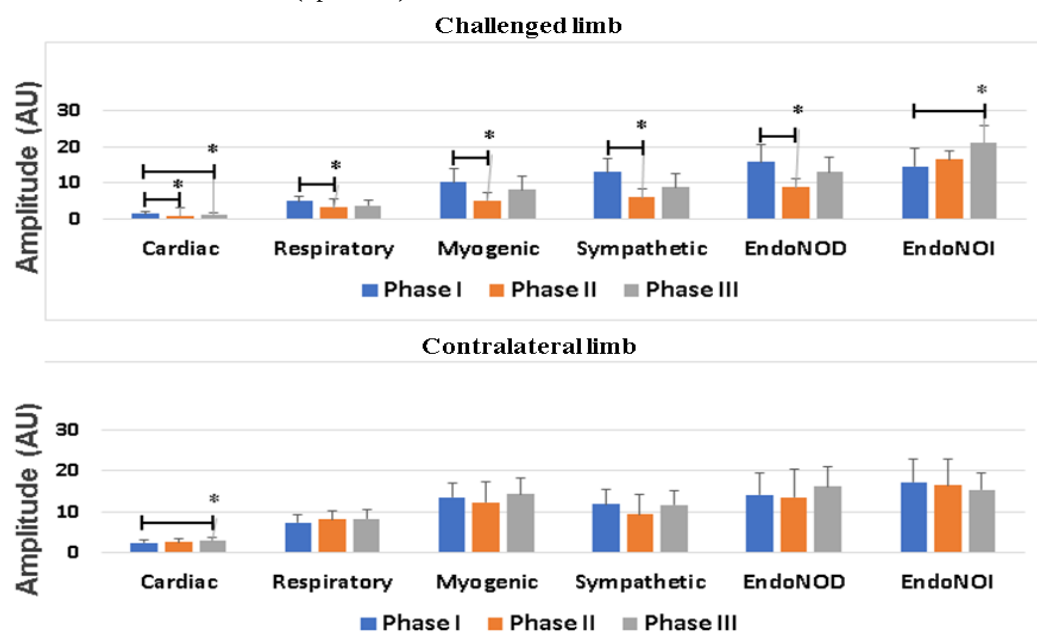
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in a young healthy panel (20). PPG and LDF signals have been shown to have comparable frequency intervals for the components described. As shown, the PAHR response to the pendent leg (Phase II) involves a marked decrease of myogenic, sympathetic and endothelial NO dependent components in both limbs, although significant differences could only be found in the test limb. The cardiac and respiratory high frequency components also change significantly in the test limb.

Table 3 - Mean lower limb perfusion changes expressed in arbitrary blood perfusion units obtained by laser Doppler flowmetry (LDF) and reflection photoplethysmography (PPG) in all participants' left and right lower limbs after single leg dropping procedure to evoke the venoarteriolar reflex (see text). Pulse values (bpm) are also shown. Data are expressed as means $\pm$ SD and medians, followed by p values where applicable (see RESULTS). Statistical comparison (Wilcoxon sign rank test) between lower limbs is shown in each phase of the protocol. *p < 0.05.

	Phase I		Phase II		Phase III		Δ II-I (%)			Δ III-I (%)		
	mean $\pm$ sd	median	mean $\pm$ sd	median	mean $\pm$ sd	median	mean	p	median	mean	p	median
LDF (BFU's)												
pendant	49.1 $\pm$ 52.9	24.7	24.1 $\pm$ 36.7	6.4	39.4 $\pm$ 43.4	20.9	-50.8	0.005*	-74.1	-19.7	0.139	-15.7
contralateral(static)	46.4 $\pm$ 49.3	26	38.3 $\pm$ 48.0	17.2	41.4 $\pm$ 51.2	19.2	-17.5	0.028*	-33.7	-10.7	0.445	-25.9
PPG (AU's)												
pendant	231.3 $\pm$ 194.5	201.3	40.0 $\pm$ 60.0	17.7	205.1 $\pm$ 199.2	154.6	-82.3	0.005*	-91.2	-11.3	0.139	-23.2
contralateral(static)	167.4 $\pm$ 123.5	180.3	128.8 $\pm$ 97.3	113.6	140.0 $\pm$ 103.8	145.7	-23	0.017*	-36.9	-16.3	0.169	-19.2
Pulse												
(bpm)	66.6 $\pm$ 8.3	66	66.7 $\pm$ 8.4	67.9	65.8 $\pm$ 7.9	66.4	0.1	0.878	1.4	-1.3	0.114	-2.3

Figure 3 - Component analysis of laser Doppler flowmetry (LDF) signals (means $\pm$ SD) obtained with a venoarteriolar reflex manouever in both lower limbs (see RESULTS). Amplitude variation (AU's) is shown from high- to lowest frequency components. Frequency bands used were cardiac (0.6–2 Hz), respiratory (0.145– 0.6 Hz), myogenic (0.052– 0.145 Hz), sympathetic (0.021– 0.052 Hz), endoNOD (0.0095– 0.021 Hz), and endoNOi (0.005– 0.0095 Hz)]. Statistical significance of variation between Phases II and I and III and I were also calculated (*p < 0.05).



Discussion

The basic concepts of hemodynamical control and regulation of peripheral perfusion are long known, however, the interaction of these different components (neural, metabolic, cardiac and vascular) to ensure hemodynamical homeostasis is still only partially understood.

Blood pressure has been used as a central indicator considering the multiple reflexes (baroreceptor, chemoreceptor, medullar, other) involved in its regulation, mediated by the autonomic nervous system. However blood perfusion or oxygen delivery also contribute to blood pressure, while cardiovascular homeostasis requires a central neuronal control of the heart and vasculature (1,3,5,10,39-42), with maintenance and proper adjustment of cardiac output, mean arterial pressure, and resistance, according to the needs of each moment. Therefore, a crucial goal, no matter the enormous quantity and multiplicity of research produced on this subject, is still to find a measurable (clinical) endpoint representing the individual's cardiovascular homeostatic capacity. Some reasons to explain this absence, justifying our focus, follow:

- sophisticated assessment tools and accumulated know-how improved reference technologies (such as Video-Capillaroscopy or Photo-plethysmography) made currently available along with state-of-the-art instruments such as functional Magnetic Resonance Imaging (fMRI), Optical Coherence Tomography (OCT), or Multispectral optoacoustic tomography (MSOT) (7,43-46).

However, their cost effectiveness and accessibility varies greatly, and only a few have been clinically validated for specific purposes (47-50). Optical – based systems using different lasers are likely the most popular systems in-use, providing non-stationary, oscillatory perfusion signals at different depths on *in vivo* skin. Related signals, including

gold-standards such as LDF, indicate perfusion rather than “flow,” such that results must be expressed in arbitrary units.

- Outcome measures are as extensive as diverse. Even when technologically related, systems provide different variables parameters and indexes, raising discussion about their significance and usefulness. Index outcomes have been also proposed as a mean of communicating an overall condition comprising the most responsive determinants, compared to functional “thresholds”. The “cardiovascular reactivity” the “vascular age” or the “cardio-ankle vascular” indexes, for example, illustrate this variety (51-53). Mechanical-based variables and parameters, such as pressure, stress, and volume, seem to provide the most consistent information. Related quantifiers such as Compliance / Distensibility, modulus (Young’s, Petersons’s) and other (pulsatility, augmentation, resistance) might also be calculated (51,54), however, the expense of the technology providing this information limits its applicability.
- Most of those quantitative “descriptors” are obtained in a quasi-static (by opposition to dynamical) instant-mode assessment ignoring the normal (adaptive) physiology, limiting the interpretation of the clinical endpoint.

Skin has been progressively recognized as an excellent target to study local responses, and regulation of skin microcirculation involves the usual influencers where effector and sensory flows interplay among endothelial, myogenic, and metabolic activities (8,29,33,38,44,55). Other approaches using the skin as a functional window were proposed (a) with posture modification, (b) after a suprasystolic pressure occlusion of an extremity (finger, hand, foot) (c) by breathing an oxygen saturated atmosphere, or (d) during a venoarteriolar reflex (6,8,15,18-23,55-57). These are well known references regarded as “practical” procedures with diagnostic potential to study microvascular

function, although the “practicality” of a hyperoxia testing or a 5 minute post-occlusive reactive hyperemia in a peripheral vascular disease patient may be controversial. Nevertheless, very few studies compared procedures using different assessment methods (19,56).

Our experimental design addresses these constraints. We use two complementary techniques (LDF and PPG), and, noteworthy for distal measurements (hands, feet), probes are placed in both limbs. Thus, our primary outcome is “perfusion” obtained with easy to use, cost effective, reproducible conditions – all of which are essential to be applicable to a clinical setting. The oscillatory nature of the signals, also known as flowmotion, might be further explored by decomposition analysis (WT) to provide a mechanistic overview of the generated signals (as discussed within the “experimental” section above). Our initial approach indicated that the one leg elevation lying supine showed equivalent impact as the post-occlusive reactive hyperemia (PORH), a common exploratory procedure in vascular physiology and pathophysiology (8,19,56) believed to be governed by local endothelial vasodilators sensory nerves and myogenic activities. With those two maneuvers, we detected a similar reduction in perfusion also present in the contralateral (non-challenged) limb. Therefore, our observations could not result exclusively from local responses, revealing instead a homeostatic reflex arc. The same adaptive response with involvement of the contralateral limb was found with VAR (20) a reflex believed to protect the microcirculatory unit from high hydrostatic pressures when venous pressure increases. VAR (58,59). VAR has been described as a local regulation reflex resulting from myogenic interactions with non-adrenergic vasoconstriction (8,58,59). Our method revealed that the VAR procedure promptly generated the same adaptive reflex to restore the systemic hemodynamics locally altered by the pendent limb. These findings suggest

that local phenomena contribute only in part to the mechanistic effects observed with POHR or VAR. No matter being locally evoked, these events trigger this PAHR (Prompt Adaptive Haemodynamical Response) which impacts systemic hemodynamics. We have shown is present with dramatic microcirculatory changes in the hindlimb ischemia mouse model challenged by hyperoxia during regenerative angiogenesis (6), and present even with light and superficial challenges such as a single-limb massage (effleurage) in human, with increased perfusion also in the contralateral non massaged limb (22, and this study). Therefore, these reflexes / responses obtained under different conditions whenever local perfusion is modified are revealed, in the absence of disease, by these immediate adaptive responses seeking to readjust local perfusion to global haemodynamical requirements. The receptors and effectors involved may be difficult to identify, but the reliable, quantifiable measurement of the perfusion outcome is practical and may be of clinical value.

In the present study we look deeper into the physiological relevance of this PAHR by reproducing those two experimental conditions— the effleurage as a “soft challenger” and the VAR as the “hard challenger” in an older group of participants. As shown (Table 1), responses following effleurage are consistent with the previously published PAHR in younger volunteers (22). The perfusion increase is detected with both LDF and PPG in both limbs, only significant in the massaged limb. Blood pressure and pulse reductions ($p < 0.05$) are also present in this group. Absolute perfusion values are much higher here compared with the younger participants and this is in opposition to some previous opinions justifying a reduction of the lower leg flow with (increased) age by a less effective baroreflex (60-63). Other studies assessing the influence of age during a post-occlusive response and matched-intensity leg exercise could not find differences between

healthy young and older adults (64). In fact, the effect of age on the distal perfusion is still an unanswered issue, with contradictory reports on capillary density (65) and endothelium structural modifications (66). Nevertheless, it is clear that peripheral microcirculatory disfunction does not result solely from local microcirculatory impairment. The same risk factors associated with the ageing process affect the macrocirculatory structures and reduce its buffering capacity, which results in an increase of pulsatile stress on microcirculation (12,67,68). This is in line with our findings which consistently include a significant increase of peripheral perfusion in older participants even if healthy, likely related to less effective endothelial and myogenic responsiveness and potentially aggravated in the presence of other pathological processes (69,70). It is relevant to point out that those studies used different technologies to measure perfusion, with measurements usually obtained from a single limb. Thus, the variability or response of the other limb (e.g., by the non detected effect of the PAHR) was not evaluated.

The WT component analysis of the LDF signal depicted a less expressive frequency profile compared with the younger group (Figure 2). Our data reveals discrete, non-significant increases in cardiac, respiratory, and myogenic components and a small reduction of sympathetic and endothelial components, especially in the massaged limb. Similar but less evident changes are noticed in the contralateral non massaged limb. Hence the soft, prolonged challenge of effleurage massage still evokes a comparable PAHR as in the younger group, but the reduced dimension and responsiveness of the system suggest its age dependency.

The PAHR following the venoarteriolar reflex was also depicted in both limbs by LDF and PPG, and involved a significant perfusion reduction in both (dependent and contralateral non-dependent) limbs as noted in the younger participants study (20) although less expressive. Comparison between these two groups revealed no significant

differences. The component analysis of PPG signals from the younger group has shown an effective reduction of myogenic sympathetic and endothelial activities which we anticipated could be related to the intense reduction of cardio-respiratory and neurogenic activities (20). In the present aged group, LDF component analysis confirmed the same profile where a significant reduction of cardiorespiratory, myogenic, neurogenic and endothelial (NO dependent) components was observed in the dependent limb (Figure 3). The same tendency was noted in the contralateral limb, where significant differences are absent except for the recovery period in the cardiac component. Hence the PAHR profile obtained in this group is similar but less expressive than the one obtained with the younger group of participants, and the differences were not as great as those observed with effleurage.

In conclusion, our results confirm the interest of PAHR as a practical, reliable reaction to characterize and quantitatively describe *in vivo* cardiovascular physiology in healthy individuals and illustrate the adaptive response to reestablish perfusion homeostasis after a localized (occlusion, limb position-change, VAR) or systemic (HLI, hyperoxia, movement) controlled microcirculatory challenge. Clearly, age seems to affect PAHR. Aging, even if in a “healthy mode,” involves a progressive reduction of effectiveness and efficiency of all homeostatic sensors and effectors. As might be expected, our mild challenger (effleurage) generated different afferent sensory input, integration and effector responses than the more intense challenger (VAR), and we can expect that pathological processes and other conditions known to affect microcirculation (e.g., smoking, obesity, specific medication, others?) will involve more expressive changes in the microcirculatory components. PAHR further allows a mechanistic view over the measurable outcome, accessing some of the most important intervenients and the

respective adaptive crosstalk mechanisms. The proposed method is easy to use, cost-effective, and reproducible for diagnostic purposes, and in our opinion, of interest for clinical application.

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Capítulo V: Conclusões

Tendo em conta as questões de investigação definidas para este projeto, assim como os objetivos e marcos delineados, descrevem-se de seguida as principais conclusões deste estudo.

A primeira questão de investigação colocada, que consistia em como avaliar, o mais próximo possível do estado fisiológico normal, o impacto hemodinâmico da massagem no membro inferior humano, levou-nos a desenhar um protocolo de massagem a ser aplicado *in vivo*, no membro inferior humano, e em pessoas saudáveis, logo o mais próximo possível do estado fisiológico normal. Neste sentido, desenvolvemos um protocolo experimental com a preocupação de garantir a sua reprodutibilidade e rigor, considerando também o total conforto e bem-estar dos participantes e garantindo a manutenção da homeostase circulatória, de modo a obter resultados comparáveis em idênticas circunstâncias experimentais e registando de modo continuo os eventos hemodinâmicos, antes, durante e após a aplicação da manobra de massagem.

Este protocolo, que implica um procedimento de massagem de um membro inferior (perna) aleatoriamente selecionado, usando o outro membro como controlo, aplicado numa direção (proximal ou ascendente numa das variantes e na direção descendente ou distal numa outra variante). O procedimento de massagem é designado por “massagem Sueca” e o protocolo da sua aplicação contém essencialmente três fases registadas contíguas. A Fase I, com registo em repouso em supinação, durante 10 minutos, a Fase II, de provocação com aplicação de massagem de perna e pé durante 5 minutos e a Fase III, de recuperação com registo durante 10 minutos. Os registos respeitam os sinais de fluxo sanguíneo com FPG e FLD, com as sondas colocadas nos dois pés em ambos os dedos 1 e 2, respetivamente, e ainda o registo de pressão arterial, medida no braço.

O nosso desenho experimental permitiu também estudar mais aprofundadamente a utilização de tecnologias de registo não invasivas e de natureza ótica, amplamente utilizadas em outros contextos, nomeadamente as tecnologias FLD, o *gold standard* e

por isso, a nossa primeira escolha e a FPG, uma tecnologia também desde há muito conhecida e recentemente melhorada com a mais moderna eletrónica. Estes foram os instrumentos que nos permitiram registar os eventos hemodinâmicos em avaliação.

A segunda questão de investigação pretendia avaliar o impacto fisiológico da massagem mesmo que localizada apenas numa zona específica do nosso corpo. Trata-se de um procedimento terapêutico “não-convencional” há muito utilizado de forma empírica ao longo dos tempos, onde tem faltado concordância sobre a demonstração clara dos seus efeitos, se existentes, no sistema cardiovascular em geral e sobre a fisiologia microcirculatória local.

Os nossos resultados permitiram evidenciar com ambas as tecnologias (FLD e FPG) o mesmo tipo de fenómeno, mostrando o impacto da massagem em ambos os membros inferiores, apesar de aplicada apenas a um dos membros, com um aumento significativo do fluxo sanguíneo no membro massajado, e igual efeito, embora mais discreto, mas sempre presente, no membro contra-lateral e não massajado.

Estes sinais de alteração de perfusão foram extensamente analisados, tendo incluído o recurso a outros métodos de análise de dados incluindo a transformada de Ondeleta (TO) dos sinais de FLD e de FPG e a análise de textura. Apesar do sentido das variações serem sempre os mesmos, em especial para a análise TO, esta mostrou componentes com distintas intensidades de registo (em ambos os casos sempre mais acentuadas no membro sujeito à manobra de massagem) tratando-se de componentes de FLD ou de FPG. Conforme demonstrámos, estas diferenças foram atribuídas às diferentes interações entre a luz e o tecido proporcionadas pelas tecnologias que utilizámos, com diferentes capacidades de penetração. A análise TO dos sinais de FLD (o *gold standard*) revelou um aumento dos componentes cardíaco, respiratório e miogénico, assim como a diminuição dos componentes simpático e endoteliais (dependente de ON e independente de ON), aquando da fase de aplicação da manobra.

Testámos ainda o potencial impacto do sentido da massagem, usando o mesmo procedimento de registo e aplicação da massagem, mas com orientações opostas (no sentido proximal e distal). Não foram observadas diferenças significativas entre estas duas variantes com a FLD ou com a FPG, pese embora o facto de as respostas hemodinâmicas terem sido mais evidentes no procedimento proximal, o mais utilizado na prática clínica, apesar da falta de evidências que até agora eram inexistentes.

Concluímos, finalmente, deste longo desenvolvimento, que a massagem, aplicada nas presentes condições experimentais, envolve mais do que uma alteração da microcirculação local, desencadeia aquilo que designamos por uma Resposta Imediata de Adaptação Hemodinâmica (ou PAHR- Prompt Adaptive Hemodynamic Response) que envolve todos os sensores e efectores que contribuem para a manutenção da homeostasia cardiovascular. Este “reflexo” está presente sempre que se alteram as condições microcirculatorias locais, como demonstrámos com o reflexo venoarteriolar ou com a alteração de postura, pelo que acreditamos que possa vir a ter um valor de diagnóstico importante.

Verificamos ainda com o estudo dos grupos de indivíduos mais velhos, que esta resposta Imediata de Adaptação Hemodinâmica, embora seja semelhante aos grupos de jovens, mas existem diferenças, sendo essas respostas menos acentuadas nestes grupos de indivíduos mais velhos.

Assim, considerando estas conclusões, podemos elevar as hipóteses formuladas à categoria de tese e afirmar que:

- O procedimento experimental que propomos demonstrou ser rigoroso, facilmente reproduzível e totalmente adequado ao estudo, com especial realce durante a própria fase de massagem, garantindo ainda o total conforto, bem-estar dos participantes, bem como a manutenção da homeostase circulatória.
- As tecnologias escolhidas para avaliar a microcirculação, nomeadamente a fluxometria por Laser Doppler (FLD) e a fotopletismografia (FPG), demonstraram ser adequadas, pois os resultados permitiram descrever com rigor quantitativamente a microcirculação periférica *in vivo*, em condições dinâmicas como as envolvidas na massagem, tendo sido

verificado que a utilização de ambas as tecnologias, permite fornecer uma análise mais sustentada sobre os fenómenos em causa.

- A manobra de massagem quando aplicada no membro inferior de indivíduos jovens e saudáveis, permite aumentar o fluxo microcirculatório no membro inferior massajado, mas igualmente no membro contralateral. Este efeito, independente do sentido com que é aplicada a massagem, influencia a hemodinâmica em geral, afetando a frequência cardíaca e a pressão arterial. Este reflexo deverá ter outra expressão em indivíduos mais velhos e em doentes com patologia vascular, pelo que acreditamos que, para além do interesse do valor terapêutico da massagem, a avaliação deste “reflexo” por si poderá ser utilizada como instrumento de avaliação da “saúde vascular”.

- A resposta hemodinâmica à massagem que identificamos neste estudo (PAHR), embora seja semelhante, é claramente dependente da idade e de menor variação nos indivíduos mais velhos.

Anexos

Anexo I – Consentimiento Informado – Massoterapia Grupo I

Anexo II – Ficha de Carga - Participante Protocolo Massoterapia

Anexo I – Consentimento Informado – Massoterapia Grupo I

 CBIOS - UDE	Código: Code	Edição nº: Edition nr	Data: Date
	Título: Consentimento Informado Title		

Dados do voluntário

Nome: _____

Data de Nascimento: __/__/____

Código de Referência: _____

Código do Estudo: _____

Informação do Voluntário Consentimento de Participação

O investigador responsável pelo presente projeto propôs-me participar num estudo de investigação desenvolvido sob responsabilidade do CBIOS - CENTRO DE INVESTIGAÇÃO EM BIOCÊNCIAS E TECNOLOGIAS DA SAÚDE.

Foi-me explicada e afirmada a minha total liberdade para aceitar ou recusar participar neste estudo.

Tendo tomado conhecimento dos resultados do exame clínico preliminar realizado neste laboratório, li e compreendi com clareza as informações que se seguem:

Objetivo do estudo

Pretende-se investigar o impacto no membro inferior nas variáveis fisiológicas da circulação periférica antes e depois da realização de um protocolo de massoterapia com a duração de uma sessão.

Resumo da metodologia

O estudo é realizado num painel de cerca de 30 voluntários jovens de ambos os géneros e sem patologia cardiovascular. Cada voluntário aclimatizar-se-á durante 20 minutos na posição de decúbito dorsal numa marquesa, realizando de seguida dois protocolos experimentais. Serão colocados no voluntário, sensores de Eletrocardiografia (ECG), Fotopletismografia e sondas de LDF e de temperatura:

 CBIOS - UDE	Código: Code	Edição nº: Edition nr	Data: Date
	Título: Consentimento Informado Title		

1. O voluntário passa 10 minutos na posição de decúbito dorsal; com o plano horizontal.
2. O voluntário passa 5 minutos na posição de decúbito dorsal; com uma sessão de massoterapia numa das pernas/pé aleatorizada/o (1º protocolo sentido ascendente e 2º protocolo no sentido descendente).
3. O voluntário passa 10 minutos na posição de decúbito dorsal; com o plano horizontal.

Cronograma geral do estudo		
Visita (Voluntário)	Motivo experimental	Duração
Novembro e Dezembro de 2016, Janeiro a Dezembro de 2017, Janeiro a Julho 2018	Realização dos protocolos experimentais	1,5 h

As visitas terão lugar no CBIOS – laboratório de Dermatologia Experimental da Universidade Lusófona de Humanidades e Tecnologias (ULHT), em Lisboa.

Compromissos fundamentais assumidos pelo voluntário
Obrigações: - Não consumir bebidas cafeinadas e alcoólicas nem aplicar produtos tópicos cosméticos nos pés e pernas 24H antes e durante a realização dos protocolos experimentais de recolha de dados; - Não tomar medicação vasoativa nos 5 dias anteriores ao dia da realização dos protocolos experimentais, incluindo o período de sessões de massoterapia. - Respeitar o mesmo horário e tipo de alimentação no dia da leitura inicial e final, assim como realizar a ingestão de alimentos com duas horas de antecedência ao início de recolha de dados.

Riscos menores previsíveis
- Eritema poderá persistir durante algumas horas nas áreas da pele onde foram colocados as sondas; - Eritema na pele da perna de teste derivado à manipulação (massoterapia) das mesmas.

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 CBios - UDE	Código: Code	Edição nº: Edition nr	Data: Date
	Título: Consentimento Informado Title		

Em caso de qualquer reação adversa ocorrida durante o estudo, tenho o direito a solicitar um exame clínico imediato. Poderei a todo o momento pedir informações complementares ao responsável pelo estudo. Foi-me ainda comunicado que o estudo, os seus objetivos e metodologias, foram ainda apreciados por uma Comissão de Ética, a qual emitiu o respetivo parecer favorável à sua realização.

Os dados que me dizem respeito (pessoais e clínicos) serão estritamente confidenciais. Autorizo que (em caso de necessidade) as áreas de pele interessadas sejam fotografadas. Não autorizo a consulta dos dados, exceto por pessoas relacionadas com o estudo e sua avaliação, incluindo monitores, auditores, Comissão de Ética e entidades regulamentares, os quais poderão aceder aos dados clínicos, em absoluto respeito pela sua confidencialidade, em conformidade com a Lei de Proteção de Dados Pessoais.

Se desejar, sou livre a qualquer momento de optar por parar a minha participação sem estar obrigado a qualquer justificação; em consonância, informarei o investigador responsável e não receberei qualquer indemnização.

Aceito, pois, participar livremente neste estudo nas condições assim especificadas.

e comprometo-me por minha honra, e por minha livre vontade a, durante este estudo e durante o período de exclusão que me foi indicado a não aderir a qualquer outro estudo a desenvolver em humanos, mesmo que realizado por um investigador deste centro ou de qualquer outro.

O meu consentimento não dilui as responsabilidades dos organizadores do estudo.

Conservo todos os direitos que me são concedidos por lei.

Feito em ____ de ____ de ____

Assinatura dos Investigadores

Assinatura do voluntário

Responsáveis

Anexo II – Ficha de Carga - Participante Protocolo de Massoterapia

Ficha de Carga - Dados do Participante

Nome: _____
Data nascimento: ____/____/____ Idade: ____
Género: ____ Grupo: ____ Pé: ____
Medições dedos pé: Grande: ____/____; ____ Indicador: ____/____; ____

Questionário clínico

1. Tem alguma doença cardiovascular?

____ Não

____ Sim - Qual ou quais? _____

2. Tem familiares em primeiro grau com doença cardiovascular?

____ Não

____ Sim - Que doenças? _____

3. É fumador(a)?

____ Não

____ Sim – Quantos cigarros fuma, em média, por semana? ____/ semana

4. Toma alguma medicação regularmente?

____ Não

____ Sim - Qual ou quais? _____

5. Tomou alguma outra medicação nas últimas 48H?

____ Não

____ Sim - Qual? _____

6. Aplicou algum produto tópico nos pés nas últimas 24H?

____ Não

____ Sim - Qual? _____

7. Toma algum suplemento alimentar regularmente?

____ Não

____ Sim - Qual ou quais? _____

8. Há quantas horas tomou a última refeição? _____

9. Pratica exercício físico?

____ Não

____ Sim – Qual a frequência por semana? ____/ semana

10. Teve febre nos últimos 5 dias?

____ Não

____ Sim

11. Consome regularmente bebidas cafeinadas?

____ Não

____ Sim - há quantas horas está sem consumir bebidas cafeinadas? _____

12. Tem ciclos menstruais regulares?

____ Não

____ Sim. Em que dia do ciclo menstrual se encontra? _____

Dados do voluntário

Nome: _____

Peso: _____

Altura: _____

Início protocolo:

P Arterial braço; FC: ____/____; ____

P Arterial tornozelo: ____/____; ____

Protocolo Massoterapia Ascendente

Descendente

Minuto

Fase I (10 minutos)

P Arterial braço; FC: ____/____; ____

FC: ____/____; ____

3'

P Arterial braço; FC: ____/____; ____

FC: ____/____; ____

5'

P Arterial braço; FC: ____/____; ____

FC: ____/____; ____

7'

Fase II (5 minutos)

P Arterial braço; FC: ____/____; ____

FC: ____/____; ____

12'

P Arterial braço; FC: ____/____; ____

FC: ____/____; ____

13'

P Arterial braço; FC: ____/____; ____

FC: ____/____; ____

14'

Fase III (10 minutos)

P Arterial braço; FC: ____/____; ____

FC: ____/____; ____

18'

P Arterial braço; FC: ____/____; ____

FC: ____/____; ____

20'

P Arterial braço; FC: ____/____; ____

FC: ____/____; ____

22'